Identification of glioma-associated antigen MUC 2-63 as CD44

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Summary Monoclonal antibody MUC 2-63 recognises neurogenic tumours and has been used successfully for radioimaging human malignant gliomas. We now show that the MUC 2-63 antigen has the same tissue distribution and molecular weight range as the CD44 antigen and confirm the identity of these two molecules in blocking studies using MUC 2-63 and the CD44 anti-framework antibody F10-44-2. Thus not only MUC 2-63 but also other anti-CD44 monoclonal antibodies should prove useful in imaging and, perhaps, therapy of brain tumours.

The CD44 molecule was originally defined by the monoclonal antibody F10-44-2 and found predominantly on lymphohaemopoietic tissues and brain (Dalchau et al., 1980; Stoll et al., 1989). In man the CD44 antigen is expressed on T and B lymphocytes, granulocytes, monocytes/macrophages and the majority of erythrocytes (reviewed in Haynes et al., 1989; Stamenkovic et al., 1989). It is acquired by medullary thymocytes during T-cell maturation in the thymus and is up-regulated on memory T cells (Dalchau et al., 1980; Haynes et al., 1983; Sanders et al., 1988). CD44 is also present at low levels on normal epithelium, but is highly expressed on carcinomas, including those of the colon (Daar & Fabre, 1983; Stamenkovic et al., 1989). In human brain the molecule has a molecular weight of 90 kDa, while a lymphoid form of 80–90 kDa (CD44H) and an epithelial form of 160 kDa (CD44E) have been described (McKenzie et al., 1982; Stamenkovic et al., 1989, 1991; Brown et al., 1991). The differences between these and other isoforms, ranging in molecular weight from 85 to 250 kDa, lie in the membrane-proximal region and result from alternative splicing of at least ten different exons and from post-translational modifications (Brown et al., 1991; Jackson et al., 1992; Screaton et al., 1992; Tolg et al., 1993).

Many functions involving cell–cell and cell–extracellular matrix interactions have been attributed to CD44, and are likely to be mediated by different isoforms (Belitsos et al., 1990; Stamenkovic et al., 1991; Jalkanen & Jalkanen, 1992). Such interactions play a role in cell development, activation and migration (Berg et al., 1989; Miyake et al., 1990; Ritter & Crispe, 1992; Haegel et al., 1994). Differential expression of CD44 variants has been observed on some epithelial, neuronal and lymphoid tumour cells compared with their normal counterparts, and between metastatic and non-metastatic tumours; this may prove useful in diagnosis and disease evaluation (Matsumura & Tarin, 1992; Koopman et al., 1993; Salmi et al., 1993). Moreover, there is compelling evidence in support of a role for CD44 in tumorigenesis in the rat, in which expression of the p-meta-1 splice variant confers metastatic potential on tumour cells that were previously non-metastatic (Günthert et al., 1991).

The monoclonal antibody MUC 2-63 was one of a panel of antibodies raised to human glioma cells in an attempt to generate antibodies useful for the typing of brain tumours. It reacted with a cell-surface molecule on gliomas, neuroblastomas and melanomas, as well as with embryonic and fetal brain, but did not recognise any of the non-neurogenic tumour cell lines studied (including breast, gastric and colonic carcinoma) and did not bind to normal brain apart from a few cells bordering on the tumour tissue (Stavrou et al., 1987). Radiolabelled MUC 2-63 was subsequently successfully used in vivo for imaging of glioma (Bergh et al., 1990;

Stavrou et al., 1991). Although preliminary biochemical analysis of the MUC 2-63 antigen yielded molecular weights from 80 to 190 kDa, the exact nature of the molecule detected by MUC 2-63 was not known (Stavrou et al., 1990).

We recently included MUC 2-63 in a panel of antibodies that was used to study tumours of the skin and colon. Surprisingly, the antigen detected by MUC 2-63 was found to be present throughout all the tissues analysed. We have therefore analysed further the expression of this antigen in both tumour and normal tissues using immunohistochemical and flow cytometric techniques and have determined the molecular weight of the molecule by Western blotting. Our data show a striking similarity to data published for the CD44 antigen. Subsequent blocking experiments with MUC 2-63 and the anti-CD44 antibody F10-44-2 confirmed the specificity of MUC 2-63 as an anti-CD44 antibody.

Materials and methods

Tissues

Human colon and skin biopsies were from the Royal Sussex County Hospital, Brighton, tonsils were from St Mary's Hospital, London, and paediatric thymus samples were obtained from children undergoing cardiac surgery at Great Ormond Street Hospital, London. Tissues for immunohistochemistry and biochemistry were snap frozen and stored in liquid nitrogen. Cryostat sections were cut at 6 μm, dried overnight, fixed for 10 min in absolute acetone, and either used immediately or stored at −20°C until use. For flow cytometric analysis, fresh thymus and tonsil were teased into single-cell suspension in phosphate-buffered saline (PBS) and washed before use. To obtain peripheral blood mononuclear cells (PBMCs), 10 ml of blood containing 200 units of heparin (Flow, UK) was layered over Ficoll-Hypaque (Sigma, UK) and centrifuged at 2,000 r.p.m. for 20 min at 4°C. The interface PBMCs were removed and washed twice before immunostaining.

Antibodies

Primary antibodies were all mouse monoclonal antibodies, used either as tissue culture supernatant or as purified Ig, diluted in Tris-buffered saline (TBS) at a concentration determined by prior titration. MUC 2-63 is an IgG1 monoclonal antibody (Stavrou et al., 1987). Two independent preparations were produced from separate cell stocks and used as supernatant (London) and purified IgG (Hamburg); these gave identical results in all experimental systems. Antibodies for FACS analysis were: anti-CD3 (1:10; Dakopatts, Denmark), anti-CD22 (1:10; Dakopatts), anti-macrophage (1:10; Dakopatts) and fluorescein-conjugated anti-CD44 (CD44-FITC, 1:5; Serotec, UK). An irrelevant isotype-matched

antibody was used as negative control in immunostaining. MR6, an IgG1 antibody that detects a 200 kDa molecule on the surface of most thymocytes, tonsillar lymphocytes and PBMCs, was used as the control for the blocking studies (Larché *et al.*, 1988). The secondary antibody for immunohistochemistry was peroxidase-conjugated rabbit anti-mouse Ig (RAM-PX, 1:20; Dakopatts). For FACS analysis, RAM-FITC (1:20; Dakopatts) was used.

Immunolabelling

Tissue sections were stained by either indirect immunoperoxidase or immunofluorescence techniques (De Maagd *et al.*, 1985; Mat *et al.*, 1990). For suspension analysis, 1 × 10⁶ cells were labelled by indirect immunofluorescence and analysed by flow cytometry (EPICS Profile, Coulter, USA) (Larché *et al.*, 1988). RAM-PX and RAM-FITC secondary reagents were preincubated in 5% human serum to remove cross-reactivity with endogenous human Ig.

SDS–PAGE and Western blotting

Cryostat sections (10 × 9 μm) were lysed in 50 μl of lysis buffer (10 mM Tris–HCl, pH 7.2, 0.15 M sodium chloride, 0.5% Nonidet P-40, 1 mM PMSF) for 15 min at 4°C. Lysates were centrifuged at 13,000 g for 4 min at 4°C. Supernatant proteins were then separated on a 7.5% polyacrylamide gel (SDS–PAGE; Laemmli, 1970) and analysed by Western blotting using MUC 2-63, followed by RAM-PX and the substrate diaminobenzidine (DAB) (Towbin *et al.*, 1979; Larché *et al.*, 1988).

Results

Immunohistochemistry

On thymus sections MUC 2-63 showed strong staining of all medullary thymocytes and small scattered groups of cortical thymocytes (Figure 1b). It also labelled blood vessels and Hassall's corpuscles, but no other thymic epithelium. On tonsil sections MUC 2-63 strongly stained B- and T-cell areas, blood vessels, epithelial cells and follicular dendritic cells; however, the germinal centre region stained less intensely than the outer areas of the follicle (Figure 1d).

In all seven normal and adenoma colon samples tested MUC 2-63 gave moderate to strong staining of both epithelium and lamina propria. Seven of the nine colonic carcinomas were also moderately to strongly MUC 2-63 positive, while in two the epithelium was only weakly positive (Table I). Similarly, all normal skin and naevi samples and 11 of 14 basal cell carcinomas (BCCs) showed strong staining with MUC 2-63, the remaining three being only weakly positive (Table I, Figure 1f). In eight of the BCCs only a proportion of the tumour cells were positive (~50%).

The staining patterns seen with anti-CD44-FITC were indistinguishable from those given by MUC 2-63 (Figures 1a, c and e).

Western blotting analysis of the MUC 2-63 antigen

Thymus lysates gave a single band at ~90 kDa. Normal colon gave a major band at ~96 kDa and a minor band at ~137 kDa which was not visible on all blots, probably because of differences in the amount of protein loaded (Figure 2). Similarly, colon adenoma showed a major (~180 kDa) and a minor (~90 kDa) band, while colonic carcinoma gave bands at ~150 kDa (major component) and ~90 kDa (minor component). Since the ~90 kDa bands on both adenoma and carcinoma were weak they could represent breakdown product of the larger, major, band; alternatively, they could represent infiltrating leucocytes. BCC also gave two bands, at ~96 kDA and ~143 kDa (not shown).

Table I Analysis of MUC 2-63 antigen expression in tumours of skin and colon

| Tissue (n)ᵃ | Labelling with MUC 2-63 monoclonal antibody | | |
	Epithelium	Dermis	Lamina propria
Normal skin	+ + +	+ + +	N/A
Naevus (3)	+ + +	+ + +	N/A
BCC (14)	+ +/+ + +	+ +/+ + +	N/A
Normal colon (3)	+ +	N/A	+ +
Colonic adenoma (6)	+ +/+ + +	N/A	+ +/+ + +
Colonic carcinoma (9)	+ /+ +/+ + +	N/A	+ +/+ + +

ᵃNumber of samples analysed. +, + +, + + + denotes intensiy of labelling (weak, moderate, strong). N/A, not applicable.

Flow cytometric analysis

The surface expression of the MUC 2-63 antigen on thymocytes, tonsillar leucocytes and PBMCs was analysed by flow cytometric analysis. Anti-CD3 (T lymphocytes), anti-CD22 (B lymphocytes) and anti-macrophage antibodies were used to define leucocyte subpopulations. MUC 2-63 stained the majority of cells in all three cell preparations (Table II), in agreement with the data obtained with tissue sections.

Since the cellular distribution and biochemical characteristics of the MUC 2-63 antigen were strikingly similar to those previously described for CD44, this raised the possibility that MUC 2-63 was an anti-CD44 antibody. To test this hypothesis, blocking experiments were performed using MUC 2-63 and the anti-CD44 monoclonal antibody F10-44-2 (CD44-FITC). Preincubation of cells with MUC 2-63 completely inhibited the subsequent binding of CD44–FITC (Table II). An isotype-matched control monoclonal antibody, MR6, which bound to >50% of the cells in each of the preparations used, had no effect on CD44–FITC binding. Similar antibody blocking data were obtained with tumour tissue using MUC 2-63 followed by CD44–FITC on frozen sections of basal cell carcinoma (Figure 1g and h).

Discussion

Monoclonal antibody MUC 2-63 was one of several reagents raised for typing brain tumours (Stavrou *et al.*, 1987). In this
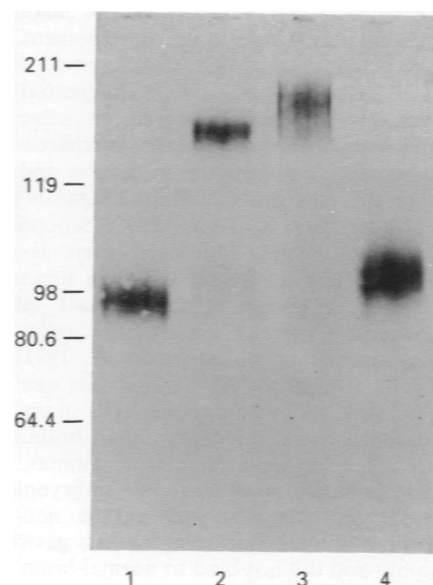

Figure 2 Western blotting analysis of MUC 2-63 antigen. Tissue lysates were run on a 7.5% SDS–PAGE gel, blotted onto nylon membrane and stained with MUC 2-63 followed by peroxidase-conjugated rabbit anti-mouse Ig and DAB. Lane 1, normal thymus; lane 2, colon carcinoma; lane 3, colon adenoma; lane 4, normal colon.
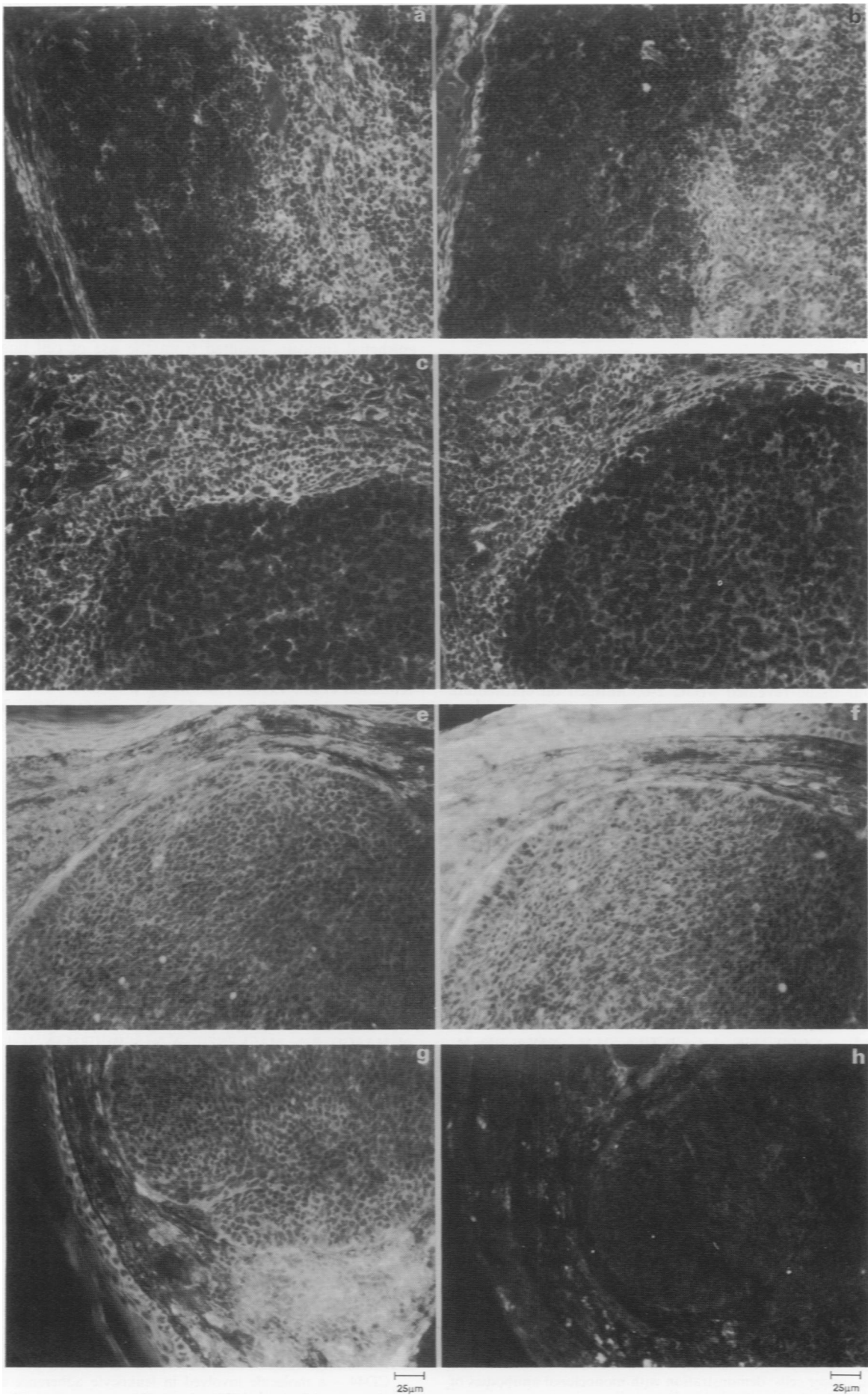
Figure 1 a-f, Immunofluorescent labelling with antibodies MUC 2-63 (b, d and f) and anti-CD44 (a, c and e) showing indistinguishable staining patterns on frozen sections of thymus (a and b), tonsil (c and d) and basal cell carcinoma (e and f). MUC 2-63 binding was detected in indirect immunofluorescence using FITC-conjugated rabbit anti-mouse Ig. CD44 was detected either by indirect immunofluorescence (a, c and e) or by direct immunofluorescence using FITC-anti-CD44 (g and h). Preincubation with MUC 2-63 blocks the subsequent binding of FITC-anti-CD44 (h), indicating that the two antibodies recognise the same target antigen. Magnification bar: 25 μm.

Table II Reactivity of monoclonal antibody MUC 2-63 with leucocytes from thymus, tonsil and PBMC and its ability to block binding of CD44-FITC

Antibody	Tonsil	PBMC	Thymus
Negative control	5.1[a]	1.5	2.7
Anti-CD22 (B cells)	55.0	9.6	2.0
Anti-CD3 (T cells)	39.3	39.6	67.3
Anti-macrophage	5.3	1.4	2.6
MUC 2-63	88.8	74.8	72.9
MR6	57.1	ND	55.7
Anti-CD44	64.5	64.7	26.9
MUC 2-63 anti-CD44[b]	1.3	0.0	0.9
MR6 anti-CD44[c]	69.4	62.9	34.7

[a]Percentage positive cells by flow cytometric anaylsis. [b]Cells were preincubated with MUC 2-63 prior to addition of anti-CD44-FITC antibody. [c]Cells were preincubated with MR6 prior to addition of anti-CD44-FITC antibody.

original study it was shown to react with gliomas, neuroblastomas and melanomas, but not with any of the non-neurogenic tumours tested. MUC 2-63 was subsequently successfully used in in vivo radioimaging (Bergh et al., 1990).

We included MUC 2-63 in a recent study of epithelial tumours of skin and colon as part of an ongoing analysis of epithelial antigen expression during tumorigenesis (Mat et al., 1990, 1993). All antibodies in this study were tested on sections of human thymus as a positive control for the immunoperoxidase staining. Surprisingly, MUC 2-63 strongly labelled small groups of cortical and all medullary thymocytes, suggesting that the MUC 2-63 antigen is acquired with T-lymphocyte maturation. This was further analysed in tonsil and PBMC preparations, in which MUC 2-63 was found to label mature T and B lymphocytes and macrophages. Analysis of tumour tissue revealed MUC 2-63 antigen on epithelium, connective tissue and infiltrating leucocytes in all samples studied, although there was some variation in the intensity and in the proportion of epithelial cells within a tumour that were labelled. The molecular weight of the molecule recognised by MUC 2-63 in these tissues was analysed by Western blotting on thymus, colon and skin lysates. These experiments showed that the antigen is expressed in two main molecular weight forms of ∼90 kDa and ∼150 kDa.

These molecular weight data were strongly reminiscent of data previously published for CD44, with a lymphoid form of 80-90 kDa and an epithelial form of 160 kDa resulting from alternative exon splicing (Stamenkovic et al., 1989; Jackson et al., 1992; Tolg et al., 1993). In addition to these two cell-specific isoforms, several minor forms ranging in molecular weight from 50 kDa to 200 kDa and variably present on different cell types have been described (Stamenkovic et al., 1989; Günthert et al., 1991; Jackson et al., 1992). We also saw additional minor bands on some Western blots.

Moreover, cell distribution data for MUC 2-63 also matched that reported for CD44. Thus both antigens are acquired during thymocyte maturation and are present on mature T and B lymphocytes, monocytes/macrophages, connective tissue and colorectal tumours (Dalchau et al., 1980; McKenzie et al., 1982; Haynes et al., 1983; Daar & Fabre, 1983; Stoll et al., 1989). Our previous failure to detect MUC 2-63 on epithelial tumours resulted from the use of cell lines rather than fresh tumour samples as used in the current study (Stavrou et al., 1987).

Our data therefore strongly suggested that MUC 2-63 detects an epitope present on all CD44 molecules. Blocking experiments using a well-characterised anti-'framework' anti-CD44 monoclonal antibody, F10-44-2, confirmed this specificity. The expression of MUC 2-63 on human malignant gliomas and neuroblastomas has important implications. Firstly, and as previously demonstrated, the molecule provides an effective target for in vivo imaging of brain tumours and, if administered intratumorally or intrathecally, may provide effective immunotherapy for these tumours (Bergh et al., 1991). Secondly, the MUC 2-63/CD44 molecule may be involved in tumour development, possibly controlling cell growth or migration via interactions with the extracellular matrix (Stamenkovic et al., 1991; Jalkenen & Jalkenen, 1992; Knudson et al., 1993). Thus MUC 2-63 and other anti-CD44 monoclonal antibodies should also provide useful tools with which to study tumorigenesis in the brain.

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