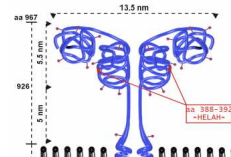


IMMUNOPHENOTYPIC AND MOLECULAR STUDY OF A PARTICULAR EXPRESSION PATTERN OF CD13 EPIOTOPE 7H5 IN CHRONIC LYMPHOCYTIC LEUKEMIA



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BACKGROUND

CD13/aminopeptidase N (APN) is a membrane-bound, zinc-dependent metalloproteinase that plays a key role the control of cell growth and differentiation, as well as tumor invasion and angiogenesis, that might be strongly modified by APN inhibitors, such as the phenolic natural product curcumin. CD13 has been widely applied in leukaemia phenotyping. However, it is rarely reported in mature lymphoid malignancies and is generally correlated to poor prognosis. We developed a novel monoclonal antibody (Mab) 7H5, which was classified as CD13 during the 6th Leukocyte Typing Workshop, Kobe, 1996 and immunoprecipitated a molecule with molecular weight of 130-170 kDa, which was suggested to recognise a new epitope. 7H5 did not inhibit the reference myeloid-associated Mabs MCS-2 (MR 17), neither LeuM7. The three Mabs were shown on peripheral blood granulocytes and monocytes but not on normal lymphoid cells, erythrocytes and platelets, yielding similar reactivity with more than 80% of acute myeloid leukaemia (AML) cases. However, the reactivity with lymphoid malignancies has been poorly studied [Kishimoto et al. (eds) *Leucocyte typing VI*. 1997, Michova et al 2003].

THE AIM OF THE STUDY

To study the phenotypic pattern and genotypic expression of Aminopeptidase N (CD13) in chronic lymphocytic leukemia (CLL) samples, as well as in other lymphoproliferative malignancies, by comparing the binding pattern of 7H5 to the reference CD13 antibody LeuM7 by direct immunofluorescent staining and flow cytometry, and to molecular data for CD13mRNA expression obtained by RT-PCR. To study the potential antileukemic activity of curcumin in CLL.

MATERIAL & METHODS

✚ Bone marrow and/or peripheral blood samples from 50 CLL patients [mean age 62 yrs ± 11 yrs, ranging 37-89 yrs] and 85 patients with precursor and mature lymphoid malignancies [mean age 52 yrs ± 15 yrs, ranging 19-76 yrs] were included in the study. Diagnosis and subclassification was based on WHO criteria (2008). Leukocyte number in the peripheral blood was $40 \times 10^9/l \pm 39 \times 10^9/l$, ranging $1.5 - 170 \times 10^9/l$.

Flow cytometric study

Direct immunofluorescent staining and flow cytometry analysis (FACSCalibur, Becton Dickinson, USA) according to the protocol of Mansour, using:

Mouse monoclonal antibody **7H5-PE** [IgG2a] [BulBio NCIPD Ltd, Sofia]

Mouse monoclonal antibody **LeuM7-PE** - Becton Dickinson [B-D, Cat N 347837]

Phenotyping panel of Mabs [myeloid: CD11c, CD13, CD14, CD15, CD33, CD64; B-lymphoid: CD10, CD19, CD20, CD21, CD22, CD23, IgM; T-lymphoid: CD1a, CD2, CD3, CD4, CD5, CD7, CD8, [B-D]; others: CD45, CD34, CD56, CD38, HLA-DR [BulBio NCIPD Ltd, Sofia]

Stained cells were run on a FACSCalibur flow cytometer (BD) and analysed by "CellQuest Pro" software as previously described. At least 10 000 events for each sample were collected and analysed.

Molecular studies of mRNA expression of CD13 by RT-PCR

Total RNA was extracted from leukemic cells using column ready to use kit. Reverse transcription: random hexamers and MMLV reverse transcriptase [Promega]. Multiplex PCR: Taq-polymerase and 30 cycles of amplification [Eppendorf] with 2 sets of primers:

CD13

CD13-S :5'-gTCCAgggTCCAggTCCAg-3'

CD13-AS:5'-TgACAgTggATATTgTgCAC-3'

B2-microglobuline - as an internal control for mRNA integrity

B2-M1(S): 5'-ACCCCACTgAAAAgATgA-3'

B2-M2(AS):5'-ATCTTCAAACCTCCATgATg-3'

Analysis by electrophoreses in 3% agarose gel, Ethidium bromide staining under UV.

MMT-dye reduction assay

The cytoreductive activity of curcumin was evaluated in primary cell cultures of mononuclear cells isolated from peripheral blood from newly diagnosed patients with CLL using the MTT-dye reduction assay.

✚ Table 1. Patterns of CD13 expression in leukaemic patients.

Diagnosis	7H5+ LeuM7+	7H5+ LeuM7-	7H5- LeuM7+	7H5- LeuM7-	Total
Chronic lymphocytic leukemia	9 [18%]	33 [66%]	1 [2%]	7 [14%]	50
Other Mature B-cell lymphomas	2 [7%]	21 [67%]	2 [7%]	6 [19%]	31
Lymphoplasmocytic lymphoma	1	6	0	0	7
Hairy cell leukemia	0	3	0	3	6
Marginal zone lymphoma	0	4	1	0	5
Mantle cell lymphoma	0	3	0	2	5
Follicular lymphoma	0	2	0	1	3
Diffuse large B-cell lymphoma	1	3	1	0	5
Precursor B-lymphoblastic leukemia	1	6	9	21	37
T-cell lymphoid neoplasms	0 [0%]	1 [6%]	0 [0%]	16 [94%]	17
Precursor T-lymphoblastic leukemia	0	1	0	6	7
T-cell lymphomas	0	0	0	10	10
Acute myeloid leukemia	44 [83%]	0 [0%]	8 [15%]	1 [2%]	53

DISCUSSION

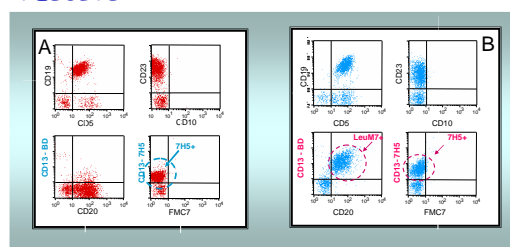
✚ Remarkably, the 7H5 anti-CD13 Mab was detected in a significant proportion of B-cell neoplasms, particularly in CLL, thus allowing for the identification of CD13, which otherwise might have remained underestimated in a total of 86 % of CLL cases. Our data showed that the Mab clones used to detect the CD13 antigen have variable immunoreactivity against cells with B-cell phenotype, which may partly explain the conflicting reports concerning the incidence of myeloid antigen expression in lymphomas. [Nakase et al 1996; Cocco et al 2005; Ahmad et al 2008; etc.].

✚ RT-PCR for CD13mRNA showed good correlation with immunophenotypic data allowing for specific identification of CD13 transcripts in LeuM7(+) and/or 7H5(+) leukemic cell populations, which has not been reported so far in CLL. Since various molecular mechanisms are involved in the regulation of CD13/APN transcription, including the c-maf and c-myc proto-oncogenes, angiogenic signals, ras signaling pathway, etc, further investigations are warranted [Mahoney et al 2007; Petrovic et al 2003, Bhagwat et al 2001, 2003; Rangel et al 2007; etc.].

✚ The incidence of CD13/APN in B-cell neoplasms and CLL, particularly, presumes that the inhibition of APN/CD13 may be an effective new molecular target therapy for these patients. Natural and synthetic inhibitors of APN activity have been characterized so far and revealed that APN is able to modulate major biological events (cell proliferation, invasion, angiogenesis). [Bauvois et al. 2006; Luan et al 2007; Zhang et al 2008; etc.].

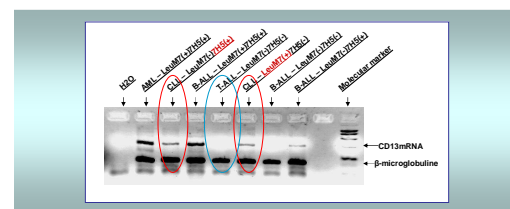
✚ Using MTT-dye reduction assay we demonstrated that curcumin as a potent CD13/APN inhibitor [Shim et al 2003] caused a concentration dependent antileukemic activity on freshly isolated and cultured mononuclear cells from CD13-pos CLL patients. These preliminary in vitro data indicate that curcumin might be useful in CLL and further investigations of curcumin+drug combinations are planned.

RESULTS



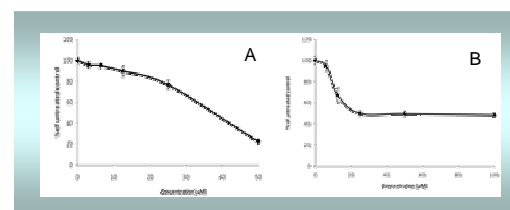
✚ Figure 1. Expression of 7H5 epitope in B-CLL.

Peripheral blood samples from B-CLL patients were stained as described in Materials & Methods section. Histograms A - a patient with a discordant CD13 phenotype (7H5+LeuM7-); Histogram B - a patient with a concordant positive CD13 phenotype (7H5+LeuM7+) of CLL B-cells.



✚ Figure 2. RT-PCR for CD13 mRNA in leukemias.

The multiplex RT-PCR performed as described revealed a heterogeneous pattern of CD13 mRNA expression in haematological malignancies: AML - acute myeloid leukemia; ALL - acute lymphoblastic leukemia; CLL - chronic lymphocytic leukemia. PCR positivity was in concordance with either 7H5 or CD13-BD positivity in CLL patients.



✚ Figure 3. MMT-dye reduction assay in CLL samples.

Curcumin caused a concentration dependent antileukemic activity on freshly isolated and cultured mononuclear cells from CLL patients. The incubation time was 72 h and cell viability was detected using the MTT-dye reduction assay. Patient (A) - with CD13-neg CLL (7H5-/LeuM7-) showing low sensitivity to curcumin; Patient (B) - with CD13-pos (7H5+) CLL, interestingly, showed greater sensitivity to lower curcumin concentrations and with the concentration increase a plateau of the efficacy was reached.