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Original Paper

Splice Variants of the Cell Surface Glycoprotein CD44 Associated with Metastatic Tumour Cells are Expressed in Normal Tissues of Humans and Cynomolgus Monkeys

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Certain isoforms of the CD44 glycoprotein family play an essential role in the metastatic spread of tumour cells. Protein expression of such CD44 isoforms has also been observed in a variety of human malignancies. In this study, we compared the expression of exon v5- and v6-containing CD44 isoforms in normal human and cynomolgus monkey (*Macacca fasciculata*) tissues. Cloning and sequencing of cynomolgus CD44 exons v5 and v6 revealed a homology of 97% and 95%, respectively, between man and monkey. Two monoclonal antibodies (MAbs) directed against an epitope encoded by human exon v5 (VFF8) and an epitope encoded by exon v6 (VFF18) were used to determine expression of CD44 isoforms in man and monkey. Immunohistochemical screening of a representative profile of normal human and cynomolgus tissues revealed that expression of exon v5- and v6-containing CD44 isoforms was almost identical in the two species. Exon v6 staining was observed only in a subset of epithelial tissues, whereas v5 staining was additionally detected on certain non-epithelial tissues. These data suggest that cynomolgus monkey could serve as a system to test the usefulness of antivariant CD44 MAbs with regard to antibody-based tumour therapy.

Key words: CD44 isoforms, splice variants, normal tissues, tumour therapy
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INTRODUCTION

THE METASTATIC spread of tumour cells is the main cause of death in cancer patients. An efficient therapeutic approach should, therefore, not only eliminate primary tumour cells, but also their metastases. Antigens that are preferentially expressed on tumour cells, but not or to only a limited extent on normal tissues, are potential targets for such an approach. Recent experiments suggest that certain isoforms of the cell surface glycoprotein CD44 could be such targets. These CD44 isoforms are generated by differential splicing, by which 10 of the 20 exons of the CD44 gene can be removed or expressed in various combinations at the RNA level [1, 2] (see also Figure 1). Hence these exons are designated "variant" exons (v1–v10).

Overexpression of certain variant isoforms of rat CD44 in an originally non-metastasising rat pancreatic carcinoma cell line leads to spontaneous metastatic behaviour of these cells [3,4]. Upon subcutaneous injection of the variant CD44-expressing tumour cells in syngeneic rats, these animals rapidly develop lymph node and lung metastases. Injection of the parental tumour cells (variant CD44-negative) leads only to formation of local tumours. An antibody which recognises exon v6 of the variant portion of the CD44 molecule is capable of retarding or even blocking the metastatic spread of the variant CD44-expressing tumour cells [5,6]. Thus, variant isoforms of CD44 are causally involved in the metastatic spread of tumour cells.

In order to evaluate a possible role of CD44 variants in metastatic disease in humans, antibodies specific for the variant portion of the human CD44 molecule have been generated [7–9]. Immunohistochemical screening of various tumour types revealed the expression of CD44 variants both on primary and metastatic tumour cells of epithelial origin, for example, colorectal, breast, gastric and cervical carcinomas [7, 8, 10–12]. Among the variant CD44 isoforms detected, those containing exons v5 and v6 dominate. Further fine analysis of CD44 expression in human tumours with three different exon v6-

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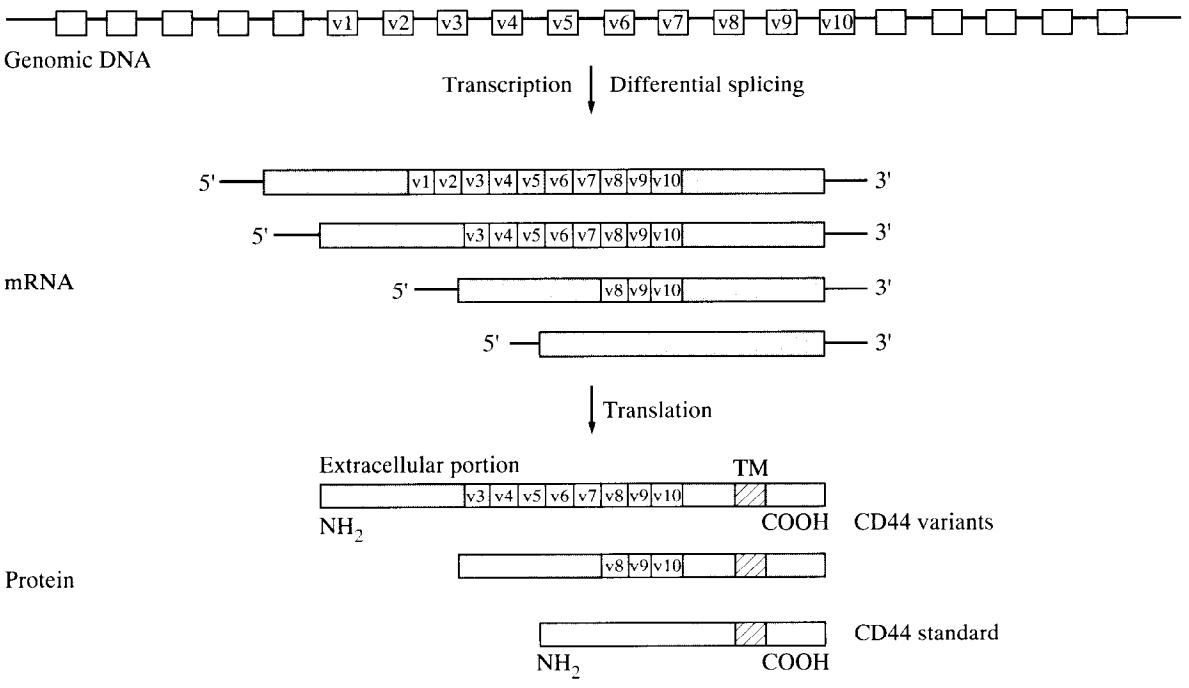

Figure 1. Schematic representation of the CD44 gene and individual splice variants. Removal of various combinations of the 10 variant exons (v1 – v10) of the CD44 gene through differential splicing leads to various mRNAs (four different possibilities are depicted). Upon translation, different variant CD44 isoforms are generated which only differ in their variable region in the extracellular portion of the molecule. The different exon and intron sequences are not drawn to scale. TM, transmembrane region.

specific monoclonal antibodies (MAbs) revealed that one of those, namely VFF18, reacted with a larger subset of the tumours than the other two (data not shown). Therefore, MAbs VFF8 (exon v5) and VFF18 (exon v6) were chosen as potential candidate vehicles for antibody-based tumour therapy.

In this paper, the homology between human and cynomolgus monkey (*Macacca fasciculata*) CD44 exons v5 and v6 was determined. Furthermore, expression of exon v5 and v6-containing CD44 isoforms in normal human and cynomolgus tissues was immunohistochemically analysed to evaluate the usefulness of this monkey as an animal model for toxicity studies.

MATERIALS AND METHODS

Cloning and sequencing of cynomolgus CD44 variant exons v5 and v6

Ten microgrammes of cynomolgus genomic DNA (kindly provided by IDEC Inc., San Diego, U.S.A.) were added to PCR reaction mixtures (50 μl) that contained 20 mM Tris/HCl, pH 8.8; 10 mM KCl; 3 mM $MgSO_4$; 0.1% Triton X-100; 200 μM deoxyribonucleotide triphosphates; 1 U Taq DNA polymerase and the CD44 exon v5 primers EBI5299 (5'-TTTAACCATCATCACAGCAG-3', 1 μM) and EBI5300 (5'-CCGACCGCCATCTTGCTTAC-3', 1 μM) located within the intron sequences adjacent to the human v5 exon. For amplification of exon v6, the primers EBI5303 (5'-AACTGATATTCTTCTCACAG-3', 1 μM) and EBI5304 (5'-CTTGTTAAACCATCCATTAC-3', 1 μM) were used, which are located within intron sequences adjacent to human exon v6. The conditions used were 94°C, 40 s; 55°C, 45 s; 72°C, 50 s for 40 cycles in a Perkin Elmer Cetus DNA thermal cycler. One microlitre of each PCR product was cloned into the TA-vector cloning system (Invitrogen, California, U.S.A.). Miniprep DNA of nine individual clones of exon v5 and 3 individual clones of exon v6 were sequenced on an Applied Biosystems automated sequencer.

Peptide ELISA

The synthetic peptides Hu1 (human v6; QWFGNRWHEGYRQT) and Cy (cynomolgus v6; QWFGNRWHEGYLQT) (kindly provided by Dr Horst Ahorn) were coated on to 96-well ELISA plates (Nunc, Denmark or ActiA, Bio Products Ltd, Wrexham, U.K., containing reactive aldehyde groups) at a concentration of 50 μg/ml (in 0.05 M $NaHCO_3$, pH 9.6) overnight at 4°C. After washing with phosphate buffered saline (PBS)/0.05% Tween 20, the wells were blocked with blocking buffer (PBS/0.5% bovine serum albumin (BSA)/0.05% Tween 20) for 1 h at room temperature, followed by one washing step. Incubation with the primary antibody (VFF18, 10 pg/ml–10 μg/ml in blocking buffer) was performed for 2 h at room temperature, followed by three washing steps. As a secondary antibody, a rabbit anti-mouse horseradish peroxidase-conjugated antibody (DAKO Corp., Copenhagen, Denmark; dilution 1:15000 in blocking buffer) was used (2 h, RT). After three washing steps, colour development was performed with TMB solution (Kirkegaard + Perry, Gathersburg, U.S.A.) and measured with a Hewlett-Packard ELISA-reader.

Tissues

Normal human tissues were selected from the files of the Department of Pathology, Academic Medical Centre, University of Amsterdam, The Netherlands. All samples were snap-frozen in liquid nitrogen immediately after removal from the patient and stored at −80°C. At least three specimens of each tissue type were examined. Cynomolgus monkey (*Macacca fasciculata*) tissues were obtained from a 15 year old female, healthy individ-

ual (kindly provided by E. Merck, Institute for Pharmacokinetics and Metabolism, Grafing, Germany). Tissues were snap-frozen in cooled 2-methyl-butane and stored at −80°C. At least three different regions of each organ were examined.

Antibodies

The generation of antivariant CD44 MAb has been described in detail elsewhere [9]. The exon specificities of the MAbs VFF8 (IgG1) and VFF18 (IgG1) were determined in ELISA and Western blot assays using glutathione S-transferase fusion proteins encoded by CD44 exons v5 and v6. MAb VFF8 recognises an epitope encoded by exon v5 and MAb VFF18 recognises an epitope encoded by exon v6. To further pinpoint the VFF8 and VFF18 epitopes, overlapping CD44v5 and v6 peptides were synthesised and the exact epitope sequence was determined (Figure 2).

Immunohistochemistry

Cryostat tissue sections (6 μm) were fixed in methanol/acetone (1:1) or pure acetone at −20°C for 10 min. Endogenous biotin (if indicated) was blocked with 0.1% streptavidin in PBS (10 min) followed by incubation with biotin (0.01% in PBS) for 10 min. Sections were washed in PBS and pre-incubated with normal goat serum (10% in PBS). After three washes in PBS, the sections were incubated with the primary antibody (VFF18: 5 μg/ml; VFF8: 10 μg/ml, EBI-1 (isotype-matched negative control; [13] 10 μg/ml in PBS/1% BSA for 1 h). For blocking experiments, MAb VFF18 was incubated with the peptide Hu1 at a concentration of 1 or 10 μg/ml for 1 h at 4°C prior to the incubation of the tissue sections. Endogenous peroxidases were blocked with 0.3% H_2O_2 in methanol and the sections incubated with the secondary biotinylated antibody for 30 min (anti-mouse IgG-F(ab^1)$_2$, DAKO Corp., Copenhagen, Denmark). For colour development, the sections were further incubated (30 min) with horseradish peroxidase that had been coupled to biotin as a streptavidin–biotin-peroxidase complex (DAKO Corp). The sections were then incubated in 3,3-amino-9-ethyl carbazole substrate (Sigma Immunochemicals) for 5–10 min, the reaction was stopped in H_2O and the sections were counterstained in haematoxylin. Evaluation of the staining was performed with a Zeiss Axiolab light microscope and the staining intensities were quantified as follows: ++, strong expression; +, weak expression; −, equivocal or no expression detectable.

a. DNA sequence comparison between human and cynomolgus CD44 variant exons v5 and v6

exon v5:

```
cyn  AT GTA GAC AGA AAT GGC ACC ACT GCT CAT GAA GGA AAC TGG
hum  AT GTA GAC AGA AAT GGC ACC ACT GCT TAT GAA GGA AAC TGG

cyn  AAC CCG GAA GCA CAT CCT CCC CTC ATT CAC CAT GAG CAT CAT
hum  AAC CCA GAA GCA CAC CCT CCC CTC ATT CAC CAT GAG CAT CAT

cyn  GAG GAA GAA GAG ACC CCA CAT TCT ACA AGC ACA A
hum  GAG GAA GAA GAG ACC CCA CAT TCT ACA AGC ACA A
```

exon v6:

```
cyn  TC CAG GCA ATT CCT AGT AGT ACA ACT GAA GAA ACA GCT ACC CAG
hum  TC CAG GCA ACT CCT AGT AGT ACA ACG GAA GAA ACA GCT ACC CAG

cyn  AAG GAA CAG TGG TTT GGC AAC AGA TGG CAT GAG GGA TAT CTC CAA
hum  AAG GAA CAG TGG TTT GGC AAC AGA TGG CAT GAG GGA TAT CGC CAA

cyn  ACA CCC AGA GAA GAC TCC CAT TCG ACA ACA GGG ACA GCT G
hum  ACA CCC AGA GAA GAC TCC CAT TCG ACA ACA GGG ACA GCT G
```

b. Comparison of human and cynomolgus exon v5 and v6 encoded protein sequences

exon v5:

```
cyn  VDRNGTTAHEGNWNPEAHPPLIHHEHHEEEETPHSTST
hum  VDRNGTTAYEGNWNPEAHPPLIHHEHHEEEETPHSTST
                          VFF8
```

exon v6:

```
cyn  QAIPSSTTEETATQKEQWFGNRWHEGYLQTPRED
hum  QATPSSTTEETATQKEQWFGNRWHEGYRQTPRED
                    VFF18
cyn  SHSTTG
hum  SHSTTG
```

Figure 2. Sequence comparison between human and cynomolgus CD44 variant exons v5 and v6 at the (a) DNA, and (b) protein level. Differences between the human and cynomolgus sequences are typed in bold. In (b), the epitope locations for MAbs VFF8 (exon v5) and VFF18 (exon v6) are indicated.

RESULTS

Sequence comparison of human and cynomolgus monkey exons v5 and v6

Sequences of cynomolgus CD44 variant exons v5 and v6 were amplified by PCR from cynomolgus genomic DNA using PCR primers deduced from the human genomic sequence [1]. The PCR products were sequenced and compared to the published human CD44 sequence (Figure 2). Both cynomolgus exon sequences v5 and v6 differ in three nucleotides each from their human counterparts. At the protein level, cynomolgus exon v5 deviates in only one amino acid and cynomolgus exon v6 in only two amino acids from the human sequence (Figure 2b). The homology between human and cynomolgus is 97% for exons v5 and 95% for exons v6.

Comparison of VFF18 binding to human and cynomolgus CD44v6 peptides

The great homology between human and cynomolgus exons v5 and v6 made it very likely that the antihuman CD44 antibodies would also recognise the cynomolgus protein. Epitope analysis for the MAb VFF8 revealed a 100% identity between the human and cynomolgus monkey epitope (Figure 2b). The epitope of MAb VFF18 differed in one amino acid between the two species (Figure 2b). To test whether the antibody VFF18 also recognises the cynomolgus protein, the cynomolgus homologue of the

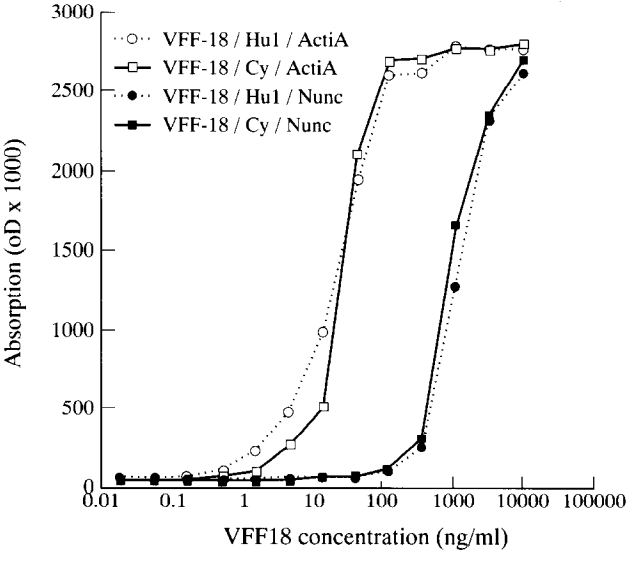

Figure 3. Comparison of MAb VFF18 binding to a human (Hu1) and cynomolgus (Cy) CD44v6 peptide. The absorption curves for two different ELISA plates (ActiA and Nunc) are shown.

human CD44v6 peptide, which is recognised by VFF18, was synthesised and the binding affinity of VFF18 to both peptides was determined in an ELISA assay (Figure 3). The result indicates that there is no difference in binding of MAb VFF18 to both the human and monkey CD44v6 peptide.

Expression of CD44 variants in human and cynomolgus normal tissues

A representative profile of normal human and cynomolgus tissues was immunohistochemically analysed in order to compare the tissue distribution of CD44v5 and v6 isoforms in both organisms. The results obtained with the antibodies VFF8 (exon v5) and VFF18 (exon v6) are summarised in Table 1. In general, antibody VFF18 shows a much more restricted reactivity than VFF8. In a subset of epithelial tissues, expression of both exons v5 and v6 was detectable (breast myoepithelium, bronchial epithelium (basal layer), pneumocytes type II, skin keratinocytes, hair follicles, sweat gland myoepithelium, epithelium of tonsil and bladder) (Figure 4). In marked contrast, none of the investigated non-epithelial tissues showed reactivity with VFF18, whereas smooth muscle cells, spleen lymphocytes (scattered cells only), brain neurons and microglia, and axons and Schwann cells in the spinal cord express CD44v5 as determined with the antibody VFF8. In most organs investigated, man and monkey display an identical pattern of CD44v5 and v6 expression. Exceptions are microglia in brain, lymphocytes in spleen (MAb VFF8), follicular epithelium of the thyroid and intercalated ducts of the pancreas (MAb VFF18) (Table 1). In cynomolgus kidney, VFF18 strongly reacted with the basement membrane of the tubules, whereas in human kidney no reaction was detectable (Figure 4i,j). This reaction was completely abolished after pre-incubation of MAb VFF18 with the peptide Hul, indicating a specific reaction of the antibody with the tubular basement membrane of cynomolgus kidney (not shown).

DISCUSSION

In situ expression of variant CD44 proteins has been reported for a variety of different human tumours [7–12, 14–16]. In non-

Table 1. Expression of CD44v5 and v6 in human and cynomolgus tissues

Tissues		VFF8 (v5) Human	VFF8 (v5) Cynomolgus	VFF18 (v6) Human	VFF18 (v6) Cynomolgus
Epithelia					
Adrenal gland		-	-	-	-
Breast	-luminal epithelium	-	-	-	-
	-myoepithelium	++	++	++	++
Colon	-crypt base	-	-	-	-
	-crypts	-	-	-	-
Kidney	-glomerular epithelium	-	-	-	-
	-tubules	-	-	-	-*
Liver	-bile ducts	-	-	-	-
	-hepatocytes	-	-	-	-
Lung	-bronchial epithelium	++	++	++	++
	-pneumocytes type II	++	++	++	++
Pancreas	-acini	-	-	-	-
	-ducts	-	-	+†	-
	-Langerhans cells	-	-	-	-
Skin	-keratinocytes	++	++	++	++
	-hair follicles	++	++	++	++
	-sweat gland myoepithelium	++	++	++	++
Stomach	-surface epithelium	+	+	-	-
Thyroid	-follicular cells	+	++	+	-
Tonsil	-epithelium	++	++	++	++
Urinary bladder	-transitional epithelium	++	++	++	++
Others					
Brain	-microglia	+	-	-	-
	-neurons	++	+	-	-
Endothelium		-	-	-	-
Fibroblasts		-	-	-	-
Kidney	-glomerular mesangium	-	-	-	-
	-glomerular endothelium	-	-	-	-
Kupffer cells		-	-	-	-
Lymphocytes	-lymph node	-	-	-	-
	-spleen	-	+‡	-	-
	-tonsil	-	-	-	-
Muscle	-heart	-	-	-	-
	-skeletal	-	-	-	-
	-smooth	++	++	-	-
Spinal cord	-axons	++	++	-	-
	-Schwann cells	++	+	-	-

-, equivocal or no expression; +, weak expression; ++, strong expression; *, Strong reaction with basement membrane; †, Intercalated ducts; ‡, Scattered cells only.

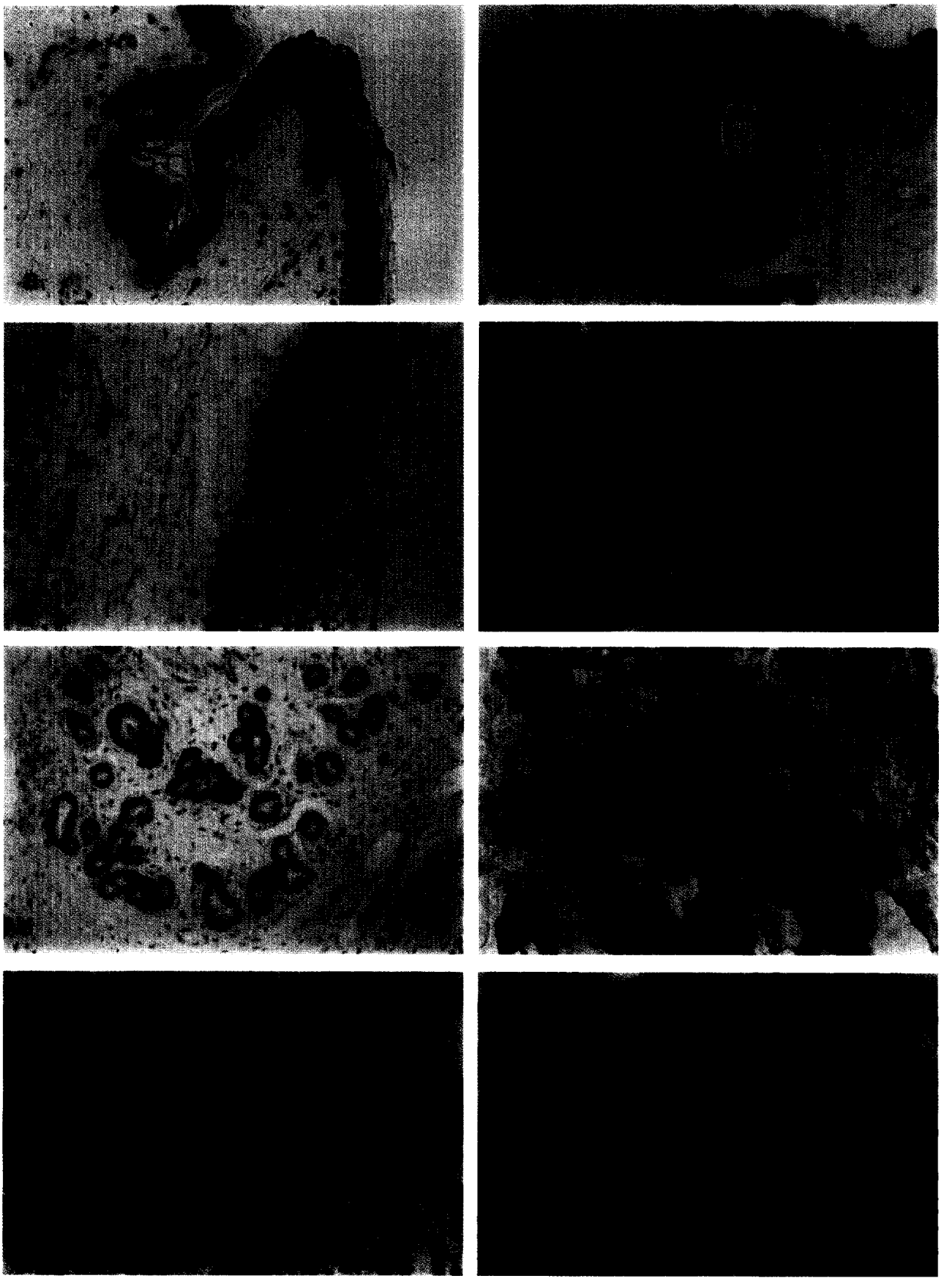

Figure 4. (a)–(h)

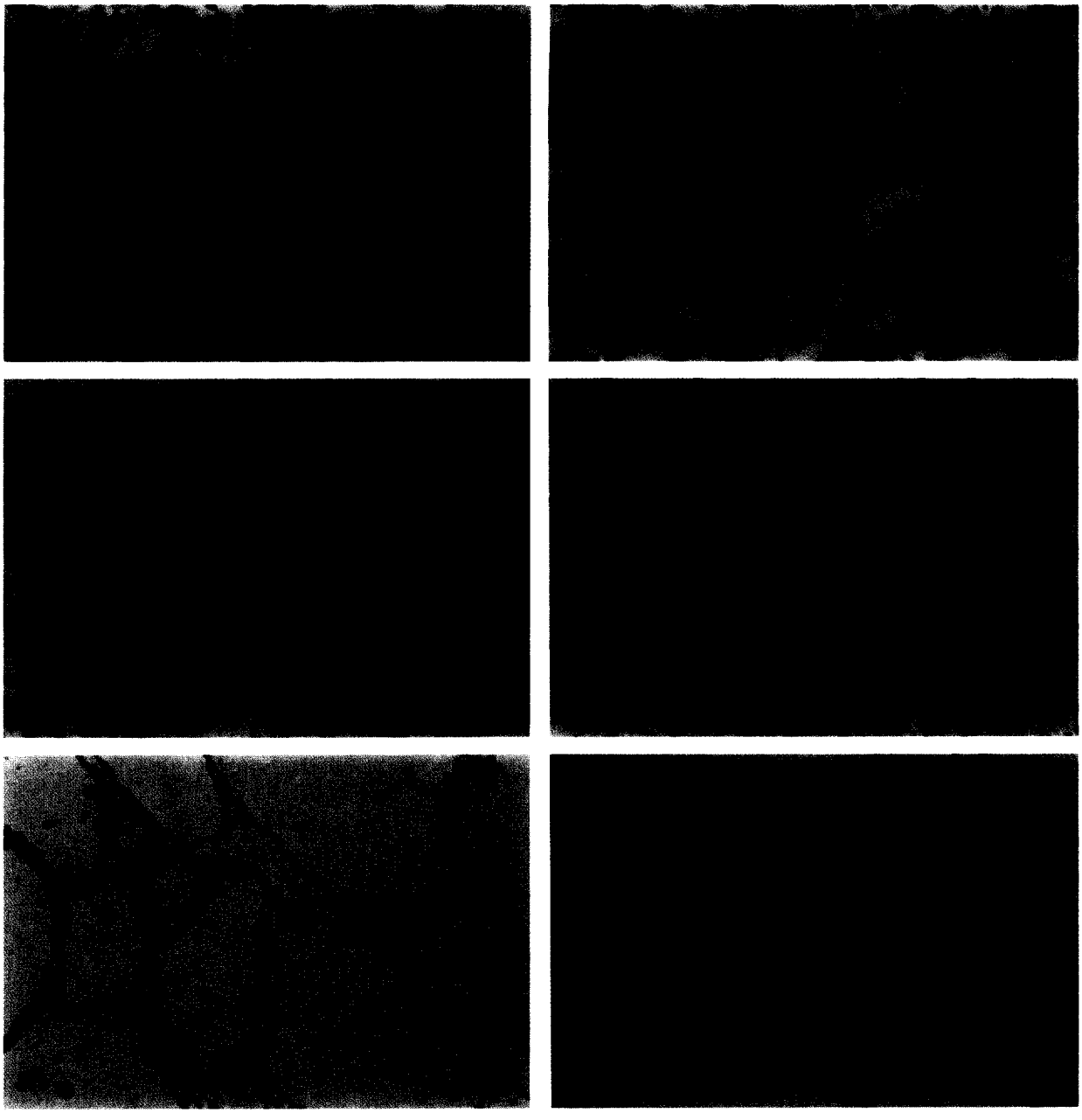

Figure 4. Immunohistochemical analysis of normal human (a, c, e, g, i, k, m) and cynomolgus monkey (b, d, f, h, j, l, n) tissues. The following tissues are shown: skin (a, b); tonsil (c, d); breast (e, f); lung (g, h); kidney (i, j); liver (k, l) and thyroid (m, n). Antibody: VFF18; counterstain: haematoxylin; original magnification: ×40 (c, e: ×20); bar represents 100 μm (c, e: 200 μm).

Hodgkin's lymphomas, only the more aggressive tumours show CD44v6 expession [9]. In colorectal tumours, exon v6-containing variants are more frequently expressed in advanced tumour stages (Dukes' C,D) than in benign adenomas [7,10]. Recent data suggest that exon v6 expression can predict overall survival in colorectal tumour patients [17]. In breast cancer, the vast majority of primary invasive tumours and lymph node metastases have been found to be CD44v5 and v6 positive [12], and exon v6 expression significantly correlates with reduced overall survival of patients [18,19]. Furthermore, expression of CD44 isoforms is not restricted to primary tumours, but also occurs in lymph node metastases, distant metastases, and bone marrow micrometastases [8,12,14,15].

Examination of normal tissues, from which CD44 variant-expressing tumours arise, revealed that expression of exons v5 and v6-containing isoforms is very restricted (breast) or does not occur (colon, lymphatic tissues) [7–10,12,16,20]. Thus, antibodies to CD44 variant proteins may be useful as specific carriers for delivery of cytotoxic drugs, toxins or radioisotopes to the tumour cells. We, therefore, extended the analysis of CD44 variant expression to other normal tissues. In parallel, we studied the expression of CD44 isoforms in the cynomolgus monkey (*Macacca fasciculata*) as a potential animal model for preclinical studies.

Cloning and sequencing of cynomolgus exons v5 and v6 revealed a high degree of similarity between man and cynomolgus monkey. The homology between human and monkey exons v5 and v6 is 97% and 95%, respectively. In contrast, the homology between human and mouse exons v5 and v6 amounts to only 55% and 51%, respectively [2]. Our *in vitro* binding study

showed that the human anti-CD44v6 antibody VFF18 binds to the cynomolgus peptide with the same affinity as to the human homologue (Figure 3). Immunohistochemical analysis revealed that CD44v5 and v6 expession in man and monkey is very similar (Table 1). In rat, the pattern of CD44v6 expression has been found to be significantly different from that in human [21]. Surprisingly, MAb VFF18 showed strong staining with the basement membrane of cynomolgus kidney, a reaction which was not observed in human kidney. Since blocking experiments with the synthetic peptide Hu1 indicate that this reaction is not caused by non-specific binding of MAb VFF18, one might speculate whether the MAb reacts with soluble forms of CD44 trapped in the cynomolgus kidney basement membrane.

Our results indicate that antibody VFF18 may be a suitable targeting vehicle, as the respective antigen encoded by exon v6 displays a more restricted pattern of expression than that encoded by exon v5. VFF18 recognises the cynomolgus protein with the same affinity as the human homologue. Moreover, the expression pattern of CD44v6 isoforms is almost identical in humans and cynomolgus monkeys. Preclinical studies in cynomolgus monkeys may provide useful information on the biodistribution and safety of this antibody.

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