Three-Dimensional Distribution Patterns of CD34 Antigen on Nonactivated Cord Blood Cells

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Abstract
Circulating immature blood cells are inhomogeneous in most cytometric parameters, including CD34 expression. This surface antigen forms patches, and we studied their spatial distribution pattern by processing staggered and digitalized microscopical images; the number, fluorescence intensity, and volume were determined in randomly identified CD34-positive cord blood cells in suspension. Quantitative fluorescence analysis of individual CD34-labeled cells was performed after acquisition of microscopical images in high resolution by using a CCD camera and adjacent deconvolution. Two major (n = 42 and n = 41) and one minor (n = 5) group of hematopoietic progenitors with different CD34+ distribution patterns were detected. All CD34 antigen patches are localized on the spherical cell membrane, and differ most significantly in fluorescence intensity (P < 10^-4), antigen volume (P < 0.004), and patch number per cell (P < 0.02). If all parameters are employed, 96.6% of CD34+ cells are correctly classified into one of the three groups as shown by discriminant analysis. If fluorescence intensity is eliminated, recognition efficiency decreases to 68.2%, indicating that this is the most powerful single parameter. While total fluorescence intensity is most characteristic for each group, total patch volume and number per cell, and heterogeneity of patch volumes and their spatial distribution also contribute significantly to reliable classification into their groups.

Key terms
CD34 antigen; human cord blood cells; three-dimensional microscopy

In clinical settings, the CD34 marker is commonly used for graft quality control (1), and measured in individual hematopoietic cells by flow cytofluorimetry (2). The range of expression of this surface antigen in the immature cells is large, and other physical parameters, like cell size and light diffraction, also display vast heterogeneity. Little is known about CD34 antigen distribution on the cell membrane; it is therefore tempting to study its distribution patterns and find out whether different types of such patterns exist. Two recent studies have employed confocal microscopy; in one study, circulating CD34+ cells were studied after adherence to glass slides (3), whereas, in another, bone marrow cells were fixed on gold support (4). In both studies cells were activated, which goes along with actin polymerization and enhanced adhesiveness (5). In nonactivated state of normal cells, however, true spatial distribution of CD34 membrane antigen is not yet visualized and evaluated so far. The aim of our study was to analyze the morphological details of the CD34 membrane patches in their three-dimensional localization on nonactivated cord blood cells in suspension by using image deconvolution.

Computer-assisted image deconvolution by CellScan was developed in 1994 (6). It reassigns out-of-focus stray light from the digitalized microscopical images of fluorescent cells to its origin (7,8). Using optical sectioning technique, stacks of such images can be produced, which represent the aspect of consecutive slices of a cell along the Z-axis. After segmentation, adjacent processing of data with Imaris software generates 3D images of the cell, and gives information about all patches in place.
These images are clear, highly resolved, and present high signal-to-noise ratio with great dynamic range (9,10) which allows to find out whether the CD34+ cells in nonactivated state are perfectly spherical, or carry the previously described membrane projections, which were interpreted as magnupodia changing shape rapidly (11,12). On the other side, acquisition of several parameters of each individual cell allows their combined statistical evaluation, which should reveal whether the CD34 distribution patterns actually identify different subsets of immature cells with characteristic 3D parameters.

**Materials and Methods**

**Cell Preparation and Immunofluorescent Staining**

After informed consent of the mothers, cord blood samples were collected in four at-term deliveries after disconnection of the umbilical cord. Briefly, cord blood samples were diluted [1/4] in phosphate-buffered saline (PBS, Sigma, Saint Quentin Fallavier, France) supplemented with bovine serum albumin (BSA, 0.5%, Sigma) and citrate (ACD, 0.6%, Baxter, Maurepas, France) at pH 7.4. Mononuclear cells were enriched by density gradient centrifugation over lymphoprep (Nycomed, Oslo, Norway). Twice washed cells were counted, and viability using trypan blue (Sigma, Aldrich) was found to be between 92 and 94%. Before staining, the cells were incubated in 2% human AB serum, and then fixed in paraformaldehyde (1% in PBS); this prevents cell adhesion, and thus maintains natural CD34 antigen distribution on the nonactivated cells. Cells were stained with anti-CD34 antibodies (class1: Immu 133, Immu 409, and class 2: QBEnd 10), being conjugated with phycoerythrin (CD34 pool mAB from Immunootech, Marseille, Luminy, France). The well of a wet epoxy chamber slide (Diagnostica Roth, Lauterbourg, France) was filled with the cell suspension, closed with a cover slide, and sealed with Eukitt (KO, Kindler, Freiburg, Germany).

**Three-Dimensional Fluorescence Microscopy Data Processing and Spatial Image Reconstitution**

A three-dimensional Olympus BX51 epifluorescence microscope, equipped with a 100×/1.25 NA immersion objective (Olympus, Creteil, France) and fitted with Scanalytics Cellscan EPR (Exhaustive Photon Reassignment) System (IPLAB 4A551-P-Scanalytics, Fairfax, VA, USA) was used to generate highly resolved 3D images from individual CD34-positive cells. Hardware consists of a light shutter for controlling exposure time, a piezoelectric positioning device for axial displacement of the objective, and a CCD camera. The system is controlled by an IBM-compatible PC fitted with a SuperCardTM vector processor for acquisition of images representing serial slices, contains software for processing of raw data, and performs deconvolution by EPR and image analysis. Scalar images were recorded using a 12-bit cooled CCD Princeton camera (CCD Kodak KAF 1400, cooled at −40°C; Princeton Instruments, Trenton, NJ). The physical pixel size was 6.8 μm × 6.8 μm and the matrix size was 1320 × 1035 pixels. Fluorescence distribution on cell surfaces was determined by optical sectioning into horizontal slices with 0.5-μm intervals, with respect to the Shannon sampling criteria (13) according to our optical conditions (NA, wavelength, oil indices); thus 40 consecutive images were acquired per suspended cell.

The Point Spread Function in our acquisition conditions is measured with fluorescent 0.260-μm beads (Molecular Probes®). These beads are a very dilute suspension (1:10,000 of the original), and a very small aliquot (10 μl) is pipetted on a cover slide; after drying, the beads stick to the surface. Twenty microliters of mounting fluid is then applied on the glass slide prior to positioning the cover slide. The beads can now be used for measuring the impulse response. To obtain a good quality 3D measurement, we adapt the time for acquisition to the central level, to use at best the dynamics of the sensor.

The digitalized images are deconvolved with the EPR software algorithm in order to remove stray light in images prior to further data processing (7). After deconvolution, segmentation was performed by simple thresholding fixed experimentally at 10% of the maximum intensity; voxels are assigned to CD34 patches, and then described in terms of intensity, volume, and spatial position. Scalar voxel values representing linear fluorescence light intensities appear in the interval of 0–4,095 gray levels in the 12-bit camera system.
Adjacently, the voxel values of all patches belonging to one cell are summed up. The physical voxel size for calculating antigen volume in $\mu m^3$ is $0.068 \times 0.068 \times 0.5$ as defined in the object plane.

Then the whole cell is reconstructed in 3D from individual antigen by Imaris Surpass software (Imaris-Imagic, Zurich CH, Switzerland). The program Imaris starts with a segmented image and congregates contiguous voxels into patches. It is thus possible to analyze the data being derived from the patches for each 3D image of an individual cell. The following features were extracted:

- Sum of patch volume per cell in $\mu m^3$,
- Sum of patch fluorescence intensities per cell in scalar voxels values,
- Number of CD34 patches per cell,
- Patch volume heterogeneity, defined as the ratio: maximal-minimal patch volume /total patch volume per cell,
- Spatial distribution of patches, given by respective 3D positions and their relative distances from the cell center in $\mu m$.

**Statistical Studies**

Stepwise statistical analysis is performed with Unistat 5.5 Statistical Package. At first, for identifying cellular subpopulations, a hierarchical cluster analysis (dendrogram and cluster graph) of all extracted parameters in combination was done. Herein, clusters are formed sequentially, starting with groups of cells showing highest similarity, and assembling those to clusters of higher order step by step. Squared Euclid clustering is employed for measuring distances, i.e. degrees of cluster similarity. This way, the existence of subgroups can be recognized. The cluster graph is a scatter diagram showing which data points belong to which cluster. Total fluorescence intensity and total patch volume are selected in 2D representation.

For probing whether parameters differ significantly between the subgroups, the Mann–Whitney test was employed because of nonnormal distribution of values. For the same reason each parameter was depicted in "box and whisker" and dot plots, with median and quartile values being indicated.

Finally, a discriminant analysis was performed to test the accuracy of the classification into subpopulations obtained by hierarchical cluster analysis. We did a canonical discriminant analysis based on the eigenvectors and eigenvalues of the proximity matrix, which thus involves an iterative algorithm. Iterations continue until either the reduction in the objective function is less than $1 \times 10^{-12}$ tolerance level, or the maximum number of 50 iterations is reached. The emerging subpopulations have been obtained using the Wilk’s $\lambda$ test, and the significance of $\lambda$ is determined from a $\chi^2$ distribution. For cell classification, the $\chi^2$ distances from all centroids are computed. The probability of the cell being a member of a subpopulation is equivalent to the significance level of this $\chi^2$ value with $m$ degrees of freedom (14).

**RESULTS**

As shown in Figure 1A, primary two-dimensional images of CD34$^+$ cells formed by optical sectioning microscopy com-
bined with acquisition control procedure contain stray light. This could decisively be eliminated by deconvolution (Fig. 1B). Spherical aberrations and background noise were corrected by instrumental optimization to obtain clear images (15).

Numerical data on CD34 patches are presented here for the first time to our knowledge (Figs. 2 and 3); they were collected in 88 consecutive and unselected CD34\(^+\) cells in cord blood samples. Statistical evaluation shows that the selected

**Figure 3.** Graphic display of all 3D parameters characterizing CD34\(^+\) cell subsets. Boxes indicate lower quartile (bottom line), median (middle line), upper quartile (top line). (A) Sum of patch fluorescence per cell expressed in gray levels (g.l. \(\times 10^{-3}\)) for intensity. (B) Sum of antigen volume converted in \(\mu m^3\). (C) Patch number per cell. All three subsets show large variation in quartiles for total, mean, and maximal values per subpopulation. (D) Spatial heterogeneity of patches. (E) Spatial distribution of patches. Similar values expressed in \(\mu m\) are found for spatial parameters which cover largely overlapping ranges with similar means.
CD34 patch parameters clearly identify subpopulations of cells and classify individual CD34⁺ cells correctly: three subpopulations with different CD34 membrane antigen pattern are distinguished by hierarchical cluster analysis in the dendrogram (Fig. 2A), two major (41 cells in subpopulation 1 and 42 cells in subpopulation 2) and a minor one (5 cells in subpopulation 3). In the cluster graph of antigen volume and intensity per cell, position of all cells belonging to a cluster are contiguous (Fig. 2B), and these populations are classified practically without overlaps. Cell frequency distributions with regard to fluorescence intensity are seen in the lower part of the figure.

Data are displayed in Figure 3 as whole box and dot plot graphs. Median values for total antigen intensity (Fig. 3A) and patch volume (Fig. 3B) increase from subpopulation 1 to 2 and 3; antigen patch numbers per cell (Fig. 3C) show slightly higher values for subpopulation 1 than for 2 and 3. Homogeneous spatial patch distribution (Fig. 3D) is indicated in the ratio measurement by low values (<7) and heterogeneous distribution by higher values; accordingly, subpopulation 1 shows the lowest median value corresponding to more homogeneous patch distribution compared to subpopulations 2 and 3. Within each subpopulation, distances of the patches from cell centers (Fig. 3E) show low variations (<0.55 μm); this suggests that all patches are localized at similar distance from the center of the cell, and that the membrane approximates an “ideal” sphere. Therefore extrusions as observed previously in activated cells (3,4) are absent in suspended cells.

For comparing these parameters in population 1 and 2, the Mann–Whitney U test was used. Highly significant differences were obtained for total intensity ($P < 10^{-3}$); also the differences in total cluster volumes ($P < 0.0037$) and in the number of patches per cell ($P < 0.018$) were significant, whereas spatial patch parameters (heterogeneity and distribution) were not ($P > 0.150$).

To test the hierarchical cluster classification of all cells in the three subpopulations, we calculated the strength of discrimination. Taking all parameters into account, 96.6% of all stem cells are correctly classified; the efficiency for classification into subpopulation 1 and 3 is 100%, and 92.9% in subpopulation 2 (3 out of 42 cells misclassified).

These results prove the effectiveness of the 3D parameters, and thus confirm the accuracy of hierarchical cluster analysis. However, if total intensity is eliminated, only 68.2% of all cells could still be correctly classified, which underlines the high efficiency of this parameter.

Characteristic CD34⁺ cells of the subpopulations pointed out in the cluster graph (Fig. 1B) are reconstructed in 3D after deconvolution (Fig. 4). In cells with low heterogeneity (cell A, ratio 0.59; cell B, ratio 0.48; and cell D, ratio 0.46) patches form isolated fragments, whereas in cells with high spatial heterogeneity they are closely neighboring each other (cell C, ratio 0.91; cell E, ratio 0.97 and cell F, ratio 0.87).

**DISCUSSION**

On all cell surfaces the antigen patches are spread out flat, thus covering the cell surface membrane at varying degrees. Interestingly, two recent microscopical studies (3,4) and electron microscopic analysis (11) on contact-activated cells observed protruding, dense CD34 pockets; these were not found in our studies, which focus on patch distribution patterns of not contact-activated progenitor cells.

Discovery of three well-separated subpopulations based on CD34 membrane antigen patterns raises the question whether these progenitors also possess specific potentials for cloning, survival, homing, and differentiation into different lineages (16–18). The CD34 molecule expressed on hematopoietic progenitor cells contains a large number of epitopes; many of them are recognized by antibodies of class 1, 2, and 3 (19). Because highest total fluorescence intensity appears to be...
present at the most immature cells, it was hypothesized that during further steps of differentiation CD34 molecules may be released into the medium, as it happens with other adhesion molecules of the cell surface (20). An alternative explanation would be that CD34 expression is downregulated during further divisions, and therefore indicates more advanced progenitor populations (21).

It will be interesting to find out whether these features match with the subsets we characterized here. Of course this necessitates sorting of the subsets as viable cells, and this can be done by applying total fluorescence intensity in flow cytometry with use of the precise ranges which we have identified. In conclusion, our study suggests that 3D fluorescence deconvolution microscopy analysis is able to distinguish subpopulations of CD34+ cells on the basis of characteristic patch patterns.

**LITERATURE CITED**


