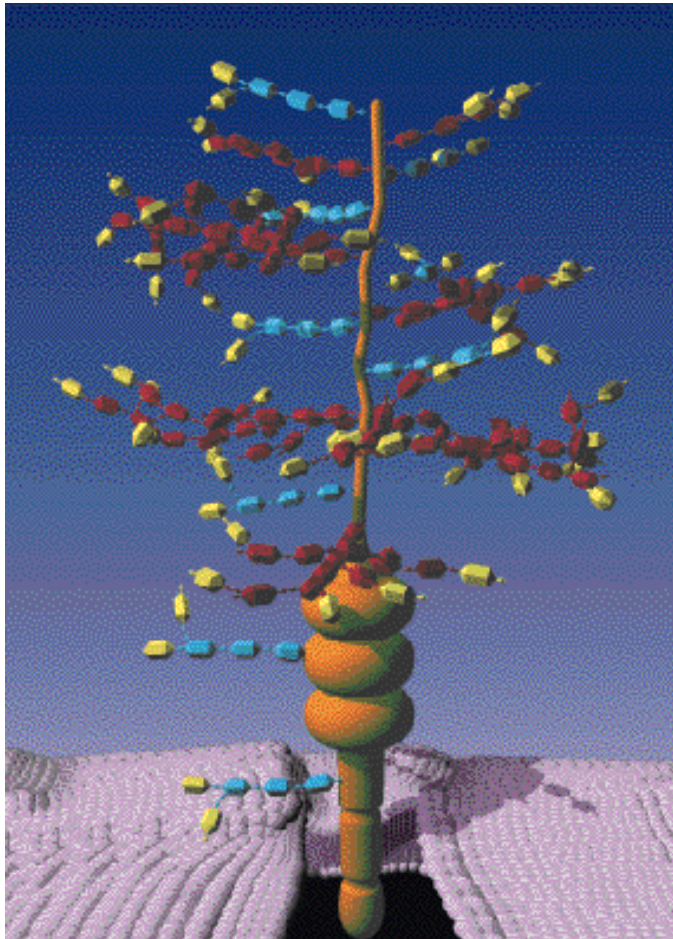


PROGENITORS



▲ Figure 1

The CD34 Antigen

The CD34 molecule is a monomeric type I integral trans-membrane glycoprotein of apparent molecular weight 105-120 kDa. The 373 amino-acid protein backbone (40 kDa) is heavily glycosylated with a maximum of 9 complex-type N-glycans and numerous highly sialylated O-linked glycans. This glycosylation pattern is characteristic of the sialomucin family, which comprise leucosialin/CD43. A key issue in CD34 biochemistry is the polymorphism of glycosylation, as demonstrated by epitope variability in immunological analysis. The gene coding for the CD34 antigen is located on chromosome region 1q32, in a region containing a cluster of genes encoding adhesion molecules. However, the CD34 amino acid sequence shows no identified homology with any known protein.

The function of the CD34 antigen in early hematopoiesis still remains elusive. The mucin-like structure of CD34 suggests a role in cellular adhesion, possibly in adhesion of progenitors and stem cells to the stromal cells. Interestingly, some CD34 monoclonal antibodies (such as Immu-133, see below) can induce homotypic cell adhesion in CD34⁺ cell lines. The 73 amino-acid cytoplasmic domain contains several potential phosphorylation sites which can potentially be phosphorylated by phosphokinase c *in vitro*. However, CD34-mediated signal transduction by this mechanism has not been demonstrated.

Many molecular features of the CD34 molecule remain to be elucidated, including :

- the nature of its polymorphism
- the identity of enzymes involved in its post-transcriptional modifications
- the identification of its putative ligand(s)
- the determination of cytoplasmic substrates for CD34-mediated signal transduction

CD34 monoclonal antibodies are uniquely important research tools to address these open questions.

CD34 Antigen in Early Hematopoiesis

The concept of a common precursor stem cell for all lineages of the hematopoietic system was initially proposed about a century ago by A. Pappenheim. Experimental validation of this concept was impossible until the development of stem cell populations having unique genetic markers. Transfer experiments with cells of this type lead to a complete hematologic reconstitution, with all mature cells expressing the original marker, confirming the hypothesis of a single hematopoietic stem cell. Primitive hematopoietic stem and progenitor cells are very rare events detected in bone marrow and peripheral blood as well as in fetal hematopoietic tissue and umbilical cord blood. This cellular compartment is in fact heterogeneous, comprising extremely primitive stem cells, progenitors committed to differentiation and morphologically differentiated precursors.

The early studies of hematopoiesis were based on tedious *in vitro* cell culture and *in vivo* transfer experiments. In 1984, a major advance was made with the discovery of a monoclonal antibody which bound a cell-surface moiety on all hematopoietic progenitors. Subsequently designated as CD34, this molecule remains the only phenotypic surface marker that identifies early hematopoietic cells. Approximately, 0.1% of normal peripheral blood mononuclear cells and 1% to 4% of human bone marrow cells express the CD34 antigen. The identification and characterization of CD34⁺ hematopoietic stem cells has opened new fields of research, and has become a major interest for both biological and clinical research.



CD34 Monoclonal Antibodies

Twelve years after the generation of the first CD34 monoclonal antibody (clone My10), more than 30 different CD34 antibodies have now been described and clustered in the HLDA workshops (see CD chart insight). Three classes of reactivity were defined for these antibodies based on the differential susceptibility of membrane binding to enzyme treatment of the CD34 antigen. The binding of CD34 antibodies is analyzed after treatment with neuraminidase, O-glycoprotease and chymopapain. This reactivity classification does not correspond to an epitope mapping of CD34 antigen. Antibodies of a given class can recognize spatially distinct epitopes. Conversely, cross-inhibition experiments show that antibodies of different classes can bind overlapping epitopes.

◆ **Class I antibodies react with epitopes sensitive to all three enzymatic cleavages. Immu-409 and Immu-133 belong to this class although both antibodies display distinct characteristics:**

▲ **Immu-409 exhibits a low affinity constant ($1.9 \times 10^7 M^{-1}$ for binding to KG1a cells). No normal progenitor cells and only a few leukemic samples are stained with Immu-409 (1). As the reactivity of Immu-409 is completely dependent on sugar moieties, it is likely that the epitope recognized by Immu-409 corresponds to a non-standard oligosaccharide structure. The antibody works in Western-blotting, immunoprecipitation and in flow cytometry (both direct and indirect immunofluorescence). Immu-409 may be useful in research studies such as epitope mapping, or dealing with CD34 polymorphism. It is not suitable for acute leukemia phenotyping.**

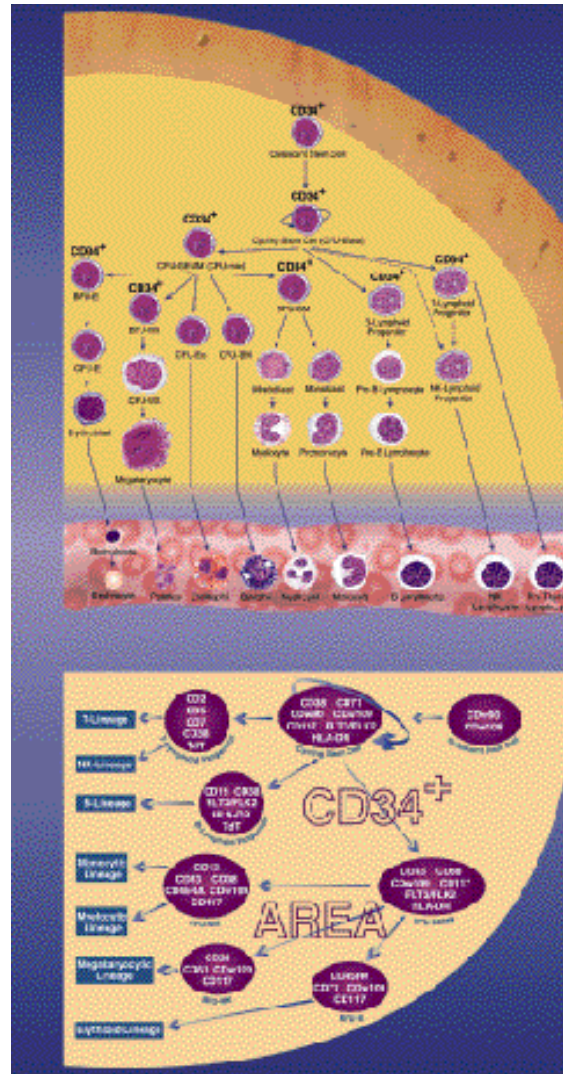
▲ **Immu-133 has an affinity constant of $3.2 \times 10^8 M^{-1}$. It recognizes an epitope located at amino acid positions 43-50, at the amino-terminus of the extra-cellular domain (3). The epitope is partially dependent on associated sugar moieties and seems to be expressed well in several circumstances. The antibody works in western-blotting, immunoprecipitation and in flow cytometry (direct and indirect immunofluorescence). Immu-133 binding induces homotypic cell adhesion (2).**

◆ **Class II antibodies react with epitopes which are resistant to neuraminidase and sensitive to O-glycoprotease and chymopapain. The well-known prototype antibody of this class is QBEnd-10:**

▲ **QBEnd-10 has an affinity constant of $1.3 \times 10^8 M^{-1}$. QBEnd-10 binds an epitope located at amino acid positions 37-55 within the peptide sequence (3). Interestingly, this sequence overlaps with that recognized by Class I antibody Immu-133, confirming the results obtained from cross-inhibition binding studies. QBEnd-10 binding induces homotypic cell adhesion, indicating the functional relevance of the CD34 region recognized by both antibodies Immu-133 and QBEnd-10. For analysis in flow cytometry, the phycoerythrin-conjugated form is particularly recommended. It is also suitable for Western-blotting, immunoprecipitation and immunohistochemistry.**

◆ **Class III antibodies react with the CD34 molecule regardless of its glycosylation state. In a comparison with Class I and Class II epitopes, the expression of Class III epitopes is generally the highest. However, subtle variations in the staining pattern of various $CD34^+$ targets may occur between different Class III antibodies (4). The Class III reactivity appears to correspond with 3 non-overlapping conformational epitopes. 581 is a remarkable Class III antibody:**

▲ **In any fluorochrome conjugation, 581 is so bright that discrimination between negative and positive cells is very clear. 581 recognizes a denaturation-resistant epitope and can be used in Western-blotting (internal data, not published), despite earlier reports that Class III antibodies are inappropriate for this application. This antibody can also be used for immunoprecipitation experiments.**





The four antibodies Immu-409, Immu-133, QBEnd-10 and 581 are representative of the present classification into three large families of epitopes. Despite current uncertainties regarding CD34 structure, CD34 antibodies are widely used for research applications such as immunological studies of hematological malignancies and stem cell enumeration. It is critical to select the appropriate antibody and the appropriate class of reactivity, depending on the particular field of application. The following sections provide some guidelines.

Studies of Hematological Malignancies

The use of flow cytometry for research into hematological malignancy is widespread (5). CD34 expression is associated with an immature phenotype and so co-expression with differentiation antigens typical of mature phenotypes can define abnormal phenotypes associated with oncogenic transformations.

However, the polymorphism of glycosylation patterns in CD34 expression is a major pitfall for the analysis of acute leukemia. Each CD34⁺ leukemic cell expresses a unique combination of CD34 epitopes and might escape recognition by certain CD34 antibodies. If the goal is to detect all CD34 positive malignant cells, then the use of a Class III antibody is a reasonable strategy. To circumvent the CD34 epitope variability, an alternative approach is to pool several antibodies directed against different epitopes.

A future challenge for research on immunological characterization of leukemia is to dissect and classify the post-transcriptional modifications of CD34 antigen in the different malignant cell types. Antibodies belonging to all three classes of reactivity should prove useful in this regard.

Enumeration of Progenitors and Stem Cells

The most important current research use of CD34 antibodies is the enumeration of hematopoietic stem cells by flow cytometry (6). There is an increasing number of studies concerning the mobilization of peripheral blood progenitor cells for autologous and allogeneic transplantation. The combination of CD34 antibodies and flow cytometry have made it straightforward to identify and enumerate the CD34⁺ progenitor cells. Several guidelines have been published in this field, including the ISHAGE recommendations (7). This paper describes a method based on CD45/Side Scatter gating combined with CD34 staining, which is well adapted to the detection of rare events such as CD34⁺ cells.

The choice of the CD34 antibody is crucial. Since the Class III epitopes are the most broadly expressed, 581 is well suited for this application. The antibody J33 has become the gold standard for CD45 detection (7). By combining 581-PE with J33-FITC and using an appropriate gating strategy, the enumeration of CD34⁺ progenitors is reproducible and objective.

Stem-Kit™ CD34⁺ HPC Enumeration Kit provides a more sophisticated and dedicated research tool. Stem-Kit comprises five reagents (a dual color 581-PE/J33-FITC, a negative control, a lysing reagent, positive COULTER Stem-Trol™ Control Cells and Stem-Count™ Fluorospheres), and allows direct determination of CD34⁺ absolute counts by flow cytometry.

Stem Cells / Progenitors Subsets

The CD34⁺ compartment, which is only a small fraction of the hematopoietic tissue, comprises cells at various stages of differentiation, including a small number of quiescent stem cells with self-renewal capabilities. Multi-parametric flow cytometry analysis can be used to analyze the heterogeneity of cell membrane molecule expression within this compartment. Especially significant are studies to determine the phenotype of the most immature compartment. That is, those cells with self-renewal capabilities, as assessed by cell culture and transfer experiments.

The CD34⁺ CD38⁻, HLA-DR⁻, CD90⁻(Thy-1)⁺ phenotype defines a subset which contains the most primitive cells. Acquisition of CD38 expression and, loss of CD90 expression occurs with lineage-commitment and differentiation of progenitor cells. Differentiation can be further assessed with the expression of lineage-specific antigens such as CD33, CD13, CD7, CD10, CD19, CD56, CD41a, Glycophorin A (Figure 2). Novel differentiation antigens with restricted tissue distribution such as an erythroblast-specific membrane antigen should also prove useful in the study of early hematology differentiation. The expression profiles of cytokine and growth factor receptors are important keys to developing an understanding of the progenitor cell regulation by cytokines and soluble mediators (8). The products of the proto-oncogene molecule c-kit (CD117) and FLT3/FLK2 (CD135) are Tyrosine-kinase growth factor receptors which are expressed on hematopoietic stem cells and are involved in the control of cellular proliferation (9, 10).

More recently, new receptors expressed on progenitor cells have been characterized. An example is the polio virus receptor (PVR, CD155) and related homologous membrane molecules named PRR. The function and the physiological ligands of PVR/PRR are not yet identified. The availability of new monoclonal antibodies to these molecules will help to open a new field of investigation in the biology of early hematopoiesis.

Insight into Stromal Cell/Stem Cell Interactions.

Another exciting aspect is the role of the internal microenvironment of the hematopoietic organs in regulating and sustaining the differentiation and self-renewal of hematopoietic stem cells. The stromal tissue is a heterogeneous compartment comprising endothelial cells with two major cell types in addition : central macrophages and reticular cells. These are both



associated with the extra-cellular matrix which consists essentially of fibronectin and heparan sulfate proteoglycans. The negatively charged heparan sulfate proteoglycans can act as a reservoir which concentrates hematopoietic growth factors, making them available for CD34⁺ progenitors. The cognate stromal cell-stem cell interactions are crucial for stem cell maintenance and homing. Both heterotypic and homotypic cell adhesion, as well as adhesive interactions with the extra-cellular matrix are involved.

The CD34⁺ cells express many adhesion molecules belonging to distinct families such as integrins (VLA-4), selectins (L-selectin), addressins (CD44) and immunoglobulin superfamily adhesion molecules (PECAM-1, CD31). The corresponding physiological ligands for these adhesion molecules can be found on both stromal cell membranes and the extra-cellular matrix. The availability of a large panel of monoclonal antibodies directed against adhesion molecules enables further investigation in this area (11-13).

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