Enhancement of the Therapeutic Index: From Nonmyeloablative and Myeloablative toward Pretargeted Radioimmunotherapy for Metastatic Prostate Cancer

Sally J. DeNardo,1 Carol M. Richman,1 Huguette Albrecht,1 Patricia A. Burke,1 Arut Natarajan,1 Aina Yuan,1 Jeff P. Gregg,1 R.T. O’Donnell,1,2 and Gerald L. DeNardo1

Abstract

Purpose: New strategies that target selected molecular characteristics and result in an effective therapeutic index are needed for metastatic, hormone-refractory prostate cancer.

Experimental Design: A series of preclinical and clinical studies were designed to increase the therapeutic index of targeted radiation therapy for prostate cancer.111In/90Y-monoclonal antibody (mAb), m170, which targets aberrant sugars on abnormal MUC1, was evaluated in androgen-independent prostate cancer patients to determine the maximum tolerated dose and efficacy of nonmyeloablative radioimmunotherapy and myeloablative combined modality radioimmunotherapy with paclitaxel. To enhance the tumor to liver therapeutic index, a cathepsin degradable mAb linkage (111In/90Y-peptide-m170) was used in the myeloablative combined modality radioimmunotherapy protocol. For tumor to marrow therapeutic index improvement in future studies, anti-MUC1 scFvs modules were developed for pretargeted radioimmunotherapy. Anti-MUC1 and anti-DOTA scFvs were conjugated to polyethylene glycol scaffolds tested on DU145 prostate cancer cells and prostate tissue arrays, along with mAbs against MUC1 epitopes.

Results: The nonmyeloablative maximum tolerated dose of 90Y-m170 was 0.74 GBq/m² for patients with not more than 10% axial skeleton involvement. Metastatic prostate cancer was targeted in all 17 patients; mean radiation dose was 10.5 Gy/GBq and pain response occurred in 7 of 13 patients reporting pain. Myeloablative combined modality radioimmunotherapy with 0.4 GBq/m² of 90Y-peptide-m170 and paclitaxel showed therapeutic effects in 4 of 6 patients and 30% less radiation to the liver per unit of activity. Neutropenia was dose limiting without marrow support and patient eligibility was a major limitation to dose escalation. Hypoglycosylated MUC1 epitopes were shown to be abundant in prostate cancer and to increase with disease grade. Anti-MUC1 scFvs binding to prostate cancer tissue and live cells were developed into di-scFv binding modules.

Conclusions: The therapeutic index enhancement for prostate radioimmunotherapy was achieved in clinical studies by the addition of cathepsin cleavable linkers to 90Y-conjugated mAbs and the use of paclitaxel. However, the need for marrow support in myeloablative combined modality radioimmunotherapy restricted eligible patients. Therefore, modular pretargeted radioimmunotherapy, aiming at improving the tumor to marrow therapeutic index, is being developed.

Although localized prostate cancer is curable, metastatic hormone-independent prostate cancer is usually fatal. Better understanding of this disease has yielded information useful in designing new therapeutic strategies (1, 2). Studies of epithelial cancer biology have provided insights into the relationship of abnormal glycoproteins and prostate cancer grade of relevance for tumor targeting (3–7).

Cancer-related aberrations in MUC1 epithelial mucin present unique epitopes that can provide targets for site-specific therapy for many epithelial cancers. Normal MUC1 is apically distributed on the surface of epithelial cells as a large, complex glycoprotein composed of a polypeptide core protected by long oligosaccharide side chains on its extracellular region (8). A 20-amino-acid sequence repeated in tandem (variable number of tandem repeats) constitutes the MUC1 core (9). In most epithelial cancers, expression of MUC1 is up-regulated, hypoglycosylated, and aberrantly glycosylated, and its cell surface distribution is no longer apical. Monoclonal antibodies (mAb) against the MUC1 mucin in breast cancer have previously been investigated using a panel of mAbs and MUC1 mucin–related synthetic peptides and glycopeptides (10). Whereas MUC1 glycoproteins have been found in the blood of cancer patients and used as

Authors' Affiliations:1School of Medicine, University of California Davis, Sacramento, California and 2Northern California Veteran’s Administration Healthcare System, Sacramento, California

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Requests for reprints: Sally J. DeNardo, Radiodiagnosis and Therapy, Molecular Cancer Institute, University of California Davis, Room 3100, 1508 Alhambra Boulevard, Sacramento, CA 95816. Phone: 916-734-3787; Fax: 916-451-2857; E-mail: sdenardo@ucdavis.edu.

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markers for disease (11), mAbs to MUC1 epitopes not shed in the blood have been used for radioimaging and radioimmunotherapy (12–15). These mAbs include those shown to target the aberrantly glycosylated MUC1 peptide core, and also mAbs m170 and 155 produced against a synthetic asialo GM1 disaccharide but reactive to Thompson-Friedenreich–related sialyl-Tn antigen (16, 17); the Thompson-Friedenreich antigen has been recently confirmed as MUC1 associated (18).

For targeted radiation therapy, such as radioimmunotherapy, to have an effect on prostate cancer, the therapeutic index, defined as the therapeutic effect on cancer cells compared with toxic effects on the most sensitive normal tissues, must be increased (19, 20). This report spans nonmyeloablative radioimmunotherapy-related responses through combined modality radioimmunotherapy trials to new developments for future therapeutic index enhancement through novel pretargeted radioimmunotherapy. Both patient radioimmunotherapy trials in prostate cancer patients and immunohistochemistry using novel scFvs on prostate cancer tissue arrays show that hypoglycosylated MUC1 epitopes provide excellent targets for hormone-independent metastatic prostate cancer radioimmunotherapy. To reduce marrow toxicity, new pretargeted radioimmunotherapy is being developed based on the MUC1 scFv epitopes in prostate cancer and using new di-scFv modular pretargeting molecules.

**Material and Methods**

**Radioimmunotherapy and combined modality radioimmunotherapy**

**Patients.** Twenty-six patients with androgen-independent progressive prostate cancer were entered on one of two radioimmunotherapy protocols, each requiring a radioimmunoconjugate quantitative imaging pharmacokinetic/dosimetry study, 1 week before radioimmunotherapy. The initial quantitative imaging pharmacokinetic/dosimetry study from each patient was used in the dosimetry analysis and radioimmunoconjugate comparison.

**Radioimmunoconjugates.** m170 monoclonal antibody. mAb m170 is a murine immunoglobulin G developed using a synthetic asialo GM1 terminal disaccharide immunogen related to the Thompson-Friedenreich antigen recently shown on MUC1 (16–18, 21). mAb m170 (current good manufacturing practice grade) from Biomira, Inc. (Edmonton, Canada) was >95% monomeric immunoglobulin G by PAGE and met U.S. Food and Drug Administration guidelines. mAb m170 is a murine immunoglobulin G developed using a synthetic asialo GM1 terminal disaccharide immunogen related to the Thompson-Friedenreich antigen recently shown on MUC1 (16–18, 21). mAb m170 (current good manufacturing practice grade) from Biomira, Inc. (Edmonton, Canada) was >95% monomeric immunoglobulin G by PAGE and met U.S. Food and Drug Administration guidelines.

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**Statistical methods.** Cumulated activity was converted to radiation dose for 22N using the Medical Internal Radiation Dose formula, Medical Internal Radiation Dose 5 values, and reference man masses (30). The radiation dose from blood to marrow and marrow-to-marrow was calculated as previously described (23, 29, 31). Only tumors with a measured volume of 10 mL or greater were used for dosimetric calculations.

**Pretargeted radioimmunotherapy development**

**Immunohistochemistry on prostate tissues.** Formalin-fixed tissue embedded in paraffin, obtained from the University of California at Davis Human Biological Specimen Repository and categorized in terms of pathology and Gleason grade (32), included a range of prostate cancers from prostatic intraepithelial neoplasia to Gleason grade 5 and benign/normal prostate tissues. The prostate tissues represented in the 197 array cores were 77 normal/benign (39%), 31 prostatic intraepithelial neoplasia and 89 Gleason grade 3 to 5 (45%).

Three well-characterized anti-MUC1 mAbs, with different epitope specificities, were chosen to evaluate the presentation of MUC1 on the prostate tissue array (Table 1); mAb m170 was not included because it does not react well with fixed tissues.

**BrE3 monoclonal antibody.** In previous studies, this mAb stained 97% of metastatic breast cancer specimens with >75% positive cells (33). BrE3 binds to glycosylated and nonglycosylated forms of MUC1.
Table 1. Characteristics of anti-MUC1 mAbs used to validate the suitability of aberrant MUC1 as a target for pretargeted radioimmunotherapy in prostate cancer

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NOTE: Because their MUC1 reactivity has been well characterized (10, 37), these mAbs were used to quantitate and characterize MUC1 on prostate tissue.

with similar affinity and its expression has been reported to be associated with breast cancer patient survival (34–37). Normal tissues were not targeted on imaging studies (12, 13). BrE3 mAb was obtained from Dr. R. Ceriani.

B27.29 monoclonal antibody. In prior studies, mAb B27.29 showed increased diffuse, cytoplasmic MUC1 expression on prostate cancer compared with normal prostate (6). Nuclear magnetic resonance studies of the binding of B27.29 to peptides with various glycosylation patterns indicate this mAb binds to the core epitope spanning the PDTRP sequence and a carbohydrate epitope (38). In a study on breast patterns indicate this mAb binds to the core epitope spanning the studies of the binding of B27.29 to peptides with various glycosylation compared with normal prostate (6). Nuclear magnetic resonance increased diffuse, cytoplasmic MUC1 expression on prostate cancer.

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Clinical Studies

Pretargeted radioimmunotherapy development
Prostate cancer MUC1 targets: epitope selection for pretargeted radioimmunotherapy molecules. Immunohistochemistry done on the tissue array with three anti-MUC1 mAbs (BrE3, B27.29, and HMFG1) gave a positive MUC1 score if 50% or more of the cells were stained (Fig. 3). The profile of BrE3 showed a strong correlation between prostate cancer stage and increase in MUC1 staining \( (P < 0.001) \). Because BrE3 recognizes predominately a peptidic epitope within the variable number of tandem repeat region of MUC1, this result suggests that hypoglycosylated MUC1 is a good marker for aggressive prostate cancer.

Anti-MUC1 scFv selection and production. ScFvs binding to biotinylated bovine serum albumin-MUC1 peptide were selected from anti-MUC1 phage display libraries \( (43, 45) \). In these libraries, the format of a full-length scFv is VH-(GaS),linker-VL. Production yields and additional characteristics of selected scFvs are indicated in Table 2. Production yields vary significantly from one scFv to another although their nucleotide sequences share 70% or greater homology \( (45) \). As deduced from their isolectric point values, these scFvs vary in net charge at pH 7.

The MUC1 binding of two scFvs, as scFv-c, E1 and G1, was characterized by comparing their prostate tissue binding patterns with those of the three anti-MUC1 mAbs \( (\text{Fig. 3}) \). As shown, the tissue binding of the E1 scFv-c increases with prostate cancer stage, whereas that of the G1 scFv-c seems less discriminating \( (\text{Fig. 4}) \). Therefore, Br-E3 and E1 scFv-c have MUC1 epitopes which become more prominent with disease progression.

Di-scFv. Soluble bivalent di-scFv were produced in \( E. \ coli \) by expression of scFv genes cloned in tandem into the expression vector. These bivalent scFvs are covalently linked. Furthermore, the di-scFv design offers additional options for the location of the extra cysteine. Di-scFv-c were produced as scFv-c-scFv or scFv-scFv-c \( (\text{Fig. 5A}) \). Di-scFv-c production yields were influenced by the presence and location of the extra cysteine; the overall trend was toward a decreased yield in comparison with di-scFvs without an unpaired cysteine. SDS-PAGE profiles of E-Tag–purified scFv-c and di-scFv-c are shown in \( \text{Fig. 5B}) \); under reducing conditions, apparent molecular weights for scFv-c and di-scFv-c are 31 and 52 kDa, respectively.

ScFv site–specific conjugation to polyethylene glycol. For site-specific conjugation of scFv modules onto a functionalized PEG scaffold, the unpaired cysteine was used. This scFv-c site–specific conjugation to PEG was evaluated in respect to several variables: \( a \) size of the PEG; \( b \) nature of the group reactive with thiol; \( c \) number of functional groups per PEG molecule; and \( d \) configuration (linear or branched) of the PEG molecule. None of the combinations tested led \( \geq 89% \) of the scFv-c PEGylation, although di-scFv-c has resulted in \( >95\% \) PEGylation with the scFv-c-scFv format. Linear PEG molecules of 2 and 5 kDa \( (\text{Fig. 6A}) \) showed the highest efficiencies for PEGylation. The conjugate of interest \( (\text{Fig. 6B}) \), di-scFv-PEG, has been shown to retain the immunoreactivity of the di-scFv. These site-specific conjugation studies to PEG-Mal indicate that the free thiol is accessible in both scFv-c and di-scFv-c configurations.

Discussion
Systemically given targeted radionuclide therapy is a modality uniquely suited to provide effective therapy for metastatic
and androgen-independent prostate cancer, if the therapeutic index can be enhanced. The series of preclinical and clinical studies presented here were designed toward this goal by selecting the aspects of MUC1 targeted/pretargeted radionuclide therapy that can be developed and combined to achieve an effective therapeutic index.

Combined modality radioimmunotherapy using $^{90}$Y DOTA-peptide-m170 with paclitaxel increased efficacy in patients with

Table 2. Characteristics of selected anti-MUC1 scFvs and their scFv-c counterparts

<table>
<thead>
<tr>
<th>ScFv</th>
<th>Source</th>
<th>Selection</th>
<th>Length (aa)</th>
<th>pI</th>
<th>Production yield (mg/L)</th>
</tr>
</thead>
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<tr>
<td>E1</td>
<td>BALB/c mice: scFv gene library</td>
<td>Biotinylated BSA-MUC1</td>
<td>258</td>
<td>5.84</td>
<td>0.25</td>
</tr>
<tr>
<td>E1-c</td>
<td>Cloning</td>
<td>Extra TGT codon</td>
<td>259</td>
<td>5.84</td>
<td>0.10</td>
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<td>G1</td>
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<td>G1-c</td>
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<td>Extra TGT codon</td>
<td>260</td>
<td>5.37</td>
<td>0.21</td>
</tr>
<tr>
<td>D5</td>
<td>NZB mice: scFv gene library</td>
<td>Biotinylated BSA-MUC1</td>
<td>259</td>
<td>8.92</td>
<td>0.7</td>
</tr>
<tr>
<td>D5-c</td>
<td>Cloning</td>
<td>Extra TGT codon</td>
<td>260</td>
<td>8.76</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Abbreviations: aa, amino acid; pI, isoelectric point; BSA, bovine serum albumin.
advanced androgen-independent, metastatic prostate cancer over that previously reported for radioimmunotherapy alone (19). Further dose escalation is possible with the use of PBSC. However, PBSC harvest is only modestly successful in this older population, thus greatly limiting eligible patients. A pretargeting radioimmunotherapy strategy with novel molecules holds more promise for achieving the needed therapeutic index. Enhanced therapeutic index for pretargeted radioimmunotherapy has been shown in animal models (46–49) and in patients (50–52). Pretargeting using various streptavidin-biotin formats has been investigated (51, 53); although highly efficient for capture of the radionuclide, they raise more immunogenicity concerns than other approaches under study (47, 49, 54).

Because pretargeting molecules have to first effectively target the tumor, MUC1 pretargeting molecules presented herein are multivalent for the tumor epitope. Over the past decade, a number of bispecific mAb and mAb fragments have been generated and methods to increase their functional affinity have been explored (55, 56). To obtain stable multimers, we used di-scFv-c modules covalently attached to a PEG scaffold. PEG was chosen because PEylation is a validated drug delivery method for extension of serum half-life (57, 58).

The selection of MUC1 as the targeting epitope for pretargeted radioimmunotherapy of metastatic prostate cancer was based on several criteria: (a) tumor targeting obtained in clinical trials with mAbs, such as m170 and BrE3 (13, 19, 20, 59, 60); (b) hypoglycosylated MUC1 provides novel tumor-specific epitopes (61); and (c) peptide mimics of the 20-amino-acid motif repeated in the MUC1 variable number of tandem repeat region promote immune responses in mice for development of anti-MUC1 scFvs (43).

**Fig. 4.** Schematic of vectors used for the expression of scFv and scFv-c. A scFv expressed from the pCANTAB 5E vector carries a COOH-terminal E-Tag (E) that can be used for affinity purification and immunodetection. When expressed from pCANTAB 5E-Cys, the same scFv contains an additional COOH-terminal cysteine (C), located just upstream of the E-Tag, providing a free thiol for site-specific conjugation.

**Fig. 5.** Di-scFv-c. A, schematic of vectors used for the expression of di-scFv-c. Di-scFv-c were expressed from scFvs cloned in tandem into either pCANTAB 5E (scFv-c-scFv) or pCANTAB 5E-Cys (scFv-scFv-c). In all cases, the scFv joining linker was the 20-amino-acid-long (G4S)4 sequence. In scFv-scFv-c, the extra-cysteine (C) is inserted at the COOH terminus on the vector backbone, whereas in scFv-c-scFv, the cysteine is inserted within the (G4S)4 linker joining the two scFvs. B, reducing SDS-PAGE profiles of scFv-c and di-scFv-c. After E-Tag chromatography purification from E. coli periplasmic extracts, scFv-c and di-scFv-c proteins were loaded onto a 4% to 12% gradient polyacrylamide gel for electrophoresis under reducing and denaturing conditions. Subsequently, proteins were visualized by Coomassie blue staining. Lane 1, D5-c scFv (10 μg of total proteins); lane 2, D5D5-c di-scFv (24 μg of total proteins); KD lane, MW standards (from top to bottom): 185, 98, 52, 31, 19, and 11 kDa. Arrows, full-length scFv-c (lane 1) and di-scFv-c (lane 2) proteins. In addition to the major full-length proteins, some degradation products are copurified by E-Tag affinity chromatography.
In contrast to the wealth of available studies on MUC1 in breast cancer, knowledge of MUC1 in prostate cancer was limited. To validate MUC1 epitope targets and select suitable scFvs from our library for pretargeted radioimmunotherapy of prostate cancer, we studied the binding of three well-characterized anti-MUC1 mAbs using immunohistochemistry on a prostate tissue array (Table 1). BrE3 abundantly stained on a prostate tissue array (Table 1). BrE3 abundantly stained on a prostate tissue array (Table 1). BrE3 abundantly stained prostate cancer and showed increased staining with higher Gleason grades. Because BrE3 predominately recognizes a peptide epitope within the variable number of tandem repeat region of MUC1, this result suggests that hypoglycosylated MUC1 is a good marker for more aggressive prostate cancer. Of the scFv-c described, E1-c had a markedly similar staining profile to that of BrE3, therefore also correlating with higher grades of disease.

Multivalent, bispecific PEG-scFv molecules designed to target MUC1 and bind the chelate have been developed in the following formats: (anti-MUC1 scFv) 2-PEG-anti-DOTA scFv or (anti-MUC1 scFv) 2-PEG-(anti-DOTA scFv) 2. In theory, such molecules can be assembled by scFv site-specific conjugation to a tri- or tetra-functionalized PEG. In practice, however, homogeneous tri- or tetra-functionalized PEG are not available and PEGylation of different scFvs to multifunctionalized PEG is not readily controlled. Therefore, we achieved tetravalency by conjugation of di-scFv-c to the PEG scaffold; di-scFv-c were produced in E. coli by expression of scFv CDNAs cloned in tandem into the expression vector. Not surprisingly, di-scFv-c production yields in E. coli were decreased in comparison with those of scFv and di-scFv without an unpaired cysteine (42, 62). Regardless of the presence of an unpaired cysteine, high production yields have been reported in yeast (63, 64). Site-specific conjugation studies of di-scFv-c to PEG-Mal indicate that the free thiol is accessible in these configurations with a scFv-c-scFv format providing >90% conjugation. PEGylation efficiency seems inversely proportional to the length of the PEG (65). The di-scFv-c module for site-specific and covalent attachment to a PEG scaffold provides flexibility in the use of various scFv units.

In summary, the research presented here reflects serial developments and target choices toward an increase of the therapeutic index for metastatic prostate cancer targeted therapy. A biodegradable linked radiometal-targeting mAb combined with paclitaxel for radiosensitization resulted in combined modality radioimmunotherapy with modest therapeutic efficacy for aggressive prostate cancer. Prostate cancer tissue immunohistochemistry revealed hypoglycosylated MUC1 up-regulation corresponding to increased disease grade, and identified scFv-c molecules suitable for pretargeted radioimmunotherapy for prostate cancer. Implementation of pretargeted radioimmunotherapy with multivalent, bispecific molecules capable of targeting abnormal MUC1 and capturing subsequently administered radiochelates holds promise for further therapeutic index enhancement in prostate cancer radioimmunotherapy.

References

15. MacLean GD, McEwan A, Noujaim A, et al. Two novel monoclonal antibodies have potential for
24. Clinical Studies


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