Technical Advance

NCL-CD10–270: A New Monoclonal Antibody Recognizing CD10 in Paraffin-Embedded Tissue

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CD10 (CALLA) antigen is expressed in a wide variety of epithelial and nonepithelial tissues, but its most significant application is in the diagnosis and classification of certain types of malignant lymphoma and leukemia. CD10 is expressed in a high percentage of cases of acute lymphoblastic leukemia (ALL), follicular lymphoma, Burkitt's lymphoma, and some hematopoietic tumors. Although the antigen is not lineage specific, CD10 expression is widely used to define subgroups within B-ALL and is a useful tool for detecting the presence of leukemic blasts in the bloodstream. Currently available monoclonal antibodies to CD10 have been found to be effective only in fresh-frozen tissue and for techniques such as flow cytometry. We have used a recombinant protein corresponding to the whole of CD10 to generate a monoclonal antibody that is effective in paraffin-embedded tissue sections. We have used this antibody to assay for the presence of CD10 on a range of normal and pathological tissues. Strong staining was seen in lymphoid germinal centers, renal tubules, glomeruli, syncytiotrophoblast, hepatic parenchymal canaliculi, B-lineage ALL, follicle center cell lymphoma, and a proportion of cases of large-B-cell lymphoma. We believe that this antibody will be of value in the characterization of malignant lymphoma, in particular the differential diagnosis of small-B-cell lymphoma and subtyping of lymphoblastic leukemia, as well as the investigation of the significance of expression of CD10 in other normal and pathological tissues. (*Am J Pathol* 1999, 154:77–82)

copy of hematoxylin and eosin (H&E)-stained standard preparations, together with a limited range of phenotypic markers. However, difficulties are sometimes encountered, and full characterization requires supplementary studies for demonstration of characteristic antigens; assessment of CD10 expression is of value in certain situations in this context.

CD10 is a 100-kd type II cell-surface metalloproteinase known by a variety of eponyms, including enkephalinase and common acute lymphoblastic leukemia antigen (CALLA). It is a member of a family of exopeptidases that includes CD13 and CD26,¹ and it functions by reducing the cellular response to peptide hormones. Identified substrates are largely neural or humoral oligopeptides agonists (reviewed in Ref. 2), and the enzyme functions to terminate signaling by degrading the ligand, analogous to the acetylcholine/acetylcholinesterase system.³ CD10 is thought to be expressed during the first stages of heavy chain gene rearrangement, and in an immunological context, it is thought that the enzyme modulates the enkephalin-mediated inflammatory response.⁴

The major expression sites of this enzyme are the brush border of enterocytes, renal tubules and glomeruli, and lymphoid precursor cells.⁵ However, the enzyme is not expressed on mature B and T lymphocytes. On neoplastic cells, the antigen is present in a high percentage of cases of acute lymphoblastic leukemia (ALL), follicular lymphoma, Burkitt's lymphoma, and some hematopoietic tumors and is a useful tool in detecting the presence of leukemic blasts in the bloodstream.⁶ Although expression of CD10 is not lineage specific, it is widely used to define subgroups within B-lineage ALL. CD10 expression on B-lineage leukemias defines the largest subgroup of ALL and typically represents a group with a good prognosis. However, the absence of CD10 defines a subgroup of ALL that is particularly resistant to treatment and therefore deserves special attention.

CD10 therefore represents a useful tool in the classification and diagnosis of malignant leukemia and lymphoma. However, currently available reagents have been

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Malignant lymphomas can generally be classified accurately on the basis of morphological features by micros-

shown to be effective only in fresh-frozen tissue and for techniques such as flow cytometry. In this paper, we describe the production and characterization of a new monoclonal antibody to CD10 that detects the antigen in formalin-fixed, paraffin-embedded tissue.

Materials and Methods

Production of CD10 Recombinant Protein

Total cellular RNA extracted from peripheral blood lymphocytes according to the method of Chomczynski and Sacchi⁷ was used as a template for reverse transcription. The reaction was primed with a specific CD10 primer (5'GGGATCCTCACCAACCCGGCACTTCTTT3') using a reverse transcription (RT) kit in line with manufacturer's instructions (Promega, Madison, WI). One-half the RT reaction mix was subsequently used as a template for 30 rounds of polymerase chain reaction (PCR) after the addition of a second CD10 primer (5'-GGGATCCGTGTGCAAACTATGTCAATGGG AATA-3') and appropriate adjustment of conditions. The amplification of a 1035-bp PCR product was confirmed by agarose gel electrophoresis before cloning into pUC57/T (MBI Fermentas, Vilnius, Lithuania). Clones were identified and characterized before subcloning into the expression vector pET15b (Novagen, Madison, WI). The resulting construct was transformed into *Escherichia coli* strain BL21, and cultures were grown to an OD₅₅₀ of 0.4 before induction of protein expression by the addition of isopropyl thiogalactoside to 1 mmol/L. The CD10 fusion protein was found to be expressed in the form of insoluble inclusion bodies. These were solubilized in 8 mol/L urea in 10 mmol/L Tris/HCl, pH 8.0. The protein was refolded to a soluble form by stepwise dialysis against decreasing concentrations of urea. Final dialysis steps were against repeated changes of 10 mmol/L Tris/HCl, pH 8.0. Refolded CD10 was purified by column chromatography on His-bind resin (Novagen), and the purity of the final protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before immunization of female BALB/c mice.

Hybridoma Production

The recombinant protein was injected into five female BALB/c mice in a series of four injections at 14-day intervals. The first consisted of 20 µg of protein per mouse in Freund's complete adjuvant, injected subcutaneously. The second was identical but in Freund's incomplete adjuvant. The third and fourth injections were in PBS and administered intraperitoneally. Mice were boosted with 60 µg of protein intravenously 4 days before fusion.

The spleen cells were fused with NS-1/1Ag4-1 myeloma cells using polyethylene glycol and plated out into hypoxanthine-aminopterin-thymidine-selective growth medium. After 10 days, supernatants from each well were assayed by enzyme-linked immunosorbent assay (ELISA), and colonies showing reactivity were transferred to separate wells in 24-well growth plates. After an addi-

tional period of growth, colonies were again assayed by ELISA, and positive colonies were tested on paraffin-embedded small bowel sections. Colonies showing positive staining of the cytoplasmic membrane were subjected to an additional three rounds of cloning by double dilution.

Western Blotting

A sample of placenta was homogenized in Laemmli sample buffer⁸ before boiling for 4 minutes. Protein extracts were fractionated by SDS-PAGE on a 7.5% gel before transfer to Hybond C nitrocellulose (Amersham, Little Chalfont, UK) as described previously. After blocking, the nitrocellulose was incubated with primary antibody (1:50). After a 60-minute room temperature incubation, the blot was washed and transferred to a 1:1000 dilution of alkaline-phosphatase-conjugated rabbit anti-mouse secondary antiserum (Dakopatts, Glostrup, Denmark) for 1 hour. The blot was developed with 5-bromo-4-chloro-indolylphosphate/nitroblue tetrazolium (NBT/BCIP) until bands appeared; the reaction was stopped by immersing the blot in 10% acetic acid.

Immunohistochemistry

The antibody was tested on routinely processed paraffin-embedded tissue by an indirect avidin-biotin complex (ABC) immunohistochemical method. Briefly, sections were dewaxed and rehydrated, and endogenous peroxidase activity was blocked with hydrogen peroxide/methanol. Antigen retrieval was carried out by pressure-cooker unmasking for 1 minute in citrate buffer (200 mmol/L citric acid, 500 mmol/L NaOH, pH 6.0). The tissue was then blocked with 1:20 normal rabbit serum in Tris-buffered saline (TBS; 140 mmol/L NaCl, 50 mmol/L Tris/HCl, pH 7.6) and incubated for 1 hour with primary antibody. After washing in TBS, a biotinylated rabbit anti-mouse secondary antibody was added at 1:500 (Novocastra, Newcastle, UK), and the sections were incubated for 30 minutes. Sections were again washed with TBS and then incubated with avidin-biotin at 1:100 (Novocastra). After a final wash in TBS, the sections were developed using diaminobenzidine in a TBS/hydrogen peroxide solution for 3 minutes. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted in distrene dibutyl-phthalate xylene. Tissues showing membrane or cytoplasmic staining of any cells were assessed as positive. In neoplastic lymphoid tissue, no attempt was made to assess the proportion of cells showing positive labeling.

Tissues

All tissues used were routinely processed formalin-fixed, paraffin-embedded specimens retrieved from the archival files of the Pathology Department, Royal Victoria Infirmary, Newcastle-on-Tyne, UK. A wide range of normal tissues was studied as shown in Table 1. Pathological tissues included 58 cases of childhood acute lymphoblastic leukemia, 13 cases of diffuse large-B-cell lymphoma, and 60 cases of small-B-cell lymphoma. The

Table 1. Immunostaining for CD10 on a Range of Normal Tissues

Tissue	Morphological feature	CD10 labeling
Brain	Cerebellum	Negative
Colon	Germinal centres (in lamina propria)	Positive
	Epithelium	Negative
Gall bladder	Brush border epithelium	Positive
Kidney	Glomeruli	Strong positive
	Tubules	Positive
Liver	Bile ducts-luminal border	Positive
	Parenchymal cells	Negative
	Cannaliculi	Strong positive
Lung	Bronchial epithelium	Negative
	Interstitial stromal cells	Positive
	Alveoli	Negative
Lymph node	Follicle centers	Positive
Muscle	Striated muscle cells	Negative
	Nerves (Schwann cells)	Positive
	Inter-fascicular stromal cells	Positive
Pancreas	Glandular acini	Negative
	Islets	Negative
	Ducts	Negative
Placenta	Syncitial trophoblast	Strong positive
	Cytotrophoblast	Positive
	Stromal cells	Negative
Prostate	Glandular epithelium	Positive
	Smooth muscle	Negative
	Prostate glands	Positive
	Prostatic fluid	Positive
Skin	Epidermis	Negative
	Sebaceous glands	Weak focal positive
	Sweat glands	Negative
	Vessels	Negative
Small bowel	Enterocyte	Strong positive
Spinal cord	Column	Negative
	Peripheral nerve (Schwann cells)	Positive
Testis		Negative
Thyroid gland	Follicular epithelium	Negative
	Vascular endothelium	Focal positive
Tonsil	Germinal centers	Positive
Umbilical cord	Stromal fibroblasts	Positive
	Vessels	Negative
	Amnion layer	Negative
Ureter	Smooth muscle	Negative
	Transitional epithelium	Negative
Uterus	Endometrium, stromal cells	Positive
	Endometrium, glands	Negative
	Myometrium	Negative

latter included 11 cases of follicle cell lymphoma, 23 cases of mantle cell lymphoma, 10 cases of marginal zone lymphoma, 12 cases of lymphocytic lymphoma, and 4 cases of lymphoplasmacytoid lymphoma. These cases were defined according to morphology assessed using hematoxylin-stained sections together with immunostaining for B- and T-cell markers CD5, CD23, CD43, and cyclin D1.

Results

Antibody Production

The recombinant protein was generated as described and used to produce an immune response in mice. After the fusion, the supernatants from each well were assayed by ELISA, and 195 colonies showed reactivity. These were transferred to fresh plates and, after an additional

period of growth, were tested again by ELISA, and 70 colonies selected for testing on paraffin-embedded small bowel sections. Colonies showing positive staining of the cytoplasmic membrane were subjected to another three rounds of cloning by double dilution and assayed by Western blot.

Specificity of the antibody for CD10 was demonstrated by ELISA, Western blotting, and the labeling patterns on a panel of normal and neoplastic tissues. A single clone was isolated that showed positivity for all tests; isotyping of the supernatant revealed that the monoclonal antibody was of the IgG1 subtype. The antibody was termed NCL-CD10-270.

Antibody Characterization

Analysis by Western blot showed that the antibody labeled a protein of ~93 kd in a placental cell lysate (see

Figure 1. Western blot for monoclonal antibody NCL-CD10–270 using a placental cell lysate. Note reactivity for CD10 at molecular weight 100,000.

Figure 1). This is slightly below the generally accepted 100-kD molecular mass of the protein but is within acceptable limits due to the variable post-translational modification of the 86-kD immature polypeptide.⁹ Analysis by immunohistochemistry revealed labeling that was principally confined to the cell membrane and cytoplasm, and antibody reactivity on a panel of normal tissues is described in Table 1. Examples of tissues showing labeling include lymphoid germinal centers, renal tubules of glomeruli, syncytiotrophoblast, and hepatic parenchymal canaliculi.

Further characterization of the antibody was carried out on pathological tissues, including small-B-cell lymphomas, diffuse large-B-cell lymphomas, and a series of childhood acute lymphoblastic leukemias.

CD10 in Childhood Acute Lymphoblastic Leukemia

These comprised a series of cases presenting to the Royal Victoria Infirmary between 1985 and 1990, who were entered into the Medical Research Council trial UKALL X. Bone marrow aspirates from all patients were tested for CD10 status at diagnosis by either immunofluorescence of cyto-centrifuge preparations using a

Coulter (J5) antibody or flow cytometry using a Becton Dickinson anti-CALLA monoclonal antibody (W8E7), which are both of proven efficacy. We have re-assessed paraffin-embedded tissue blocks of bone marrow trephines from all patients using NCL-CD10–270. The results showed a strong positive correlation using Fisher's exact test ($P = 0.001$; Table 2).

CD10 in Other Pathological Tissues

Of 13 cases of large-B-cell lymphoma, 4 proved positive for CD10. In small-B-cell lymphoma, labeling for CD10 was observed in 10 of 11 cases of follicle center cell lymphoma and 2 of 23 cases of mantle cell lymphoma. Cases of lymphocytic, marginal zone, or lymphoplasmacytoid lymphoma all proved negative. These results are described in greater detail with results for other markers and molecular studies in a separate publication (submitted for publication).

Discussion

The monoclonal antibody NCL-CD10–270 has been generated using a recombinant protein immunogen corresponding to the whole of the CD10 molecule. The specificity of this antibody is supported by reactivity with a molecule of the appropriate molecular weight in Western blot assays. The antibody has proven to be highly efficient for immunostaining in paraffin-embedded tissue. The predicted widespread distribution of the antigen at various sites has been observed. The strongest immunostaining was observed in renal tubules and glomeruli, brush borders of the intestine, and lymphoid germinal centers. These results are in keeping with the wide tissue distribution of CD10, which has been previously described.^{10–12}

CD10, also known as the common acute lymphoblastic leukemia antigen (CALLA) is used in the characterization of acute lymphoblastic leukemia. CALLA positivity in ALL is associated with a better response to treatment and/or prognosis.^{13,14} We have carried out a comparative study in bone marrow trephines using cases previously assessed for CALLA status by flow cytometry or immunofluorescence, and an excellent correlation of results was obtained. Of the 47 cases originally diagnosed as CALLA-positive, 45 also showed labeling with NCL-CD10–270 (Table 2). Four of nine cases that were initially diagnosed as being CD10 negative were found to be positive using NCL-CD10–270. Although this figure is unexpectedly high, some variability is to be expected as the threshold values for these methods are likely to be different, and the discrepancies may represent greater sensitivity of our technique. This antibody may therefore prove to be of significant diagnostic utility in immunophenotyping acute leukemias in bone marrow trephine biopsies. The antibody may serve as a useful addition to the limited panel of antibodies currently available,¹⁵ although these are of proven efficacy for flow cytometric or fresh-frozen tissue applications only.

Figure 2. Staining of normal and pathological tissues with NCL-CD10-270 using a standard ABC immunohistochemical method; magnification, $\times 200$ unless otherwise stated. **A:** Lymph node, reactive hyperplasia. Note strong staining of most cells in germinal centers and general absence of staining of cells in the paracortex. **B:** Normal human kidney. Note staining in glomerular epithelial cells and luminal aspect of ascending/descending tubules. **C:** Normal human small intestine. Note strong immunostaining of brush borders of enterocytes. **D:** Prostate gland, hyperplasia. Note intense staining of prostatic glandular epithelial cells, maximal at luminal aspect of the glands. **E:** Lymph node, follicular lymphoma (magnification, $\times 40$). The follicle center cells show intense labeling in contrast to near complete absence of staining of reactive cells between the follicles. **F:** Bone marrow, acute lymphoblastic leukemia (magnification, $\times 400$). The tumor cells show intense cytoplasmic labeling with membrane accentuation in some cells.

In a study of CD10 expression in a series of small-B-cell lymphomas using NCL-CD10-270, we found strong staining in follicle center cell lymphoma; occasional mantle cell lymphomas demonstrated labeling, but all cases of lymphocytic, marginal zone, and lymphoplasmacytoid lymphoma were negative. These results are in accordance with known characteristic immunoprofiles of these tumors derived from previous studies using antibodies effective only in frozen tissues¹⁶ and will be discussed in greater detail in a forthcoming publication. Diagnosis of

small-B-cell lymphoma is usually straightforward using H&E-stained preparations together with limited immunostaining. However, some cases present diagnostic difficulty, and the specificity for CD10 demonstrated here for certain subtypes indicates that NCL-CD10-270 may be of particular value in the differential diagnosis of these cases.

Of 13 cases of large-B-cell lymphoma assessed, 4 were shown to be CD10 positive, but the clinical and biological significance of this is unknown. It is possible

Table 2. Immunolabeling with Monoclonal Antibody NCL-CD10-270

NCL-CD10-270 labeling	Childhood ALL CD10 status at diagnosis	
	CD10 positive	CD10 negative
Positive	45	4
Negative	2	5

Immunolabeling using monoclonal antibody NCL-CD10-270 on formalin-fixed, paraffin-embedded bone marrow trephine samples involved by acute lymphoblastic leukemia compared with CD10 status assessed at diagnosis by flow cytometry or immunohistochemistry shows a clear correlation of 89% ($P = 0.001$, Fishers exact test).

that some of these CD10-positive cases may represent high-grade transformation of follicle center cell lymphomas, which would have significant therapeutic implications for these patients. Additional work is needed to address this question; to our knowledge, there have been no previous investigations with respect to the possible prognostic value of CD10 in this subgroup of lymphomas.

In conclusion, we consider that monoclonal antibody NCL-CD10-270 should prove of value in the diagnosis and characterization of malignant leukemias and lymphomas, in particular, small-B-cell lymphomas and acute lymphoblastic leukemias, and may also be useful in the investigation of CD10 expression at other sites in normal and pathological tissues.

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