Development and Characterization of Anti-Renal Cell Carcinoma × Antichelate Bispecific Monoclonal Antibodies for Two-Phase Targeting of Renal Cell Carcinoma

Marion H. G. C. Kranenborg, Otto C. Boerman, Jeannette C. Oosterwijk-Wakka, Mirjam C. A. de Weijert, Frans H. M. Corstens, and Egbert Oosterwijk

Abstract

To test a two-step approach for radioimmunotargeting of renal cell cancer, quadroma cells secreting antichelate × anti-renal cell carcinoma bispecific antibodies were obtained by somatic cell fusion. Five monoclonal antibodies against the chelate 1,4,7-triazacyclononane-1,4,7-triazaheptane-N,N',N''-pentae­
acidic acid (DTPA) were produced and characterized. Competitive binding assays indicated that the anti-DTPA antibodies reacted with DTPA chelated with indium, yttrium, chromium, iron, or zinc. The affinity constants of the anti-DTPA antibodies for 111In-DTPA ranged from 0.19 to 0.23 nM⁻¹. Using different chelates, a remarkable chelate specificity of the anti-DTPA antibodies was demonstrated. The chelates recognized by the antibodies DTPA₁, DTPA₂, and DTPA₄ share a N(N')-diacetic acid group, whereas the chelates recognized by DTPA₃ share a N'-acetic acid group, suggesting the presence of different essential structures within the DTPA molecule that determine the reactivity of the antibodies.

Five anti-DTPA antibody-producing hybridomas were used for somatic cell fusion with hybridoma G250 directed against renal cell carcinoma, resulting in three bispecific antibody-producing quadroma cell lines. The bispecific monoclonal antibodies were purified from ascites fluid using protein A affinity chromatography followed by hydroxyapatite chromatography and/or cation exchange chromatography. Of the total IgG amount present in the ascites fluid, 10–15% represented the bispecific antibodies. These bispecific antibodies will allow testing and optimization of a two-step approach for radioimmunotargeting of chelated radionuclides.

Introduction

Although radiolabeled antitumor antibodies can target tumors selectively in vivo, optimal visualization is delayed for several days, because the antibody has to clear from the blood pool and normal tissues. In addition to obscuring visualization of tumors, sustained levels of radiolabeled antibody in the blood and normal tissues increase toxicity to nontargeted tissues and limit the amount of radioactivity that can be administered in radioimmunotherapy.

The efficacy of radioimmunodetection or radioimmunotherapy might be optimized by: (a) enhancing tumor uptake; or (b) reducing background activity. The use of antibody fragments with faster blood clearance (1–3) or antiantibodies to reduce serum levels of circulating antibody (4–6) has resulted in reduction of background activity. Alternatively, reduction of background activity might be accomplished by pretargeting protocols. In these, the antitumor antibody is administered and allowed to clear from the circulation, after which the radionuclide is given as a low-molecular-weight ligand. The main advantage of these protocols is the very rapid excretion of the small ligand when not targeted to the tumor. The avid interaction between biotin and streptavidin has been exploited in such a multistep approach (7–10). Analogously, pretargeting protocols may make use of bispecific antibodies and radiometal-labeled chelates. In this ap­
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Materials and Methods

Antibodies and Chelates

Production of Protein-Chelate Conjugates. KLH (Pierce, Rockford, IL) and BSA (Sigma Chemical Co., St. Louis, MO) were conjugated with the cyclic anhydride of DTPA (Sigma) according to the method of Hnatowich et al. (17). After PD10 (Pharmacia, Uppsala, Sweden) chromatography to remove uncleaved DTPA, excess InCl₃ (Merck, Darmstadt, Germany) or YCl₃ (Aldrich Chemical Co., Milwaukee, WI) was added. Free In³⁺ or Y³⁺ was removed by PD10 column chromatography.

BSA, fibrinogen (ICN Biochemicals, Costa Mesa, CA), and calf lens protein (a kind gift from Dr. W. de Jong, University of Nijmegen, Nijmegen, the Netherlands) were conjugated with ITC-Bz-DTPA (kindly provided by Dr. G. Griffiths, Immunomedics, Morris Plains, NJ) according to the method of Ruegg et al. (18). BSA and calf lens protein were conjugated with ITC-Bz-DOTA (kindly provided by Dr. O. A. Gansow, National Cancer Institute, Bethesda, MD) according to the method of Ruegg et al. (18).

Preparation of 111In-DTPA. Five μl 111InCl₃ (Mallinckrodt, Petten, the Netherlands) and 2 μl InCl₃ (2.3 ng/μl) were diluted in 50 μl 40 mm HCl and mixed with 850 μl aqueous DTPA solution (0.05 μM/ml) and 100 μl 0.1 M sodium acetate buffer (pH 6), resulting in a final pH of 3.0. Following 30 min of incubation at room temperature, the solution was neutralized with 17 μl 0.1 M NaOH. Complexing of In³⁺ was assessed by paper chromatography on Whatman I paper with methanol-water (55:45) as an eluent. The specific activity of the 111In-DTPA was 700–800 Ci/μmol.

Anti-DTPA Antibodies. BALB/c mice were immunized with an emulsion of 50 μg In-DTPA-KLH or Y-DTPA-KLH in complete Freund's adjuvant (CFA) and boosted 3 weeks later. Sera were collected 2 weeks after the last immunization.

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2 To whom requests for reprints should be addressed.

The abbreviations used are: MAb, monoclonal antibody; DTPA, 1,4,7-triazacyclononane-N,N',N''-pentae­
cidic acid; bs, bispecific; KLH, keyhole limpet hemocyanin; ITC-Bz, isothiocyanato-benzyl; DOTA, 1,4,7,10-tetraazacyclododecane-N,N',N''-N''-tetraacetic acid.

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deficient hybridoma G250 variants were isolated by bromodeoxyuridine (Boehringer Mannheim, Germany) selection. Anti-DTPA hybridomas were fused with hybridoma G250 at a 1:1 ratio and seeded in soft agarose (30 g/liter; Sigma) containing RPMI 1640 medium with hypoxanthine-thymidine supplement (GIBCO-BRL). Growing colonies were picked, grown in low-protein hybridoma medium (GIBCO-BRL) with hypoxanthine-aminopterin-thymidine supplement (GIBCO-BRL). Growing colonies were picked, grown in suspension, and tested for the production of bsMAbs by a double-specificity ELISA. Positive clones were recloned at least twice.

**ELISAs**

In all the assays described in this section, the following conditions were used, unless indicated otherwise. Wells of 96-well microtiter plates (Costar, Cambridge, MA) were coated overnight at 4°C, and remaining protein binding sites were blocked for 1 h with 50 mM phosphate buffer (pH 7.4)/150 mM NaCl/BSA (5 g/liter). Wells were rinsed with the same buffer. Peroxidase sites were blocked for 1 h with 50 mM phosphate buffer (pH 7.4)/150 mM NaCl/BSA. Serially diluted Cr-DTPA, Fe-DTPA, Zn-DTPA, Y-DTPA, or In-DTPA-BSA were subcloned at least twice in soft agarose (30 g/liter; Sigma) supplement (GIBCO-BRL, Gaithersburg, MD).

Production of bsMAbs. Enzyme-deficient hybridomas were isolated to enable selection of fused cells (21). Hypoxanthine guanine phosphoribosyl transferase deficiency was introduced in the anti-DTPA MAb-producing hybridomas by 8-azaguanidine (Sigma) selection. The anti-renal cell carcinoma MAb G250 was used as the antitumor antibody in these studies. MAb G250 and its subclass variants are directed against the renal cell carcinoma-associated antigen G250 that is expressed homogeneously in 75% of renal cell carcinomas, whereas expression in normal tissues is restricted to gastric mucosal cells and cells of the larger bile ducts (22). MAb G250 has shown excellent targeting of renal cell carcinoma in patients (23). Thymidine kinase-deficient hybridoma G250 variants were isolated by bromodeoxyuridine (Boehringer Mannheim, Germany) selection. Anti-DTPA hybridomas were fused with hybridoma G250 at a 1:1 ratio and seeded in soft agarose containing low-protein hybridoma medium (GIBCO-BRL) with hypoxanthine-aminopterin-thymidine supplement (GIBCO-BRL). Growing colonies were picked, grown in suspension, and tested for the production of bsMAbs by a double-specificity ELISA. Positive clones were recloned at least twice.

**Direct Antigen-binding Assay.** Wells coated with 400 ng DTPA-BSA, 600 ng ITC-Bz-DTPA-B fibroblasts, or 300 ng ITC-Bz-DOTA-calf lens protein were incubated with the culture supernatant, washed, and incubated with peroxidase-conjugated rabbit antisera to immunoglobulins (Dakopatts, Glostrup, Denmark).

**Competitive Antigen-binding Assay.** Wells were coated with In-DTPA-BSA. Serially diluted Cr-DTPA, Fe-DTPA, Zn-DTPA, Y-DTPA, or In-DTPA-BSA was added simultaneously with anti-DTPA tissue culture supernatant. After 3 h of incubation, wells were washed and incubated with peroxidase-conjugated rabbit antisera to immunoglobulins (Dakopatts, Glostrup, Denmark).

**Double-Specificity ELISA.** Wells coated with 400 ng In-DTPA-BSA were incubated with peroxidase-conjugated rabbit antisera to immunoglobulins (Dakopatts, Glostrup, Denmark).

**Determination of Isotypes.** The isotypes of the anti-DTPA MAb were determined in an ELISA with mouse subclass IgG-specific antisera (Southern Biotechnology Associates, Inc., Birmingham, AL).

**Determination of Affinity Constants.** Affinity constants for 111In-DTPA were determined by Scatchard analysis (25). Microtiter wells coated with rabbit antisera to immunoglobulins (Dakopatts) were incubated with excess anti-DTPA MAb or bsMAb. Increasing amounts of 111In-DTPA were added, and after 1 h at room temperature, wells were washed and counted.

**Purification of MAbs.** Ascites fluids were cleared by centrifugation and diluted 1:1 in 3 M NaCl/1.5 M glycine (pH 8.9) and subjected to protein A affinity chromatography. Immunoglobulins were eluted by sequential application of 0.1 M sodium citrate buffers (pH 6, 5, 4, and 3).
midline kinase-deficient hybridoma G250 resulted in viable quadroma colonies. Quadromas G250 (IgG1) × DTY4, G250 (IgG1) × DTXn2, and G250 (IgG1) × DTXn1 produced bsMABs, as evidenced by reactivity in the double-specificity ELISA. Fusion of G250 (IgG2b) with DTXn3 and G250 (IgG1) with DTXn3 resulted in quadroma cells that did not produce detectable levels of bsMABs, as judged by the double-specificity assay. The IgG molecules produced by the quadroma G250 (IgG2b) × DTXn3 and G250 (IgG1) with DTXn3 resulted in quadroma cells with intact DTIn3 sites and nonfunctional G250 sites, as determined in ELISAs determining solid-phase binding to either NUH82 or BSA-DTPA using anti-IgG1 or anti-IgG2b as the tracer antibody, respectively.

Purification of Antibodies. BsMAB G250 × DTY4 was purified from ascites fluid using protein A chromatography followed by cation exchange chromatography (Fig. 2). Protein A-bound bsIgG was eluted at pH 5 (Fig. 2A) and subjected to cation exchange chromatography. Application of a LiCl gradient resulted in four protein peaks (Fig. 2B), which were analyzed for G250 × DTY4 activity. bsG250 × DTY4 antibody eluted at 223 mM LiCl. The cation exchange-purified material represented 10–15% of the IgG eluted from the protein A column. The protein peak eluting at 206 mM LiCl contained immunoglobulins with intact IgG1 × IgG2a heavy-chain pairing, as shown by a mixed-isotype ELISA, but incorrect heavy-light-chain pairing, as shown by the negative reactivity in the double-specificity assay, resulting in nonfunctional binding pockets.

bsMAB G250 × DTXn2 was purified from ascites fluid using protein A chromatography followed by hydroxylapatite chromatography and cation exchange chromatography. After protein A chromatography, the material eluting at pH 6 contained bsMAB. This material separated into four peaks on hydroxylapatite chromatography, with the bsMAB eluting at 133 mM phosphate. Additional separation of bsMAB from contaminants was achieved by cation exchange chromatography, with G250 × DTXn2 activity eluting at 165 mM LiCl. The cation exchange chromatography-purified material represented 10–15% of the initial amount of protein A-bound IgG.

BsMAB G250 × DTXn1 was purified from ascites fluid using protein A affinity chromatography followed by cation exchange chromatography. Protein A-bound bsIgG eluted at pH 5 and was subjected to cation exchange chromatography. This material separated into seven peaks, with the bsMAB eluting at 276 mM LiCl. The cation exchange chromatography-purified material represented 10–15% of the protein A-bound IgG.

Discussion

Two-step strategies using bsMABs may optimize radioimmunotargeting of tumors in vivo. The main advantage of this approach is the very rapid excretion of the low-molecular-weight radiolabeled ligand when not targeted to the tumor. Several preclinical and clinical studies have shown that two-phase targeting results in high tumor:nontumor ratios shortly after injection of the radiolabel (11–15). To investigate the possibilities of two-phase radioimmunotargeting, we have developed five hybridomas producing MABs reactive with DTPA and fused these with the hybridoma G250, producing a MAB reactive with renal cell carcinoma, to obtain bsMABs.

The affinity constants of the anti-DTPA MABs for binding 111In-DTPA were in the same order of magnitude (0.19–0.23 nM−1) as reported for other antichelate MABs (12, 26).

All anti-DTPA MABs reacted also with DTPA loaded with different metals. For all anti-DTPA MABs, with the exception of DTIn3, In-DTPA was bound most efficiently, irrespective of the immunogen used (In-DTPA-KLH or Y-DTPA-KLH). In the competitive binding assays, the affinity for binding In-DTPA was 10–100-fold higher than the affinity for binding DTPA loaded with other metals. Similar results have been obtained by Reardon et al. (26) and Bosslet et al. (27).

They showed that the affinity of their antichelate antibodies for chelates labeled with indium was at least 100-fold higher than for chelates labeled with other metals. Involvement of In3+ in the antibody-antigen interaction may cause the higher affinity of the MABs for the In3+-labeled chelates (28).

The anti-In-DTPA MABs displayed a remarkable chelate specificity. The chelates recognized by DTIn1, DTIn2, and DTIn4 (DTPA and...
ful assembly of bsMAb, factors other than the isotype of the parental cell line were important. The amount of IgG present in the ascites fluid. This is in concordance with previous reports that the production of functional bsMAb is dependent on the choice of the parental cell line. Therefore, it seems that for successful assembly of bsMAb, factors other than the isotype of the parental cell line are important.

Individual purification protocols were developed for every bsMAb. bsMAb G250 × DT4 was purified using protein A affinity chromatography followed by cation exchange chromatography. The amount of bsMAb obtained after cation exchange chromatography was 10–15% of the total amount of IgG present in the ascites fluid. bsMAb G250 × DTIn3 was purified using protein A affinity chromatography followed by hydroxylapatite chromatography and cation exchange chromatography. Similarly, bsMAb G250 × DTIn2 also represented 10–15% of the total amount of IgG present in the ascites fluid. After protein A affinity chromatography and cation exchange chromatography, bsMAb G250 × DTIn1 was shown to be 10–15% of the amount of IgG present in the ascites fluid. This is in concordance with total random association of the two heavy and light chains (provided that the heavy and light chains of both parents are expressed co-dominantly) as suggested by Suresh et al. (29).

We have developed five anti-DTPA MAbs reactive with a broad range of chelated metals. Fusion of the anti-DTPA-producing hybridomas with the G250 hybridoma resulted in three quadrums cell lines producing bsMAbs. These purified bsMAbs will allow testing and optimization of a two-phase targeting protocol in nude mice models.

References