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M10 CD34 cluster workshop report

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A total of 18 murine monoclonal antibodies (mAb) was submitted to the CD34 cluster workshop and evaluated by 16 laboratories. All but one antibody was verified as recognizing CD34 antigen, the most direct evidence being reactivity with cells transfected with CD34 cDNA and binding to CD34 protein (coated on to beads or in Western blots). These 17 CD34 antibodies all reacted also with tissue fibroblasts and with vascular endothelium. The epitope specificity of the 17 antibodies could be classified into three broad classes (I, II, III) according to differential sensitivity of the epitopes to enzymatic cleavage, paraffin processing (of tissue sections), and reactivity with high endothelial venules (HEV). Reactivity with leukaemic cells was very variable with some evidence for discordant expression of CD34 epitopes on individual cells.

CD34 antibodies define an antigen selectively expressed on haemopoietic progenitor cells [1-3], vascular endothelium, and some tissue fibroblasts [4,5]. The CD34 cell surface molecule has been biochemically characterized [6,7], the human [8-11] and mouse cDNAs and gene [12,13] cloned and sequenced, and the human CD34 gene localized to chromosome 1q32 [14; reviewed in 15,16]. CD34 is

an unusual glycoprotein, similar in some respects to leucosialin (CD43) [17] in being rich in O-linked carbohydrates and sialic acid, suggesting a mucin-like structure [7,8,15,16]. These features, plus ultrastructural localization in vascular endothelium microprocesses and modulation reciprocally with adhesion molecules *in vitro* [18] and in inflammatory reactions *in vivo* [19] have suggested that CD34 may play a role in modulating cell adhesion [15]. A recent report suggests that lymph node HEV CD34 may provide an adhesive ligand for L-selectin [20].

The availability of CD34 antibodies has greatly facilitated experimental and clinical studies on human haemopoietic progenitors, candidate stem cells, and leukaemic cell populations [21-31]. Several antibodies have been allocated to the CD34 cluster in previous Workshops [32] and more candidate CD34 antibodies are becoming available. In view of the widespread clinical use of these reagents for bone marrow manipulation, their commercialization, and the evidence that different CD34 antibodies may recognize distinct epitopes, a careful characterization and comparison of all such antibodies is of considerable importance. The Workshop format provides the ideal opportunity for such studies.

Table 1 Details of the antibodies submitted to the CD34 cluster workshop

Workshop mAb				
Code	Clone name	Donor	Isotype (Ig)	Preparation
MA2	Immu-133	Hirn	G1	Protein (1 mg/ml)
MA3	Immu-409	Hirn	G2a	Protein (1 mg/ml)
MA7	14G3	Lansdorp	G1	Protein (1 mg/ml)
MA8	BI-3C5*	Tindle	G1	Ascites
MA11	CD34-9F2	Knapp	G1	Ascites
MA22	HPCA2* (8G12) [†]	Warner	G1	Protein (1 mg/ml)
MA25	43A1	Bühring	G3	Ascites
MA27	581	Gaudernack	G1	Ascites
MA28	570	Gaudernack	G1	Ascites
MA29	553	Gaudernack	G1	Ascites
MA30	563	Gaudernack	G1	Ascites
MA53	MD34.3	van der Schoot	G1	Protein (1 mg/ml)
MA55	MD34.1	van der Schoot	G2b	Protein (1 mg/ml)
MA56	MD34.2	van der Schoot	G1	Protein (1 mg/ml)
MA65	QBEnd 10* (IOM-34) [†]	Jacob	G1	Protein (1 mg/ml)
MA74	4A1	Hagiwara	G1	Protein (1 mg/ml)
MA80	9066	Gee	G1	Protein (1 mg/ml)
MA81	9069	Gee	G1	Protein (1 mg/ml)

*Evaluated and verified as CD34 antibodies in the previous Workshop, [see 32 for summary]. mAb BI-3C5 and QBEnd10 are discussed in references 2 and 4, respectively.

[†]Some of these antibodies are already commercialized under different names, e.g. QBEnd10 (IOM-34) or have commercial names that differ from original publication, e.g. HPCA2 (8G12).

Table 2 Arbitrary codes for participants who tested the CD34 antibodies

Participant code	Participant group
1	Greaves
2	van der Schoot
3	Knapp
4	Merle-Béral
5	Sutherland
6	Gaudernack
7	Bühring
8	Garrido
9	Inglés-Esteve
10	Lansdorp
11	Campos
12	Razak
13	Parravicini
14	Invernizzi
15	Castoldi
16	Springer

Results and discussion

CD34 cluster verification

Table 1 lists the submitted mAb, their Ig isotype, form (ascites or IgG), and laboratory of origin. Aliquots of these antibodies were distributed to 16 groups

(Table 2) who performed a variety of tests relevant to antibody specificity/cluster analysis. Preliminary screening in most laboratories was by flow cytometric analyses of the staining of CD34-positive cell lines KG1 or KG1a, coupled, unfortunately in only two cases, with other known CD34-positive or CD34-negative cell lines. These data were in accord with the CD34 reactivity of all of these antibodies with the possible exception of MA28 (570), which appeared to have a broad pattern of cellular reactivity including cell lines such as K-562 and RS4;11 that have no detectable stable mRNA for CD34 (group 1 [15]). Although this analysis was based on objective criteria (for example, staining intensity by flow cytometry), the data should be considered as essentially qualitative as no standardized control was used. We recommend that such controls are incorporated in any future CD34 workshop. In addition, the data generally apply to staining obtained with high concentration/saturated levels of antibody binding thus obscuring considerable differences in titre (which varied from 1:200 to >1:2000; data available on request from MFG).

Very few laboratories reported detailed testing of the candidate CD34 antibodies against bone marrow or any source of normal CD34-positive progenitor cells. This, in retrospect, was a deficiency in the Workshop study design and should be rectified in any

Table 3 Staining reactions of CD34 Workshop mAb on transfectant cells, protein-coated beads, and Western blot analysis on uncleaved CD34 antigen

Workshop mAb Code	Clone name	Reactivity* on (participating group)							Western blot efficiency (gp110) (5)
		COS/HUCD34 cells (1)		CD34 ⁺ L murine cells (10)		CD34 ⁺ NIH3T3 cells (7)		CD34 protein- coated beads (3)	
		-/+	+	-/+	+	-/+	+		
MA2	Immu-133	-/+	++	++	+	++	++	++	
MA3	Immu-409	-	+	+	-	+	+	++	
MA7	14G3	+	+	+	+	+	+	+	
MA8	BI-3C5	+	+	+	+	+	+	++	
MA11	CD34-9F2	++	-/+	-/+	+	+	+	+	
MA22	8G12	++	++	++	+	+	+	+	
MA25	43A1	+	-/+	-/+	+	+	+	++	
MA27	581	++	++	++	+	+	+	-	
MA28	570	-	-	-	-	-	-/+	-	
MA29	553	++	++	++	+	+	+	-	
MA30	563	++	++	++	+	+	+	+	
MA53	MD34.3	++	++	++	+	+	+	++	
MA55	MD34.1	++	++	++	+	+	+	++	
MA56	MD34.2	++	++	++	+	+	+	++	
MA65	QBEnd10	++	++	++	+	+	+	++	
MA74	4A1	++	++	++	+	+	+	++	
MA80	9066	++	++	++	+	+	+	++	
MA81	9069	++	++	++	+	+	+	++	

* -, No reactivity; -/+, very weak or equivocal staining/reactivity; +, weak staining/reactivity; ++, strong staining/reactivity; +++ very pronounced staining/reactivity.

Table 4 Epitope specificity of CD34 mAb as determined by enzyme cleavage

Epitope class*	Antibody	Participant groups
I Sensitive to neuraminidase, chymopapain, and glycoprotease	MA2, MA3, MA7, MA8	1, 2, 3, 4, 5
II Resistant to neuraminidase. Sensitive to chymopapain and glycoprotease	MA25, MA53, MA55, MA56, MA65, MA74, MA80, MA81 [†]	1, 2, 4, 5, 6
III Resistant to neuraminidase, chymopapain, and glycoprotease	MA11, MA22, MA27, MA29, MA30	1, 2, 4, 5, 6 [‡]

*Neuraminidase from *Vibrio cholerae* (except participant group 1 which used *Clostridium perfringens*). CD34 antibodies recognizing class I epitopes show greatly diminished binding to neuraminidase-treated cells (e.g. KG1 or KG1a) and fail to detect desialylated CD34 in Western blots. Glycoprotease from *Pasteurella haemolytica* (33).

[†]Antibodies MA53, MA55, MA56, and MA81 were found to be slightly sensitive (partial loss of staining intensity) to chymopapain and/or neuraminidase by three groups (2,3,5), but not others. Some groups (eq 2) but not others (eq 1) suggested that partial sensitivity to neuraminidase should qualify an epitope as class I. Further studies are required to resolve this issue.

[‡]Group 6 used only glycoprotease.

subsequent Workshop. Lansdorp reported quite variable reactivity with bone marrow mammalian cells with the known light scatter properties of CD34-positive progenitor cells from strongly staining mAb MA2 (Immu-133), MA8 (BI-3C5), MA11 (CD34-9F2), MA25 (43A1), and MA55 (MD34.1); other antibodies give either intermediate level staining (mAb MA22 (8G12), MA53 (MD34.3), MA56 (MD34.2), MA80 (9066), and MA81 (9069) or no staining at all (MA3 (Immu-409)). In general, this pattern parallels the titre of the antibodies (as determined by flow cytometry against KG1/KG1a cells—group 1).

Definitive tests for CD34 specificity were considered to be: (1) reactivity with otherwise CD34-negative cells transfected with CD34 cDNA (note, however, that since different cells may not all process or glycosylate the CD34 protein backbone identically, failure to react with a CD34 cDNA transfected cell line does not rule out that an antibody recognizes a form of CD34; see Table 3, for example, with MA11 (CD34-9F2)); (2) reactivity with purified CD34 protein; and (3) as confirmation, reactivity with the appropriately sized molecules by Western blot.

The data obtained (Table 3) provided three main conclusions.

1. All but MA28 (570) are antibodies that recognize CD34 protein.
2. Choice of recipient cell type for cDNA transfectants can influence expression of the antigen (or its epitopes).
3. Not all antibodies can recognize CD34 glycoprotein in Western blots. In particular, antibodies recognizing class III epitopes (see further below)

Table 5 Cross-blocking experiments for mAb in the CD34 cluster workshop

Cross-reacting Workshop mAb*	Participant group [†]
Staining mAb HPCA2 (8G12) (class III)	
MA22, MA27, MA29, MA30	7
MA22, MA27, MA29, MA30	4
MA22, MA27, MA29, MA30 (MA25, MA74, MA80) [‡]	8
MA22, MA27, MA29, MA30	6
MA22, MA27, MA29, MA30	2
MA22, MA25, MA27, MA29, MA30	11
Staining mAb QBEnd10 (IOM-34) (class II)	
MA65	7
MA65, MA74, MA80	4
MA53, MA55, MA56, MA65 (MA25, MA74, MA80) [‡]	8
Staining mAb ICH3 (class II)	
MA28, MA53, MA56, MA80	6
Staining mAb MA25 (class II)	
MA74	7
Staining mAb MA55 (class II)	
MA53, MA56, MA74	7
MA8 [†] , MA53, MA56 (MA25, MA65, MA74, MA80, MA81) [§]	2
Staining mAb MA56 (class II)	
MA8 [†] , MA53, MA55 (MA25, MA65, MA74, MA80, MA81) [§]	2

*KG1/KG1a cells were reacted with the Workshop mAb first and then with directly conjugated staining mAb. Note: Participant group 8 used leukaemia specimens.

[†]Participant group 9 performed blocking with a series of synthetic peptides, none of which blocked staining of the mAb.

[‡]mAb in brackets blocked both HPCA2 and QBEnd10 to some degree.

[†]Cross-blocking of MA55 and MA56 by MA8 [see Dercksen *et al.* M10.2] was not confirmed by other participant groups.

[§]mAb in brackets show partial blocking of staining antibody.

mAb code (epitope class)	Vascular endothelium (capillaries)			Stromal fibroblasts [†] frozen section	HEV [‡] frozen section
	Frozen section	Paraffin section	Plastic section		
MA2 (I)	+	+	-	+	-
MA3 (I)	+w	+w	-	+	-
MA7 (I)	-•	-•	-	+	-
MA8 (I)	+	+	-	+	-
MA11 (III)	+	-	-	+	+
MA22 (III)	+	-	-	+	+
MA25 (II)	+	+	-	+	+
MA27 (III)	+	-	-	+	+
MA29 (III)	+	-	-	+	+
MA30 (III)	+	-	-	+	+
MA53 (II)	+	+	-	+	+
MA55 (II)	+	+	-	+	+
MA56 (II)	+	+	-	+	+
MA65 (II)	+	+	-	+	+
MA74 (II)	+	+	-	+	+
MA80 (II)	+	+	-	+	+
MA81 (II)	+	+	-	+	+
Tested by group	1, 13	1, 13	1	1,13	16

*See Parravicini *et al.* [M10.4] for further details. w, Weak staining; •, non-specific staining all over section.

[†]See Parravicini *et al.* [M10.5] for further details.

[‡]HEV, high endothelial venules. See Girard and Springer [E6.9] for details. Antibodies scored as positive (+) varied considerably in intensity of staining. MA80 and MA81, for example, gave only weak staining. Note: HEV showed staining with MA28 as well.

are very poorly reactive, presumably because these epitopes are sensitive to denaturation.

Epitope specificity

Previously published studies [for example, 6,16,33] including the last CD34 workshop [32] have established that different CD34 antibodies recognized different epitopes that can be distinguished by their differential sensitivity to enzymatic cleavage with neuraminidase, chymopapain, and glycoprotease from *Pasteurella haemolytica*. Table 4 summarizes the data for neuraminidase, chymopapain, and glycoprotease for which there was good overall agreement and confirmation of some earlier results (for example, for MA8 (BI-3C5) and MA65 (QBEnd10)).

Several groups (2, 4, 6, 7, 8, and 11 in Table 2) attempted to interrelate epitopes by cross-blocking experiments. Cross-blocking data was generally in accord with this classification of epitope reactivity. Thus using QBEnd10 CD34 antibody as a staining antibody standard, its class II type reactivity was reported to be blocked only by class II antibodies,

although not by all class II antibodies: group 7 reported blocking by MA65 only; group 4 reported blocking by MA65, MA74, and MA80; and group 8 reported blocking by MA53, MA55, MA56, and MA65 and partial blocking by MA25, MA74, and MA80. Using the CD34 antibody HPCA2 as a staining antibody standard, the class III type reactivity was reported to be blocked only by class III epitope reactive CD34 antibodies MA22, MA27, MA29, and MA30 (but not MA11). See Table 5 for details.

No cross-blocking was reported as having been tested using a class I epitope reactive antibody.

This classification into three epitope classes or groups probably underestimates the extent of antigenic, epitopic diversity on the extracellular domains of CD34. Evidence indicative of further epitope variation within the three major classes defined by enzymatic cleavage includes the following.

- Cross-blocking by some, but not all, antibodies (subject to the caveat that this assay is dependent upon relative titre and affinity of the antibody used) as detailed above.

Table 7 Summary of reactivity of CD34 mAb in tissue sections

Epitope specificity (class)	Vascular endothelium (capillaries)			Fibroblasts		HEV frozen section
	Frozen section	Paraffin section	Plastic section	Frozen section	Paraffin section	
I	+	+	-	+	+	-
II	+	+	-	+	+	+
III	+	-	-	+	-	+

Table 8 Summary of tests that may identify CD34 epitope classes

Epitope specificity (class)*	Enzyme sensitivity			Reactivity [§]		
	Neuraminidase [†]	Chymopapain [‡]	Glycoprotease [‡]	VE/F paraffin section	HEV frozen section	Western blot
I	+	+	+	+	-	+
II	-	+	+	+	+	+
III	-	-	-	-	+	-

*Some antibodies do not fit unambiguously into this scheme. In particular, although MA53, MA55, MA56, and MA81 appear to detect class II epitopes, they may have some sensitivity (although less than class I epitopes) to neuraminidase and chymopapain (see Table 4 and footnotes). Also MA81 is inefficient at detecting desialylated CD34 gp150 Western blots.

[†]Assessed by recognition of CD34 on neuraminidase-treated cells and of desialylated CD34 in Western blots.

[‡]Assessed by recognition of CD34 on enzyme-treated cells.

[§]VE, vascular endothelium; F, tissue fibroblasts, HEV, high endothelial venules (of tonsil).

- Discordant reactivity of antibodies within an epitope class (I, II, III) with leukaemic samples, six groups (1, 4, 6, 11, 14, 15) reported antibody reactivity of leukaemic samples and four documented some discordant reactivity although without any clear patterns emerging.
- Variable detection of class I epitopes as denaturation-resistant epitopes by Western blotting (Sutherland).
- Discordant reactivity with haemopoietic cells from other species. Lansdorp reported reactivity of two class III antibodies (MA29, MA30) with rhesus monkey bone marrow cells and weak reactivity of two class II antibodies (MA53, MA56).

Reactivity in tissue sections

Previous studies have demonstrated that several CD34 antibodies react in frozen and/or paraffin-embedded sections with vascular endothelial cells and fibroblasts [4,15,34]. Three of the participating groups further evaluated this reactivity and the data is summarized in a simplified qualitative form in Tables 6 and 7.

These data confirm the reactivity of CD34 antibodies with vascular endothelium and tissue fibroblasts and further reveal variation between the

three classes of epitopes with respect to sensitivity to tissue processing. The failure of antibodies to class III epitopes to recognize vascular endothelial cell-associated CD34 in paraffin sections parallels their failure to bind in Western blots (see above) reinforcing the likelihood that class III epitopes are denaturation-sensitive. Reactivity of high endothelial venules (HEV) with CD34 antibodies is a new observation that also provides an example of discordant epitope expression. The apparent lack of sialic acid-dependent CD34 epitopes on HEV is interesting in relation to the suggestion that CD34 molecules on HEV may function as a ligand for leucocyte L-selectin [20].

Conclusions

Seventeen of the 18 mAb submitted to the CD34 cluster workshop are verified as clustered with CD34 joining those described in the previous Workshop [32]. The most significant contribution from this Workshop is, however, the more detailed insight into epitope specificity of the antibodies and the variable or discordant expression of CD34 epitopes under different conditions. The variable properties of the three major epitope classes of CD34 are summarized in Table 8. Discordant expression *between* epitope

classes on individual cells is observed with HEV [see Girard and Springer, E6.9] and on leukaemic cells [see Titley *et al.*, M10.6; Gaudernack and Egeland, M10.7; Traoré *et al.*, M10.8]. This variation has implications both for the possible function of the CD34 glycoprotein on haemopoietic stem cells and progenitors, endothelial cells, and fibroblasts, and for the choice of CD34 mAb for identifying or isolating CD34-positive cells. Other considerations may be titre and affinity. For histopathological observations in paraffin sections, and for Western blot immunochemical analyses, class II specific antibodies appear best. Epitope variation on normal stem cells should be evaluated as a matter of some urgency.

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