Published in final edited form as:

Angew Chem Int Ed Engl. 2010 February 15; 49(8): 1451–1455. doi:10.1002/anie.200906232.

Drug-Free Macromolecular Therapeutics: Induction of Apoptosis by Coiled-Coil Mediated Crosslinking of Antigens on Cell Surface

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Abstract

A new paradigm was designed for apoptosis induction mediated by the biorecognition of coiled-coil motifs at the Raji B cell surface. The heterodimerization of complementary peptides, one bound to Fab' antibody fragment, the other as grafts to HPMA copolymer, results in crosslinking of CD20 target antigens, and consequently, initiation of apoptosis.

Keywords

apoptosis; biorecognition; CD20; coiled-coil; HPMA copolymer

Molecular biorecognition is at the center of all biological processes and it forms the basis for the design of precisely defined smart systems, including targeted therapeutics, imaging agents, biosensors, and stimuli-sensitive and self-assembled biomaterials. The self-assembly of hybrid materials, composed of synthetic and biological macromolecules, is mediated by the biorecognition of biological motifs.[1] We have previously designed self-assembling hybrid hydrogel systems composed of a synthetic *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer backbone and coiled-coil peptide motifs; the results showed that it is possible to impose properties of a well-defined coiled-coil peptide onto a whole hybrid hydrogel.[2] Recently, we designed a pair of oppositely charged pentaheptad peptides (CCE and CCK) that formed antiparallel coiled-coil heterodimers and served as physical crosslinkers.[3a] HPMA graft copolymers, CCE-P and CCK-P (P is the HPMA copolymer backbone), self-assembled into hybrid hydrogels with a high degree of biorecognition.[3a,b]

We hypothesized that this unique biorecognition of CCK and CCE peptide motifs could be expanded past biomaterials design and applied to a living system and mediate a biological process. This would provide a bridge between the designs of biomaterials and macromolecular therapeutics.

To verify this hypothesis we chose to study the induction of apoptosis in CD20 positive cells. CD20 is one of the most reliable biomarkers for B cell non-Hodgkin's lymphoma (NHL),[4a,b] which functions as a cell cycle regulatory protein[4c] that either controls or

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

functions as a store operated calcium channel. CD20 also forms dimers and tetramers[4d] constitutively associated in lipid rafts of the cell membrane.[4b] It is a non-internalizing antigen that remains on the cell surface when bound to a complementary antibody.[4e] However, crosslinking of CD20-bound antibodies with a secondary antibody results in apoptosis.[4f] To exploit this phenomenon, we designed a system composed of CCE and CCK peptides, Fab' fragment of the 1F5 anti-CD20 antibody, and HPMA copolymer (Figure 1). The exposure of CD20+ Raji B cells to Fab'-CCE resulted in the decoration of the cell surface with multiple copies of the CCE peptide via antigen-antibody fragment biorecognition. Further exposure of the decorated cells to HPMA copolymer grafted with multiple copies of CCK resulted in the formation of CCE-CCK coiled-coil heterodimers on the cell surface. This second biorecognition event induced crosslinking of CD20 receptors and triggered apoptosis of Raji B cells.

The peptides CCE and CCK were prepared by solid phase peptide synthesis[3a]. Their purities and identities were confirmed by RP-HPLC and MALDI-TOF mass spectrometry (see Supporting Information (SI), Figures S1 and S2).

The HPMA graft copolymer, CCK-P, was synthesized in a three-step process (Figure 2a, for details see SI).[3a] First, HPMA was copolymerized with *N*-(3-aminopropyl)methacrylamide, and optionally *N*-methacryloylaminopropyl fluorescein thiourea via free radical polymerization. Then the amino groups at side chain termini were converted into maleimido groups by reaction with succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). At last, CCK was attached to the copolymer by thiol-maleimide chemistry through the *N*-terminal cysteine of CCK. The number average molecular weight, M_n , of P was 99.6 kDa and the average number of CCK grafts per macromolecule was 8.94 (the copolymer abbreviated as (CCK)₉-P). The murine 1F5 anti-CD20 IgG2a antibody[5] was prepared from the anti-CD20 hybridoma clone 1F5 (ATCC, Bethesda, MD) in a CellMax bioreactor (see SI) and enzymatically digested with lysyl endopeptidase. The F(ab')₂ fragment was isolated on a Protein G column.[6a,b] After labeling with Rhodamine Red-X, F(ab')₂ was reduced by tris(2-carboxyethyl)phosphine (TCEP) and then conjugated with CCE to produce the Rhodamine Red X-labeled Fab'-CCE conjugate, Fab'-(CCE)₁ (Figures 2b and S3).

The biorecognition of Fab'-(CCE)₁ and (CCK)₉-P was first evaluated by circular dichroism (CD) spectrometry. A pronounced coiled-coil signal (minima at 208 and 222 nm) was observed upon mixing of CCE/Fab'-(CCE)₁ and (CCK)₉-P (Figure 3c). Dynamic light scattering (DLS) measurements revealed that within 1 h after mixing, the effective diameter of particles in the equimolar mixture of Fab'-(CCE)₁ and (CCK)₉-P significantly increased (Figure S4).

Human Burkitt's NHL Raji B cells were used to demonstrate the biorecognition of coiled-coils at cell surface and apoptosis induction as a result of CD20 crosslinking. They were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO₂ (v/v). Exposure of Raji B cells to Fab'-(CCE)₁ decorated the cell surface with the CCE peptide as shown by confocal fluorescent microscopy image (Figure 3a). On the contrary, exposure of Raji B cells to (CCK)₉-P did not result in detectable deposition of the graft copolymer at the cell surface at experimental conditions used (Figure 3b). The exposure of Raji B cells to a high avidity multivalent construct prepared by premixing of Fab'-(CCE)₁ and (CCK)₉-P resulted in excellent binding to the cell surface (Figure 4c1-c3). This was in agreement with our studies of the interaction of CD20 with multivalent HPMA copolymer-Fab' conjugates.[6b] Finally, exposure of Raji B cells, pre-decorated with CCE peptide, to (CCK)₉-P (consecutive exposure) resulted in (CCK)₉-P attachment to cell surface (Figure 3d1-d3). This suggests that coiled-coil

heterodimers formed at the cell surface with concomitant crosslinking of CD20 receptors. This demonstrates outstanding biorecognition of CCE bound to cell surface with CCK attached as grafts to the HPMA copolymer. Apparently, the proteins present in FBS did not interfere with the antiparallel heterodimerization. For details on biorecognition studies, more images, and additional controls, see SI and Figures S5-S7.

Three assays have been used to demonstrate apoptosis induction following crosslinking of CD20 antigens: caspase 3 activity assay, Annexin V/propidium iodide assay, and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. The caspase 3 assay was used to evaluate the time dependence of apoptosis induction (Figure 4). Exposure of cells to individual components, Fab'-(CCE)₁ or (CCK)₉-P, resulted in a very low percentage of cell death, independent of the incubation interval. However, a time-dependency was observed for the coiled-coil based apoptosis induction systems: premixture of Fab'-(CCE)₁ + (CCK)₉-P and the consecutive exposure of cells to Fab'-(CCE)₁ followed by (CCK)₉-P. The premixture of Fab'-(CCE)₁ and (CCK)₉-P exhibited the highest levels of apoptosis after 6 h of incubation, whereas for both consecutive systems, Fab'-(CCE)₁ + (CCK)₉-P and (control) 1F5 + goat antimouse (GAM) secondary antibody, the extent of apoptosis increased with increasing incubation time, being the highest at 24 h. This probably relates to different levels of destabilization of plasma membrane integrity following exposure to a multivalent high avidity conjugate vs. a monovalent Fab'-(CCE)₁ a conjugate.

In further experiments the systems were compared at time intervals related to maximum apoptosis, i.e. 6 h for premixture and 24 h for consecutive addition. The extent of apoptosis could be increased by manipulating the concentration of the components. The increase of Fab'-(CCE)₁ concentration from 0.5 to 1 μ M increased the amount of apoptotic cells from 21 % to 32% in the clinically relevant consecutive system (Figure 5a).

Results of Annexin V (Figure 5b) and TUNEL (Figure 5c) assays corroborated the efficacy of the system in the presence of FBS. A comparable level (~30%) of apoptosis induction was achieved by the consecutively added Fab'-(CCE)₁ (1 μ M) and (CCK)₉-P ([CCK]₉-P=25 μ M) in comparison with 1F5+GAM (~40%) and premixed Fab'-(CCE)₁+(CCK)₉-P (~40%). The apoptotic levels observed in our experiments are comparable to those obtained after exposure of Raji B cells to multivalent antibody conjugates: HPMA copolymers containing multiple Fab' fragments,[6b] dextran - antibody (Rituximab) conjugates,[7a] and Rituximab dimers.[7b]

Control experiments validated the hypothesis that coiled-coil heterodimerization of CCE with CCK with concomitant crosslinking of the CD20 antigen is responsible for apoptosis induction. The levels of apoptosis observed after exposure of Raji B cells to: a) mixture of Fab' + CCE + CCK + P-NH₂; b) mixture of Fab'-(CCE)₁ + P-NH₂; and c) mixture of Fab' + (CCK)₉-P were very low, similar to untreated cells (Figure 5a, b, c; Controls 1, 2, and 3).

Further optimization of the system might result in even higher apoptosis levels. Some of the factors to be studied include: optimization of the concentration and timing of consecutive addition of components, the use of D-amino acid sequences, design of shorter sequences, the switch of peptides (evaluation of Fab' - CCK + CCE-P), and the insertion of a spacer between the Fab' and the peptide. Several factors contributed to the successful design of the new drug-free therapeutic system: a) The 1F5 antibody: its binding to CD20+ cells does not induce apoptosis; a secondary GAM antibody[8] is needed; b) The design of the CCE and CCK sequences[3a] (Figure S1). The antiparallel heterodimer formation reduces the steric hindrance of the polymer chain during binding of (CCK)₉-P to Raji cells decorated with CCE motifs and enhances the probability of "in-register" alignment of the CCE-CCK heterodimer; c) The HPMA copolymer employed for the conjugation of multiple copies of

one of the peptides (CCK). HPMA copolymers have been widely used in drug delivery systems;[9] their biocompatibility has been proven in animal models[10a-c] and in clinical trials;[10d] d) The choice of the CD20 antigen: CD20 is expressed on most NHL malignant cells as well as on normal B cells. However, it is not expressed on stem cells and mature plasma cells. Consequently, normal numbers of B cells can be restored after treatment.[11]

We have presented a new paradigm in apoptosis induction mediated by the biorecognition of coiled-coil forming peptide segments on the cell surface. The fact that biorecognition of coiled-coils at cell surface occurred in media containing 10% FBS indicates the specificity of the CCE-CCK interaction and bodes well for future in vivo experiments and for the development of efficient drug-free macromolecular therapeutics. The important feature of this design is the absence of low molecular weight cytotoxic compounds. The concept of drug-free macromolecular therapeutics could be expanded by using different components in the design. For example, the Fab' fragment can be replaced by antigen binding saccharides[12a] or by peptides[12b,c] selected by combinatorial methods.

Acknowledgments

This study was supported in part by NIH grant EB005288 (to JK). KW acknowledges a fellowship from the American Foundation for Pharmaceutical Education (AFPE).

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Figure 1. Induction of apoptosis in Human Burkitt's NHL Raji B cells by crosslinking its CD20 antigens, mediated by antiparallel coiled-coil formation at cell surface. The simplified scheme is not drawn to scale.

Figure 2.
a,b) Synthetic schemes for (CCK)₉-P and Fab'-(CCE)₁; **c)** CD spectra of CCE, CCK, (CCK)₉-P, equimolar mixtures of CCE and (CCK)₉-P, and Fab'-(CCE)₁ and (CCK)₉-P. Concentrations: [CCE]=[CCK]=[Fab'-(CCE)₁]=50 μ M; [(CCK)₉-P]=5.60 μ M. Data were acquired at 25 °C in PBS (pH 7.4).

Figure 3.

Biorecognition of Fab'-CCE and CCK-P on Raji B cell surface. (**r₁-r₂**) Reference cells under transmitted light. (**a**) Exposure of cells to Fab'-CCE₁ (0.5 μ M, Rhodamine Red-X labeled) resulted in decoration of cell surface with CCE; (**b**) Exposure of cells to CCK₉-P ([CCK]=25 μ M, FITC labeled) did not result in staining; (**c₁-c₃**) Exposure of cells to a premixture of Fab'-CCE₁ (0.5 μ M) and CCK₉-P ([CCK]=25 μ M); (**d₁-d₃**) Consecutive exposure of cells to Fab'-CCE₁ (0.5 μ M) followed 1 h later by CCK₉-P ([CCK]=25 μ M). (**a**, **c₁**, and **d₁**) red channel for Rhodamine Red-X; (**b**, **c₂**, and **d₂**) green channel for FITC; (**c₃** and **d₃**) Overlay of red and green channels. Images of individual cells are shown.

Figure 4.

Time dependence of apoptosis induction in Raji B cells by caspase 3 activity assay.

Untreated: Untreated cells; **Fab'-(CCE)₁:** Single component control at 0.5 μ M; **(CCK)₉-P:** Single component control ([CCK]=25 μ M); **1F5+GAM:** 1F5 Ab (0.2 μ M) 1 h followed by secondary antibody GAM (10 μ g/mL); **Premixed:** Premixture of Fab'-(CCE)₁ (0.5 μ M) and (CCK)₉-P ([CCK]=25 μ M); **Consecutive:** Consecutively added Fab'-(CCE)₁ (0.5 μ M) followed 1 h later by (CCK)₉-P ([CCK]=25 μ M). Results are presented as means $\pm$ SD (n=3).

Figure 5.

Coiled-coil mediated induction of apoptosis of Raji B cells as assessed by: **a)** Caspase 3 activity; **b)** Annexin V/propidium iodide assay; and **c)** TUNEL assay. The concentrations of 1F5 and GAM were 0.2 μM and 10 μg/mL, respectively. Concentrations of Fab'-(CCE)₁ and CCK in (CCK)₉-P were 0.5 and 25 μM, respectively for **P1** and **C1** in two experiments of the caspase 3 assay; 1 and 25 μM, respectively for all other experiments. **P-ctrl1**: a premixture of Fab' (1 μM), CCE (1 μM), CCK (25 μM), and P-NH₂ (2.80 μM); **P-ctrl2**: a premixture of Fab'-(CCE)₁ (1 μM) and P-NH₂ (2.80 μM); **P-ctrl3**: a premixture of Fab' (1 μM) and (CCK)₉-P (2.80 μM); **C-ctrl1**: Fab' (1 μM) and CCE (1 μM) for 1 h, and then CCK (25 μM) and P-NH₂ (2.80 μM); **C-ctrl2**: Fab'-(CCE)₁ (1 μM) for 1 h, then P-NH₂ (2.80 μM); **C-ctrl3**: Fab' (1 μM) for 1 h, then (CCK)₉-P ([CCK]=25 μM / [P-NH₂]=2.80 μM). Results are presented as means ± SD (n=3).