Expression of aminopeptidase-n (CD 13) in normal tissues and malignant neoplasms of epithelial and lymphoid origin

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Abstract

Aims—To provide a detailed knowledge of the distribution of the CD13 molecule, also known as the protease aminopeptidase-N, on both normal tissues and malignant neoplasms of epithelial and lymphoid origin.

Methods—CD13 antigen was examined by immunocytochemistry, using a recently produced antibody (VS5E) alongside a commercially available anti-CD13 monoclonal antibody. The VS5E recognising CD13 was produced by immunising a doxorubicin resistant breast cancer cell line (MCF-7-Adr). A striking feature of this antibody was that it stained the doxorubicin resistant cells but not the parental cell line. Both antibodies were tested on a broad range of normal tissues and three common types of epithelial malignancy (colon $n = 28$, lung $n = 30$, breast $n = 35$), and 12 cases of Hodgkin's and 52 of non-Hodgkin's lymphomas.

Results—CD13 was expressed on many tissue and cell types outside the haematopoietic system. In particular it was present on breast epithelium and in 20% (seven of 35) of breast carcinomas, but absent in normal and neoplastic colonic and bronchial tissues and lymphomas.

Conclusions—This study provides not only detailed information about the expression of the CD13 antigen, but also raises the important possibility that CD13 expression may correlate with drug resistance in breast carcinomas.

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The myeloid cell surface antigen CD13, also known as the protease aminopeptidase-N, is a 150 kilodalton surface glycoprotein^{1,2} encoded by a gene located on the long arm of chromosome 15 at bands q25-26. This 967 amino acid integral membrane protein has a large extracellular carboxy-terminal domain containing a pentapeptide motif, characteristic of members of the zinc binding metalloprotease superfamily. The amino terminal intracellular hydrophobic segment can potentially act both as a signal for membrane insertion and as a stable membrane spanning segment.²

Interest in the CD13 antigen arose when it was discovered that myeloid cell lines expressed it. Although CD13 is absent from

the surface of normal B and T lymphocytes and most lymphocytic malignancies, it is now well established that increased CD13 expression is detected in acute myeloblastic leukaemia (AML) and in some cases of acute lymphoblastic leukaemia (ALL). A high level of CD13 expression in these two types of neoplasm is correlated with a poor prognosis, in that there is a reduced success rate in inducing remission as well as a shortened survival time, particularly in patients with ALL.³

CD13 expression is not restricted to myeloid cells and related neoplasms. Non-haematopoietic cells such as fibroblasts, osteoclasts, and the epithelium that line the renal proximal tubules, intestine, and bile duct canaliculi are also CD13 positive.⁴ CD13 expression has also been detected in hepatocellular, gall bladder, and renal carcinoma cell lines.^{5,6} Mesenchymal tumours of smooth muscle, fibrous, synovial, osteogenic, and peripheral nerve sheath origin are also CD13 positive.⁷ Reports of CD13 expression in a few cases of neuroblastoma, fallopian tube carcinoma, and in some ovarian and cervical carcinoma cell lines have also been made.⁸

CD13 is identical with human aminopeptidase-N,⁹ a zinc binding protease that binds to the membrane through an N-terminal segment. It is expressed in many tissues and mainly on the brush border membrane of the small intestine, on proximal tubules, on synaptic membranes of the central nervous system, and on the surface of granulocytes and macrophages.¹⁰⁻¹³

Recently we produced monoclonal antibodies using the cell line MCF-7-Adr which is a doxorubicin resistant breast cancer cell line. One monoclonal antibody, VS5E, was selected as staining only the doxorubicin resistant cells but not the parental cell line. Characterisation of VS5E showed that it recognised CD13, raising the possibility that this antigen might be involved in some aspect of drug resistance. To investigate this it is necessary to have a detailed knowledge of the distribution of CD13 on both normal and neoplastic tissues. The present study was designed to provide this on a range of normal tissues and in five types of neoplasms.

Methods

Fresh tissue samples from 13 normal tissues (adrenal, brain, breast, bronchus, colon, kidney, liver, nasal mucosa, pancreas, skin, testes, tongue and tonsil) and from five types of neoplastic tissue (12 cases of Hodgkin's

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Figure 1 Immunoblot of a membrane preparation from MCF7 AdR cells. Lane A shows that VS5E recognises a band of molecular weight 150 kilodaltons. Lane B is a negative control stained with an irrelevant antibody (anti-bromodeoxyuridine).

Table 1 Monoclonal antibodies, VS5E and WM47, bind CD13 epitopes

Transfected cells	VS5E	WM47
COSS + CD13 construct + pSV2neo	+	+
COS + pSV2 neo	-	-

disease, 52 non-Hodgkin's lymphomas, 35 breast, 30 lung and 28 colon carcinomas) were used. Different types and grades of lymphoma, such as chronic lymphocytic leukaemia, and B or T large cell lymphomas, and three types of lung neoplasm: squamous cell carcinoma; adenocarcinoma; and small cell carcinoma were included.

VS5E was produced as detailed below.

IMMUNISATION WITH ANTIGEN

The source of antigen was a membrane preparation of MCF-7-AdR cells. About 4×10^5 cells were injected intraperitoneally into a Balb/c mouse, together with Freund's complete adjuvant. Eleven days later the mouse was given a booster injection of about 1.6×10^6 cells, with non-complete adjuvant. The booster was repeated again seven days later.

Three days after the last injection cell suspensions were prepared from the spleen of the mouse and were fused with cells from the myeloma cell line NS1, using polyethylene glycol to induce cell fusion, as described by Pulford *et al.*¹⁴

BIOCHEMICAL ANALYSIS OF THE VS5E EPTOPE

Cell cultures (MCF-7-AdR) were washed twice with phosphate buffered saline (PBS) and then harvested with 0.25% trypsin and 0.02% EDTA. The cell suspension was washed in culture medium and centrifuged for 5 minutes at 1000 rpm at 4°C. The cells were washed twice in PBS and centrifuged for a final spin at 2000 rpm for 10 minutes at 4°C. The cell pellet was solubilised with an equal volume of 1% (v/v) Triton X-100(Sigma) in 10 mM TRIS-HCl (pH 8) for 20 minutes on ice, to yield integral membrane and associated proteins. This was then spun at 200 rpm for 15 minutes at 4°C, the supernatant fluid collected, and used either immediately for analysis by (PAGE) polyacrylamide gel electrophoresis or stored as membrane extract at -70°C.

SDS-PAGE AND IMMUNOBLOTTING

Sodium dodecyl sulphide (SDS) PAGE was performed as follows. Samples were suspended in buffer containing Dithiothreitol and boiled for 2 minutes, allowed to cool, and loaded on to a gel (5.5% stacking gel, 6.5% running gel). Molecular weight markers were run at the same time. Proteins were transferred electrophoretically using a Transblot (Biorad) apparatus to nitrocellulose membranes and the protein detected by applying the antibody followed by a rabbit-anti mouse peroxidase conjugate (Dako) and visualised using diaminobenzidine with metal ion enhancement (fig 1).

WM47 (anti CD13) was produced by Favaloro *et al*⁸ (commercially supplied by Dako).

To test the reactivity of the two monoclonal antibodies used in this project to the CD13 antigen, COS cells (monkey kidney fibroblasts) were either transfected with a cDNA encoding CD13, together with the selectable marker pSV2 neo, or with pSV2 neo alone, and the cells selected for G418 resistance. Transfectants were then exposed to the VS5E antibody and the goat anti-mouse fluorescein isothiocyanate (FITC) conjugate. The cells were separated on fluorescence activated cell sorting (FACS) and the 10% highest fluorescing cells were collected.

The immunohistochemical staining was performed using the alkaline phosphatase-anti-alkaline phosphatase method (APAAP), as has been described before. Cryostat tissue sections (6 µm thick) were fixed in acetone for 10 minutes at room temperature, left to dry overnight, and either stained immediately or stored at -20°C until required. The primary antibodies were added to the dry sections in a moist chamber for 30 minutes. Anti-mouse immunoglobulins (Dako code Z 259 at 1 in 50 dilution with TBS and APAAP complex (Dako code D 651 at 1 in 50 dilution in TBS) were added and incubated for 30 minutes for each one. This step was repeated twice (the second and third incubations for 10 minutes only) to enhance the intensity of the final staining. The alkaline phosphatase substrate was applied afterwards for 15-20 minutes, and the sections, after being washed with TBS and tap water, were counterstained with haematoxylin and mounted on a suitable aqueous mounting medium. All antibody incubations were

Figure 2 VS5E immunostaining of (A) COS cells transfected with the full length cDNA of CD13 with the selectable marker pSV2 neo and (B) with the selectable marker pSV2 neo alone.

Table 2 Expression of CD13 in non-haematopoietic tissues

Adrenal	+ Reticular connective tissue
	- Endothelium
	- Epithelium
Brain	+ Endothelium of cerebral capillaries
Breast	+ Epithelium of some secretory ducts
	+ Basement membrane of secretory ducts
	+ Interlobular connective tissue
Bronchus	+ Epithelium of secretory ducts of bronchial glands
	+ Basement membrane
	- Respiratory epithelium
Colon	+ Macrophages
	- Mucosal epithelium
Kidney	+ Epithelium of proximal convoluted tubule ducts
	+ Distal convoluted tubules
Liver	+ Epithelium of bile ducts
	+ Bile canaliculi
Nasal mucosa	+ Epithelium of secretory ducts
	+ Connective tissue stroma
	+ Smooth muscle
	- Endothelium
Pancreas	+ Epithelium of exocrine ducts
	- Islets
Small intestine	+ Epithelium
Skin	+ Macrophages
	+ Basement membrane
	+ Macrophages
	- Endothelium
	- Epithelium
Testis	+ Leydig cells
	+ Spermatozoa-Sertoli cells
Tongue	+ Basement membrane
	+ Stroma
Tonsil	+ Basement membrane
	+ macrophages

followed by two one minute washings in TBS.

Results

TRANSFECTED CELLS

The reactivity of VS5E and WM47 to COS cells either transfected with a full length cDNA encoding CD13 (positive control), or with the pCDM8 vector (negative control), is shown in table 1 and fig 2. These controls

were performed to ensure that the two monoclonal antibodies used in the project reacted specifically with the CD13 antigen. As the table indicates, VS5E and WM47 react only with the COS cells containing the CD13 construct.

NORMAL TISSUES

The distribution of CD13 in 13 different normal tissues was investigated using VS5E and WM47. The two antibodies displayed identical reactivities in all tissues. The results are summarised in table 2. CD13 was expressed universally by macrophages, fibroblasts, smooth muscle cells and stromal cells. CD13 was absent from the keratinised squamous epithelium of the skin and the pseudo-stratified respiratory epithelium of the bronchus, but was found on both the secretory epithelium of the glands and ducts of the exocrine pancreas, nasal polyp, hepatic bile ducts, bronchial glands, breast, small intestine and the reabsorptive epithelium of the renal proximal convoluted tubules. CD13 was expressed by exocrine glandular secretory epithelium, but the extent of the expression varied. At least 80% of the exocrine glands and ducts of the pancreas, mucous glands of the nasal mucosa and the respiratory tract, and the hepatic bile ducts expressed CD13. The antigen was completely absent from colonic epithelium.

CD13 expression did not occur on the endocrine secretory cells of the islets of Langerhans in the pancreas or on the Leydig cells in the testes.

CD13 was not expressed by any endothelium in systemic capillaries, but was clearly present on the luminal side of cerebral capillaries (fig 3).

Figure 3 Staining of normal tissues with anti-CD13 antibody VS5E: (A) breast; (B) kidney; (C) pancreas; (D) brain.

Table 3 Expression of CD13 in different neoplasms

Tissue	No of cases positive with VS5E and WM47
Hodgkin's disease	0/12
Non-Hodgkin's lymphomas	0/52
Breast	7/35
Colon	0/28
Lung	0/30

NEOPLASMS

To investigate whether CD13 was expressed by neoplasms other than the well characterised leukaemic cell lines, five different malignant neoplasms were studied, three of which were of non-haematopoietic origin. CD13 was expressed by breast neoplasms in seven of 35 (20%) of the cases but was not expressed by Hodgkin's disease cells and non-Hodgkin's lymphomas, colon, or lung carcinomas. In most of the positive breast cases every tumour cell expressed CD13, but in a few cases CD13 was expressed focally (table 3) (fig 4).

Discussion

CD13 was expressed in many different normal tissues. It was found on macrophages, stromal cells, smooth muscle cells and fibroblasts within basement membranes and connective tissue throughout all the tissues examined. The wide distribution of this enzyme suggests that it performs functions that may be site dependent.¹⁰ It has been suggested that in the pancreas and liver CD13, which is identical with aminopeptidase-N, an ectoenzyme with a metalloprotease activity, may be essential for the breakdown of the tripeptide glutathione, which is present in high concentrations. CD13 was expressed by the reabsorptive epithelium of the proximal convoluted tubules of the kidney, but not by the absorptive epithelial lining of the colonic mucosa. In a recent study, however,¹⁵ aminopeptidase-N expression was detected in the normal colonic epithelium and in 30% of the colonic carcinomas by immunofluorescence. A possible explanation could be the difference in epitope specificity of the antibodies used.

It has been reported that CD13 is expressed in the small intestine, where it is

thought to have an important role in peptide digestion. Intestinal brush border aminopeptidase-N constitutes 8% of the total protein and is thought to play a part in the final stage of peptide digestion, with a preference for neutral amino acids. In this particular case the enzyme has a nutritional protein processing role.¹⁶ CD13 is the major receptor for different types of human coronaviruses which cause upper respiratory tract and intestinal infections.^{17 18}

CD13 was expressed by the endothelium of the cerebral capillaries, but not by the endothelium of the systemic capillaries. Whether this finding reflects morphological and functional differences between capillaries of the systemic and cerebral circulations is not known. What is known, however, is that CD13 collaborates with CD10 (neutral endopeptidase) to inactivate bioactive peptides on synaptic membranes of the central nervous system, including enkephalins and endorphins that are involved in signal transduction at the cell membrane.^{19 20}

For patients with ALL, survival studies show that expression of CD13 is positively correlated with a reduced success rate in inducing remission and a shortened survival. The production of a new monoclonal CD13 antibody (VS5E) aroused interest in CD13 as a potential marker of drug resistance. VS5E was produced using a membrane preparation of MCF-7 AdR, a breast cancer derived doxorubicin resistant cell line. This new monoclonal antibody stained MCF-7 AdR but not the parental cell line (unpublished observations). The most interesting finding of this study is that while most breast carcinomas do not express CD13, a few (20%) are positive, presumably reflecting the positivity of normal breast epithelium. It would be interesting to determine whether this subset of CD13 positive breast carcinomas exhibit an altered response in vivo to chemotherapeutic agents.

We are now undertaking a large study of breast cancer cases to confirm these findings and to attempt to elucidate whether there is a correlation between CD13 expression and a response to treatment and ultimately to prognosis.

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Figure 4 Staining of breast cancer with anti-CD13 antibody VS5E showing (A) positive case and (B) negative case.

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