Cytometry Part B (Clinical Cytometry) 72B:211–214 (2007)

Technical Innovations

Erythrocyte Morphology Automated Analysis: Proposal for a New Prediction Tool of Essential Hypertension Diagnosis

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Background: Erythrocyte morphology has already been studied in essential hypertension (EH) and cell membrane alterations have been observed. Relationships among red cell rheological, biochemical, and morphological properties still appear complex and are not clearly understood.

Methods: Erythrocyte morphology study was carried out by using the novel automated method we have recently developed. The morphometric parameters derived from optical microscope images were elaborated with image processing software (NIH Scion Image) to construct an application for the principal component analysis (PCA) to achieve a reliable and objective statistical method that would discriminate among erythrocyte morphologies of the considered groups. Three groups of subjects were studied: healthy (n = 30), healthy with familial EH (n = 25), and EH suffering subjects (n = 26).

Results: Our results show that morphological modifications are evident in both erythrocytes from EH and from healthy with familial EH subjects as compared to the controls. PCA showed remarkable morphological alterations in EH patients. In fact, the PCA explains for the 86.271% of the total variance that can be considered an excellent result.

Conclusions: The results suggest that the use of this automated easy and inexpensive method for the detection of cell shape abnormalities is of high value in the early EH prediction. © 2007 Clinical Cytometry Society

Key terms: essential hypertension; principal components analysis; EH early prediction; cell imaging; erythrocytes; predictive medicine

The study of cell shape transformation of human erythrocyte is of great hematologic interest because several clinical conditions are associated with erythrocyte shape changes (1–3). Modifications of cytoskeletal composition and/or organization can alter erythrocytic properties and shapes, which are responsible for the onset of hemolytic damage (4). The red blood cell membrane skeleton mostly determines the shape (discoid), deformability (rheologic properties), and durability (half-life and resistance to shear stress) of the erythrocyte. For example, the reduced deformability, which goes along with erythrocyte aging, is considered to be among the factors limiting the survival of old erythrocytes. Indeed, the process of reversible erythrocyte shape changes is a property that is limited in aged human erythrocytes (5). Thus far, the definition of cell shape is routinely performed by subjective microscopy evaluation, which is long, difficult to estimate, and strongly dependent on the operator's expertise (6–11). At the best of our knowledge, no other attempt of automated erythrocyte morphological analysis has been proposed other than our previously reported analytical system (12). In the present study, we elaborate and integrate the proposed original model to automatically define erythrocyte cell shape variations by using suitable morphometric parameters acquired from optical microscope images elaborated with an image processing

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Received 4 May 2006; Accepted 7 August 2006

Published online 31 January 2007 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cyto.b.20147

Table 1
Characteristics of the Subjects Used in the Study

	Age (years)	Sex (M/F)	Diastolic blood pressure (DBP)
Healthy	36.85 ± 4.84	18/12	76.32 ± 8.44
EH	39.88 ± 7.90	18/8	124.64 ± 16.05
Familial EH	36.92 ± 6.67	14/11	74.12 ± 7.05

software (NIH Scion Image) to discover as much information as possible on cell shape definition. The acquired digital image parameters have been then analyzed by the Principal Component Analysis (PCA) which allowed us to analyze multivariate data, enabling us to establish "metric" differences between groups under investigation on the basis of their characterizing multivariate data (13).

MATERIALS AND METHODS

Subjects and Sample Collection

Thirty healthy (normotensive), 25 healthy with familial essential hypertension (EH), and 26 EH subjects were studied. The characteristics of the subjects were studied by the "Dipartimento di Medicina Interna I" of the University of Roma "Tor Vergata" and the most relevant feature are listed in Table 1.

The hypertensive subjects were of new onset and were not on pharmacological antihypertensive treatment. Exclusion criteria were: age younger than 35 or older than 60 years, body mass index greater than 28 kg/m^2 for men or 30 kg/m^2 for women, clinical evidence of diabetes, target-organ disease (renal or heart failure, coronary or cerebrovascular disease), or any other illness requiring chronic medical treatment. Hypertension was defined as a diastolic blood pressure (DBP) greater than 95 mm Hg on an unrestricted diet and in the absence of treatment; normotension was defined as a diastolic pressure consistently less than 90 mm Hg at two different clinical visits. Patients between 90 and 95 mm Hg were excluded for confounding diagnosis, being not possible to exclude the presence of an hypertensive status. Systolic blood pressure (SBP) and fifth-phase DBP were measured three times at 2-min intervals with a standard mercury sphygmomanometer. Written informed consent was obtained from all participants. Patients' data management was performed according to the Italian privacy law (A3 and A4 D.lgs n. 196; 2003).

Blood was drawn and collected by venipuncture from the subjects into sterile tubes containing heparin as the anticoagulant. Whole blood was diluted with 1:2 (v/v) paraformaldehyde solution (of a 4% final concentration) and stored at 4°C for image analysis.

Morphologic Evaluations and Multiparameters Acquisition

Erythrocyte images acquired by a phase-contrast Nikon Optiphot microscope coupled with a Hamamatsu 5985 camera were processed by NIH Scion Image 1.61 on a Macintosh 6100/66 computer to estimate shape parameters.

We used the program set scale option tool to perform spatial calibration, so that the results were represented in inch calibrated units (for area measurements) and optical density calibrated units (for peak evaluation). The program rectangular option was selected for every erythrocyte because it allows parameter estimation. We selected the following parameters to be measured by the image processing software as the most representative of cell shape: CI (chromogenic index), DI (dimension index), BI (biconcavity index), and DP (density profile). We derived these indexes from the mean grey density of the selection, the area of the selection, the standard deviations of CI values, and the integrated mean density parameters, respectively. The measurements of these indexes were used for PCA analysis to achieve a reliable and objective statistical method that can discriminate among erythrocyte morphologies of the considered groups. The CI, DI, BI, and DP were calculated from a total of 1,753 erythrocytes (628 healthy; 467 EH and 658 familial EH) being this number highly representative for the statistics applied.

Statistical Analysis

To detect relationships between the groups characterized by different morphologies, we evaluated a PCA using the previously described multiparameters. The PCA is a procedure for analyzing multivariate data with the aim of reducing the dimensionality of the data and allowing the visualisation of a large number of variables in a two-dimensional plot (13). In our set of experiments, we had three groups of erythrocytes obtained from different subjects: healthy, familial EH, and EH. A diagram of the values obtained from cells of each group was plotted in the bidimensional space, defined by the 1st and 2nd principal component functions.

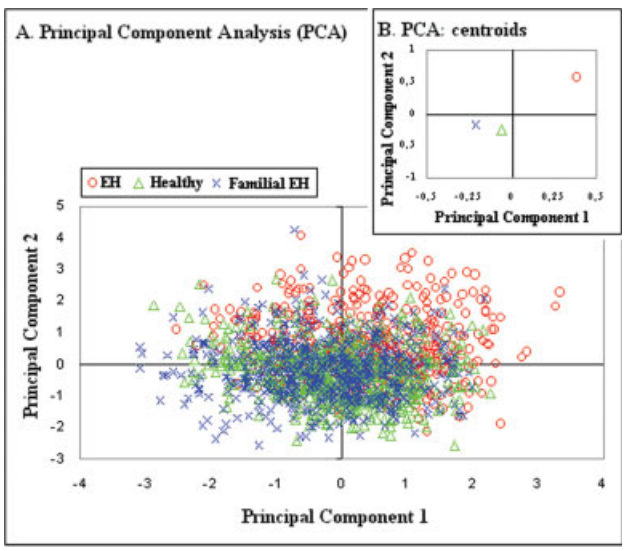

FIG. 1. Principal Components Analysis (PCA) using the multiparameters. **A**: Three groups of subjects (healthy, EH, and familial with EH) were plotted in the bidimensional space, defined by the 1st and 2nd principal component functions. **B**: A diagram of the centroids (indicating the mean values obtained from cells of each group) in the same bidimensional space was also plotted.

Table 2
Summary of Variance Explained by Principal Components

Principal component	Eigenvalues	Variance (%)	Cumulative variance (%)
1[a]	2.306	57.65	57.65
2[a]	1.145	28.62	86.27
3	0.426	10.66	96.93
4	0.123	3.07	100.00

[a]The principal components extracted by the model.

Table 3
Component Score Coefficient Matrix[a]

Variables	1st principal component	2nd principal component
DI (dimension index)	−0.036	0.961
CI (chromogenic index)	0.923	−0.239
BI (biconcavity index)	0.934	−0.054
DP (density profile)	0.762	0.401

[a]It shows coefficients by which variables are multiplied to obtain factor scores.

A multivariate analysis of variance (MANOVA) was performed in order to compare the groups, with respect to the variables extracted from image analysis. Moreover, a univariate Anova was applied for each variable, followed by a post hoc comparison among the three groups performed by least significance difference (LSD) test. The significance level was set at $\alpha = 0.05$. Statistical analyses were performed with SPSS 12.0. (Statistical Package for Social Sciences).

RESULTS

After blood sample collection from three different groups of subjects: healthy, familial EH, and EH (Table 1), we performed erythrocyte morphology analysis considering the selected parameters for cell shape (CI, DI, BI, and DP). In these three groups of erythrocytes a diagram of the values obtained from cells of each group was plotted in the bidimensional space, and a graph defined by the 1st (PC1) and 2nd principal component (PC2) functions was plotted (Fig. 1A). To better visualize the different positions of the groups studied, we also reported a diagram of the centroids to indicate the mean values obtained from cells of each group in the same bidimensional space (Fig. 1B). These modifications are clearly distinguishable in the graph.

The analysis we performed allowed us to identify the most significant variables and to characterize the variables discriminating the groups considered. The PCA, for both the PC1 and PC2 explains for the 86.27% of the total variance, that can be considered an excellent result (Table 2). Generally an explained variance higher than 65% for 1 and 2 principal components is considered a good result, while a value higher than 80% is considered excellent (14). As shown in Table 3, the 1st principal component was highly correlated with CI and BI and the 2nd principal component was highly correlated with DI (the variables correlated with the two principal components are identifiable by the highest score coefficients in absolute value). The multivariate (MANOVA) test showed a significant difference among the three groups ($P < 0.001$). Furthermore, as shown in Table 4, the application of an univariate Anova for each variable, followed by a post hoc comparison indicated that among the three groups there are highly significant differences. In particular, the DI and DP indexes of EH subjects are statistically different from the healthy and familial EH groups, indicating that EH subjects have erythrocytes with higher DI and higher DP than the other groups. Familial EH presents CI index values higher than both healthy and EH groups. Interestingly, the BI index is significantly different between all the groups.

Table 4
Univariate Anova Applied for Each Variable and Post Hoc Comparison Among the Three Groups (Healthy, Familial EH, and EH) Performed by Least Significance Difference (LSD) Test

			Anova	
	Mean ± Std. error	Comparison	*F*	*P*
DI (dimension index)				
Healthy	0.357 ± 0.002	*	119.08	<0.0005
Familial EH	0.359 ± 0.002	*		
EH	0.397 ± 0.002			
CI (chromogenic index)				
Healthy	74.415 ± 0.303	°	13.73	<0.0005
Familial EH	75.404 ± 0.367			
EH	73.012 ± 0.298	°		
BI (biconcavity index)				
Healthy	40.309 ± 0.268		79.16	<0.0005
Familial EH	38.549 ± 0.283			
EH	43.892 ± 0.332			
DP (density profile)				
Healthy	9.581 ± 0.203	#	67.78	<0.0005
Familial EH	9.633 ± 0.209	#		
EH	13.047 ± 0.281			

The same symbols inserted in the ''Comparison'' column are indicating that there is no significant difference between the groups (by LSD).

Thus, our results clearly indicate that, compared to the controls, morphological modifications are evident in both erythrocytes from EH and from healthy with familial EH subjects.

DISCUSSION

The study of cell shape transformation of human erythrocyte is of great hematologic interest because several clinical conditions are associated with erythrocyte shape changes (1–3). Some other authors have evaluated erythrocyte shape in EH patients but with conflicting results. They have not clearly associated a specific morphology to the pathology and have never taken into account the shape changes in familial EH subjects (1,10,15,16). We were able to detect significant different erythrocyte morphologies from healthy, familial EH, and EH groups. We used an automated analysis for erythrocyte shape estimation. This allowed us to use a large number of cells, to consider many morphological parameters and consequently to detect slight shape modifications. The morphological modifications observed in the groups analyzed indicate that the groups are distinguishable. Considering our previous study, these results indicate that familial EH may tend to have erythrocytes less biconcave than healthy subjects (12), while EH subjects may tend to have erythrocytes more biconcave than healthy subjects. Furthermore, all the indexes resulted from EH group have similar characteristics previously observed in target cells (12). Our ongoing studies aimed at demonstrating such hypothesis with an internal reference model, seem in agreement with this assumption. In addition, the fact that the familial EH group showed different erythrocyte morphology than healthy support the hypothesis that EH might be a genetic predisposition (17,18). This analysis can be used to predict EH pathology, since the healthy with familial EH group evidenced erythrocyte morphology alterations.

The combination of the present method with new developing sophisticated techniques would be of high value in the analysis of cell morphology for EH prediction and for early diagnosis of other erythrocyte morphology related pathologies as well.

ACKNOWLEDGMENTS

We thank Dr. Roberto Abbondanza of the REL Department of ENEA, for his support in the management of patients data following the A3 and A4 of the D.lgs n.196;2003 on privacy.

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