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ABUNDANT TYROSINE RESIDUES IN THE ANTIGEN BINDING SITE IN ANTI-OSTEOSARCOMA MONOCLONAL ANTIBODIES TP-1 AND TP-3

Application to radiolabeling

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The variable (V) genes of TP-1 and TP-3 MAbs have been cloned and sequenced. Because of the potential use of these antibodies in the diagnosis and treatment of osteosarcoma, it is important to determine the presence and position of amino acid residues that may react with radiolabeling within the V domains. In this article, location of the tyrosine residues is determined using the knowledge of immunoglobulin structures in general. The TP-1V domains have a total of 19 tyrosines, whereas TP-3V domains have 18, with approximately half of these located within complementarity determining regions (CDRs). Thus, if equal reactivity of all tyrosines is assumed, smaller fragments of MAbs have a high probability of being radiolabeled at one of these sites with possible resultant loss of antigen binding.

Osteosarcoma (OS) is the most common malignant primary tumor of bone. When treated with surgery alone, OS has a poor prognosis due to micrometastases present at the time of primary diagnosis. Neoadjuvant chemotherapy has dramatically improved the outcome (1). However, 40% of these young patients still succomb to the disease due to regrowth of chemotherapy-resistant tumor cells. Most importantly, OS patients belonging to the 'poor prognostic group' can be identified early in the course of the disease, when they still have a low tumor burden. Hence, adjuvant immunotherapy and targeted radiotherapeutic strategies can be explored as alternative treatments, and studies with novel agents, such as radiolabeled monoclonal antibodies (MAbs) (2) and the macrophage stimulating agent muramyl tripeptide encapsulated into liposomes (3), are ongoing.

MAbs are ideal as carrier molecules for radionuclides because of their specificity and affinity for their target antigen. The murine MAbs TP-1 (γ2a) and TP-3 (γ2b) (4) recognize two different epitopes on a tumor-associated antigen expressed on both canine and human OS cells (5, 6). The tumor targeting of radiolabeled TP antibodies has been examined in vivo in various animal models (7–10) and in patients suffering from OS (11). Specific tumor uptake was observed in both primary and metastatic OS. Thus, the TP antibodies seem promising as targeting vehicles for various radioisotopes suitable for detection and treatment of OS.

The labeling of MAbs with either metal or halogen radionuclides involves coupling to reactive side-chains of amino acids such as lysine ε amino groups, or direct substitution on tyrosine residues. Reactive residues are less frequent in smaller MAb fragments, such as Fab ($V_L - C_L$/$V_H - C_H1$) and scFv ($V_L - V_H$). These fragments have a more rapid blood clearance and a high tumor:blood ratio (12, 13) combined with improved tumor penetration. In particular, scFv can penetrate into tumors more rapidly than larger molecules (14), and therefore may be preferred in the treatment of solid tumors.

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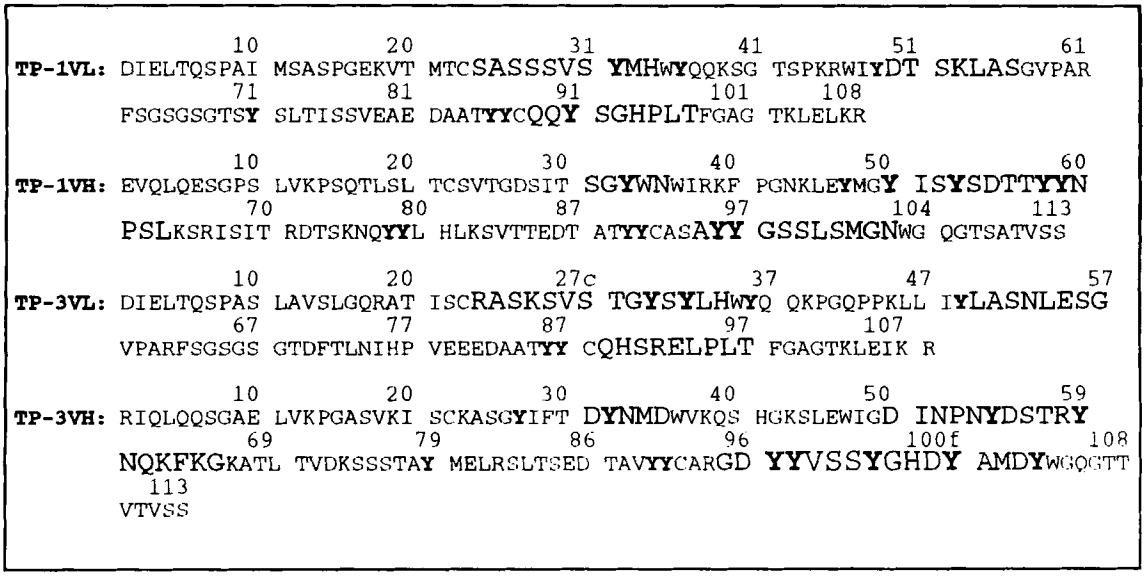

Fig. 1. Protein sequences of TP-1 and TP-3 heavy and light chain variable domains. CDR sequences are indicated by larger letters and tyrosine residues are highlighted. The numbering is in accordance with Kabat et al. (21). The amino acids are written as one-letter symbols.

The number of domains in MAb fragments is much less than in an intact IgG, which has 12 domains as opposed to 2 in scFv. Assuming an equal number of reactive residues in each domain, the number of reactive groups and the probability for labeling will decrease accordingly, that is, by a number of six. Thus, it is essential to know the positions of the available reactive residues involved in radiolabeling. This is particularly important for MAb fragments, as modification of amino acids in the complementarity determining regions (CDRs) may affect the antigen recognition.

Several conjugation strategies have been developed which have been reviewed by Pietersz et al. (15), whereas, in this context, the proteins to be labeled have received little attention. Many immunoglobulins have been well characterized on both the DNA and protein levels, and to this date the crystal structure has been solved for 52 immunoglobulins (PDB entry: immunoglobulins) obtainable from Brookhaven and Leeds crystallographic databases. This knowledge can be utilized in predicting the accessibility of individual amino acids for labeling. Also, the likelihood of interference of the attached label with antigen recognition can be deduced from this information. Since it is not possible to direct the label to a particular amino acid at a specific location on the molecule, evaluation of accessibility and interference with antigen binding prior to labeling smaller MAbs fragments is of importance.

Previously, we have reported the cloning and sequencing of the TP-variable genes, focusing on the location and accessibility of the lysine residues (16). Herein, we investigate the positions of the tyrosine residues in the TP-variable domains and predict their accessibility for labeling. This is of direct relevance to our ongoing clinical studies which utilize these MAbs following radioiodination of tyrosine residues (11).

Material and Methods

Amplification of the TP-variable genes

Preparation of mRNA from hybridomas and first-strand cDNA synthesis were performed as described by the manufacturer (Invitrogen). cDNA was amplified with 'universal primers' (17) by Taq polymerase (Promega) in a polymerase chain reaction (PCR) at the following temperatures: melting at 94°C, 45 s, primer annealing at 50°C, 1 min, and primer extension at 72°C, 1 min. Owing to low yields, reamplification of purified (Wizard DNA Clean Up System, Promega) PCR product was performed with Vent polymerase (New England Biolabs) using an annealing temperature at 60°C. In both reactions, 30 rounds of temperature cycling were performed in a Hybaid Thermal Cycler.

Cloning, sequencing and sequence analysis

Purified reamplified PCR products were ligated into M13mp18/19 (18), and transformed into Ca-competent *E. coli* MV1190 (BioRad). A single-stranded template was prepared from clones containing the insert, and sequenced (19). The TP-variable gene sequences were analysed using the UVGCG programme (20), confirming that they were V gene segments, and translated into amino acids. On the basis of the primary sequence, CDRs and FRs were iden-

Table

Position of tyrosine residues. The numbering is in accordance with Kabat et al. (21). The frequency percentages for tyrosines in other V gene sequences of same subgroups are indicated in parentheses. Residues at 95% frequency and above are conserved

Subgroup	TP-1V_H 1(A)	TP-1V_L VkVI	TP-3V_H II(A)	TP-3V_L VkIII
FR1	–	–	27(97%)	–
CDR1	33(95%)	32(97%)	32(89%)	30(30%)
	–	–	–	32(42%)
FR2	47(24%)	36(85%)	–	36(80%)
	–	49(96%)	–	49(84%)
CDR2	50(84%)	–	54(2%)	–
	53(87%)	–	59(97%)	–
	58(50%)	–	–	–
	59(83%)	–	–	–
FR3	78(45%)	71(91%)	79(90%)	86(100%)
	79(22%)	86(99%)	90(99%)	87(78%)
	90(100%)	87(99%)	91(79%)	–
	91(98%)	–	–	–
CDR3	96(14%)	91(1%)	97(41%)	
	97(22%)	–	98(29%)	–
	–	–	100b(19%)	–
	–	–	100f	–
	–	–	102(80%)	–
FR4	–	–	–	–

tified as described (21) (Fig. 1), and the subgroups to which the TP-sequences belonged were determined.

Results

The TP-variable (V) regions were analysed with regard to the location of tyrosine residues (Fig. 1). A total of 19 tyrosines are present in the TP-1 V regions, whereas 18 tyrosines can be found in the TP-3 V regions (Table). The location of tyrosines was further examined with regard to the canonical structure model (22, 23), which describes a limited number of main-chain conformations for five of the six antigen-binding loops, excluding the third region in V_H domains, their size being different from those of CDRs.

The tyrosine residues in complementarity determining regions (CDRs)

The TP-1 V domains have a total of 9 tyrosines within the CDRs, 5 being within the limits of the canonical structures. These are Tyr-32 and 91 in the V_L chain, and Tyr-53, 96 and 97 in the V_H chain.

In contrast, the TP-3 V domains have a total of 10 tyrosines within the CDRs, of which 8 are within the limits of the canonical structures. These are Tyr-30 and 32 in the V_L chain, and Tyr-32, 54, 97, 98, 100b and 100f in the V_H chain of which 4 are within the limits of the third V_H loop. In addition, Tyr-27 in framework region 1 (FR1) is part of the first hypervariable loop in the V_L domain in the canonical structure model.

The primary sequence of TP-1V_H shows great homology to HyHEL-10, an anti-hen eggwhite lysozyme antibody (24), of which the crystal structure of the antibody complexed with antigen (25) has been analysed. The analysis of the complex shows that Tyr-50, 58 and 59 in CDR2 are in contact with both the antigen and the opposite domain (26). Tyr-50 is almost completely buried, whereas Tyr-58 and 59 are partially or mostly exposed. Hence, we assume this to be true for the tyrosines at the same positions in the TP-1V_H chain. Also, a tyrosine at position 33 is present in the TP-1V_H sequence in CDR1. Residues at this position are observed to be almost completely buried (26).

In the TP-3V_H domain, Tyr-59 in CDR2 and Tyr-102 in CDR3 may or may not be in contact with the opposite domain. The observation is that Tyr-59 is mostly buried, whereas Tyr-102 is mostly exposed (26). Thus, it appears that Tyr-50 and 33 in the TP-1V_H domain and Tyr-59 in the TP-3V_H domain would not be readily accessible for labeling.

The tyrosine residues in framework regions (FRs)

The TP-V_L chains have both Tyr-36 and 49 in FR2 and Tyr-86 and 87 in FR3. In addition, TP-1 has a tyrosine residue at position 71 in FR3. Tyr-36, 49 and 87 are involved in the V_L–V_H interaction (27). They may therefore be partially buried, and not so accessible for labeling. Tyr-71 and 86 are both, according to Padlan (27), inward-pointing or buried framework residues. The residue at position 71, most commonly Tyr or Phe, is in contact with the residue at position 29 of the first hypervariable loop in the V_L domain (22).

The TP-V_H chains have Tyr-79, 90 and 91 in FR3. In addition, TP-1 has a Tyr-47 in FR2 and a Tyr-78 in FR3. According to Padlan (27), Tyr-78 and 90 are buried, whereas Tyr-47, 79 and 91 may or may not be involved in the V_L–V_H interaction. In known three-dimensional structures, the residues at positions 47 and 91 are always implicated in this interaction (27). The tyrosine at position 79 is not reported to be buried and may therefore be accessible for labeling.

Discussion

Iodine-131 remains the most frequently used radionuclide for radioimmunotherapy. To date, all clinical studies have involved electrophilic substitution methods which result in the positioning of the iodine ortho to the hydroxyl group on tyrosine residues (28). Because of the complexity of antibody molecules, the relative reactivity of each tyrosine to iodination has not been measured directly. However, studies with smaller proteins indicate that 50- to 100-fold differences in tyrosine iodination are probably not uncommon (29, 30). The relative reactivity of tyrosine residues reflects their microenvironment on the antibody

molecule, with proximity to bulky amino acids and hydrophobic domains, as well as accessibility to solvent being critical factors.

The current study suggests that about 50% of the tyrosines in the TP-V regions are within the CDRs which form the antigen-binding site. Most of these are exposed, with the exception of Tyr-50 and 33 in the TP-1V_H chain and Tyr-59 in the TP-3V_H chain, and are probably readily labeled as opposed to those present in the FRs in antibody fragments. Also, according to the canonical structure model, the residues forming the loops are involved in antigen binding (22) and iodine labeling of these tyrosines will presumably interfere with antigen binding. This may have an undesirable effect on the specificity of the molecule, rendering it incapable of being used a a targeting molecule. Indeed, iodine labeling of tyrosines in TP-$F(ab')_2$ fragments has been performed, reducing the immunoreactivity to 50%, whereas intact TP-MAbs have about 70% immunoreactivity (7), thus lending support to our observations.

From the analysis, it appears that most of the tyrosine residues found within the TP sequences are usually present in other sequences of the same subclasses (Table). We sought to explore whether this was generally the case among all the other subclasses of the murine immunoglobulin V genes. Examination of the different subclasses revealed that V_L sequences contain an average of six tyrosines, distributed in the following manner: one in CDR1, one in CDR3 and Tyr-36, 49, 86 and 87. The V_H sequences contain an average of 12 tyrosines, which are distributed in the following manner: one or two in CDR1, one to four in CDR2, several in CDR3 and Tyr-79, 90 and 91.

The fact that all the CDRs, apart from CDR2 in the variable light chains, have tyrosine residues, implies that this residue is important in the antigen binding. Tyrosine, as well as serine, threonine, methionine and tryptophan, contains one dipole creating non-covalent interaction which mediates the mutual recognition of complementary molecular surfaces (lock and key system) (31). In this regard, it is important to note that the pK value of the phenolic hydroxyl on tyrosine is decreased as a consequence of ortho substitution of radioiodine for hydrogen. The magnitude of this effect depends on the nature of the protein. For example, in thyroglobulin (isoelectric point 4.7) the apparent pK values for tyrosine and monoiodotyrosine are 11.35 and 9.3 respectively, while in lysosome (isoelectric point 11.1) pK values of 10.5–12.8 and 7.9 respectively, are observed (32). Thus, by altering the pK values of phenolic hydroxyl groups, radioiodination could interfere with the ability to form hydrogen bonds and compromise interaction with complementary cationic functionalities.

Other neutral polar residues which are capable of forming hydrogen bonds are serine, threonine, asparagine and glutamine, of which serine and threonine are well represented in the CDRs of the TP-MAbs. As internal residues, they usually form hydrogen bonds with each other or with the polypeptide backbone (31).

Our previous study on the location of the lysine residues reveals that only one lysine, Lys-27 in TP-3V_L, was within the limits of the canonical structures (16). If labeling of this lysine residue affects the targeting properties of the molecule, it can readily be exchanged for a different amino acid. In the case of tyrosines, there are too many present in the loops, making it difficult to identify which labeled tyrosine affects the specificity. Also, exchanging tyrosines with other residues will probably affect the specificity of the molecule because of alterations in hydrogen formation, when there are so many present in the loops. Hence, we believe that labeling of the lysine residues, as opposed to tyrosines, is a more sensible approach.

In conclusion, by using the knowledge of immunoglobulin fine structure, we made predictions concerning the position of reactive residues accessible for labeling. About 50% of the tyrosines are located in the CDRs, making them highly susceptible to labeling, whereas those in FRs are often inaccessible. The predictions made for the position of tyrosine residues in TP-1 and TP-3 are valid for other murine immunoglobulins as well, and indicate that reactions involving tyrosines may not be suitable for labeling of smaller MAb fragments. In tumor-targeting it is essential to retain high affinity for the antigen, thus enabling the fragments to bind quickly to their target as otherwise they will be disposed of rather quickly. By using Fab and scFv, the affinity to the target is already reduced because they are univalent. Hence, further reduction of affinity and specificity due to potentially avoidable radiolabeling factors should be avoided.

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REFERENCES

1. Link MP, Goorin Am, Horowitz M, et al. Adjuvant chemotherapy of high-grade osteosarcoma of the extremity. Updated results of the Multi-Institutional Osteosarcoma Study. Clin Orthop 1991; 270: 8–14.
2. Bruland ØS, Pihl A. Immunoscintigraphy and radioimmunotherapy. Useful approaches in the management of osteogenic sarcoma? In: Novak J, ed. Frontiers of osteosarcoma research. Hogrefe & Huber (ISBN 0-88937-113-x), 1993: 149–59.
3. Kleinerman ES, Maeda M, Jaffe N. Liposome-encapsulated muramyl tripeptide: a new biologic response modifier for the treatment of osteosarcoma. Cancer Treat Res 1993; 62: 101–7.
4. Bruland ØS, Fodstad Ø, Funderud S, Pihl A. New monoclonal antibodies specific for human sarcomas. Int J Cancer 1986; 38: 27–31.

5. Bruland ØS, Fodstad Ø, Stenwig AE, Pihl A. Expression and characteristics of a novel human osteosarcoma-associated cell surface antigen. Cancer Res 1988; 48: 5302–9.
6. Haines DM, Bruland ØS. Immunohistochemical detection of osteosarcoma-associated antigen in canine osteosarcoma. Anticancer Res 1989; 9: 903–7.
7. Bruland ØS. Fodstad Ø, Skretting A, Pihl A. Selective localisation of two radiolabeled antisarcoma monoclonal antibodies in human osteosarcoma xenografts. Br J Cancer 1987; 56: 21–5.
8. Fjeld JG, Bruland ØS, Benestad HB, Schjerven L, Stigbrand T, Nustad K. Radio-immunotargeting in immunocompetent animals using intraperitoneal diffusion chambers containing human tumor cells. Br J Cancer 1990; 62: 573–8.
9. Haines DM, Bruland ØS, Matte G, Wilkinson AA, Meric SM, Fowler JD. Immunoscintigraphic detection of primary and metastatic spontaneous canine osteosarcoma with $F(ab')_2$ fragments of osteosarcoma-associated monoclonal antibody TP-1. Anticancer Res 1992; 12: 2151–7.
10. Page RL, Garg PK, Garg S, Archer GE, Bruland ØS, Zalutsky MR. PET imaging of osteosarcoma in dogs using a fluorine-18-labeled monoclonal antibody Fab fragment. J Nucl Med 1994; 35: 1506–13.
11. Bruland ØS, Fodstad Ø, Aas M, et al. Immunoscintigraphy of bone sarcomas—results in 5 patients. Eur J Cancer 1994; 30A, 1484–9.
12. Colcher D, Bird R, Roselli M, et al. In vivo tumor targeting of a recombinant single-chain antigen-binding protein. J Natl Cancer Inst 1990; 82: 1191–7.
13. Adams GP, McCartney JE, Tai MS, et al. Highly specific in vivo tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single-chain Fv. Cancer Res 1993; 53: 4026–34.
14. Yokota T, Milenic DE, Whitlow M, Schlom J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res 1992; 52: 3402–8.
15. Pietersz GA, Rowland A, Smyth MJ, McKenzie IF. Chemoimmunoconjugates for the treatment of cancer. Adv Immunol 1994; 56: 301–87.
16. Olafsen T, Bruland ØS, Zalutsky MR, Sandlie I. Cloning and sequencing of V genes from anti-osteosarcoma monoclonal antibodies TP-1 and TP-3: Location of lysine residues and implications for radiolabeling. Nucl Med Biol 1995; 22: 765–71.
18. Orlandi R, Güssow DH, Jones PT, Winter G. Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. Proc Natl Acad Sci USA 1989; 86: 3833–7.
18. Messing J. New M13 vectors for cloning. Methods Enzymol 1983; 101: 20–78.
19. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74: 5463–7.
20. Devereux J, Haeberli P, Smithies O. A comprehensive set of sequence analysis programs for the VAX. Nucl Acids Res 1984; 12: 387–95.
21. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. Sequences of proteins of immunological interest. 5th ed., Publich Health Service, NIH Washington DC, 1991.
22. Chothia C, Lesk AM. Canonical structures for the hypervariable regions of immunoglobulins. J Mol Biol 1987; 196: 901–17.
23. Chothia C, Lesk AM, Tramontano A, et al. Conformations of immunoglobulin hypervariable regions. Nature 1989; 342: 877–83.
24. Smith-Gill SJ, Mainhart C, Lavoie TB, Feldman RJ, Drohan W, Brooks BR. A three-dimensional model of an antilysozyme antibody. J Mol Biol 1987; 194: 713–24.
25. Padlan EA, Silverton EW, Sheriff S, Cohen GH, Smith-Gill SJ, Davies DR. Structure of an antibody-antigen complex: crystal structure of the HyHEL-10 Fab-lysozyme complex. Proc Natl Acad Sci USA 1989: 86: 5938–42.
26. Abergel C, Tipper JP, Padlan EA. Structural significance of sequence variability in antibody complementarity-determining regions. Res Immunol 1994; 145: 49–53.
27. Padlan EA. Anatomy of the antibody molecule. Mol Immunol 1994; 31: 169–217.
28. Zalutsky, MR. Radiohalogenation of antibodies: chemical aspects. In: Srivastava SC, ed. Radiolabeled monoclonal antibodies for imaging and therapy. New York: Plenum Press, 1988: 195–212.
29. Seon BK, Roholt OA, Pressman D. Reactivity of tyrosine residues in the constant portion of type K Bence Jones protein. Biochim Biophys Acta 1970; 200: 81–8.
30. Dube SK, Roholt OA, Pressman D. Relative reactivity to iodination of tyrosine residues in alpha-chymtrypsin. J Biol Chem 1966; 241: 4665–9.
31. Schulz GE, Schirmer RH. Principles of protein structure. New York: Springer-Verlag, 1978.
32. Wolff J, Covelli I. Iodination of the normal and buried tyrosyl residues of lysozyme II. Spectrophotometric analysis. Biochemistry 1996; 5: 867–71.