Flow Cytometry for Separation of Keratinocyte Subpopulations from the Viable Epidermis*

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Human epidermal cell suspensions were analyzed and sorted with flow cytometry. The desmosome and differentiation-related KM48 monoclonal antibody was used for indirect immunofluorescence and permitted staining of keratinocytes at various stages of the cell maturation. Intensity of the staining correlated with the degree of differentiation. Three sorting gates were chosen to obtain subpopulations which varied distinctly in KM48 expression.

The flow cytometry-sorted cells were characterized by their ultrastructural appearance and by the bullous pemphigoid antigen expression. According to the ultrastructure criteria, about 50% of the cells obtained from the "IF negative" gate were basal layer keratinocytes (45.5% expressed bullous pemphigoid antigen); 90% of the "intermediate" gate cells were spinal layer keratinocytes, and over 80% of the cells sorted through the "strongly IF positive" gate were of the granular layer type. The method of keratinocyte separation proposed allows samples to be obtained for further biochemical and functional studies on keratinocyte subpopulations in normal and pathologic skin. J Invest Dermatol 87:480-484, 1986

The epidermis is a self-renewing tissue in which constant cell differentiation and replacement are subject to complex regulatory mechanisms. Cell interactions involve many epidermal cell (EC) subpopulations and are not, as yet, fully understood.

The methods of study of the epidermis include in situ histochemistry, autoradiography, and immunocytochemistry. These methods have been recently supplemented and improved by the introduction of new approaches which permit separation of the various cell populations for individual examinations and experiments. Studies on epidermal differentiation require, for example, methods for keratinocyte selection according to the degree of cell maturation. Various attempts have been made in this direction. Cell sorting has proved to be valuable for studies on Langerhans cell populations [2-6] and may therefore provide an interesting alternative to sequential trypsinization [7,8], "panning" on antibody or collagen-coated surfaces [8,9], density gradient separation [10-15], velocity sedimentation [16], and rosetting [17].

To date, fluorescence-activated cell sorting has been applied for enrichment of basal keratinocytes from EC suspensions. Régnier et al [18] took advantage of bullous pemphigoid antigen (BPA) expression by basal cells. The selected fluorescent subpopulation contained 68% BPA-positive cells. However, the accessibility of the antigen depended on fixation, and the nonfluorescent population, when submitted to air-drying, turned out to contain also 51% basal cells. Stuquet al [19] obtained highly purified fractions of basal keratinocytes after enrichment on collagen-coated surfaces followed by elimination of contaminating suprabasal cells by means of selective flow cytometry sorting. This method allowed separation of unstained basal keratinocytes but all the eliminated suprabasal cells were labeled with the antikeratin KL1 monoclonal antibody (McAb) which is specific for terminally differentiating keratinocytes [20,21]. Further, cell sorting with KL1 McAb required complete keratinocyte permeabilization which rendered impossible further functional studies on the collected subpopulations.

In the present report we propose a method for flow cytometry separation of keratinocytes belonging to the 3 living epidermal layers from viable EC suspensions. The discriminating marker used in our study is the KM48 McAb. It has been demonstrated by the quantitative immunogold method to label the keratinocyte membrane antigen which is expressed in quantities proportional to the extent of keratinocyte differentiation [22].

MATERIALS AND METHODS

Epidermal Cell Suspensions Epidermal cell suspensions were prepared from normal human skin (plastic surgery of breast and abdomen) by trypsinization (0.3% trypsin at 37°C for 1 h) according to the method described previously [22,23]. Viability, as determined by trypan blue exclusion, was >85%. In some experiments destined for electron microscopy (EM) the suspensions were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 2 h and subsequently washed in PBS at 4°C overnight.

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Abbreviations:
BPA: bullous pemphigoid antigen
EC: epidermal cell
EM: electron microscopy
FAS: forward angle light scatter
IF: immunofluorescence
LC: Langerhans cell
Mc: melanocyte
McAb: monoclonal antibody
PBS: phosphate-buffered saline
SB: stratum basale
SC: stratum corneum
SG: stratum granulosum
SS: stratum spinosum
SSB: keratinocytes with intermediate SB/SS morphology

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The forward angle light scatter (FAS) which mainly concerns cell diameter, and the intensity of green fluorescence (GREEN) which concerns the expression of the membrane antigen recognized by the specific McAb. The data were stored in a list mode to allow further analysis of a single parameter (using histogram distribution) and the gating of areas of interest by computer facilities. Determinations (as shown in Results) were based on 10^6 cells per sample. Analyses of 3 x 10^4 per sample were also performed to confirm the patterns of distribution.

There were 6 cell-sorting experiments. Cell separation was performed at a rate of approximately 1000 cells crossing the laser beam per second. How the sorting gates were chosen will be described in Results. It was possible to separate the cells through only 2 gates at a time. However, the results will be given together for all the 3 gates used in order to simplify the presentation.

Post Cell-Sorting Analysis of the Samples

Electron Microscopy: Samples of flow cytometry–sorted ECs were fixed with 2% glutaraldehyde in sodium cacodylate buffer for 10 min, washed in sodium cacodylate/glucose buffer (10 min), post-fixed with 1% osmium tetroxide for 10 min, routinely dehydrated with ethanol, and embedded in epoxy. Ultrathin sections were counterstained with uranyl acetate/lead nitrate and observed under EX 1200 Jeol electron microscope. The morphology of each EC examined was evaluated and percentages of cells belonging to various EC subpopulations were calculated on a basis of at least 200 cells per sample.

Cell Type Evaluation: Cell types were evaluated as follows: LC = Langerhans cells characterized by Birbeck granules, clear cytoplasm, typical nucleus; Mc = melanocytes filled with numerous melanosomes and sparse intermediate filaments (no keratin bundles); SB = keratinocytes of stratum basale type—relatively little differentiated, with loosely arranged keratin filament bundles, numerous melanin granules, generally small; SS = stratum spinosum keratinocytes, larger in size, with a well-arranged network of keratin filament bundles anchored in numerous desmosomal cytoplasmic plaques, and presenting occasional keratinosomes; SSB = keratinocytes which could not be included in either SB or SS group by EM criteria—small cells of intermediate morphology; SG = stratum granulosum keratinocytes easily distinguishable by their characteristic jellyfish-like shape, large size, thick cell walls, and presence of keratohyaline and membrane-coating granules; SC = stratum corneum keratinocytes (corneocytes)—flat anucleated cells with thick cornified envelope and homogeneous contents.

Double Immunofluorescence: Drops of the cell-sorted suspensions were smeared on glass slides and air-dried. The slides were incubated with the secondary antibody—a bullos pemphigoid serum (1:100)—for 30 min at room temperature, washed in PBS for 2 x 10 min, and reacted with the goat antihuman IgG rhodamine conjugate (Tago Inc., Burlingame, California) at 1:20 dilution for 30 min. Phosphate-buffered saline was substituted for bullos pemphigoid serum in controls. Percentages of BPA-positive cells (red IF) were counted and the persistence of the KM48-dependent green fluorescence was verified in each sample using a fluorescence microscope equipped with appropriate UV radiation filters. The results were based on a count of at least 300 cells in 3 different experiments.

RESULTS

Indirect IF with KM48 McAb: Epidermal cells in suspension were stained to a variable extent, displaying fine, patchy patterns of membrane fluorescence (Fig 2). Some completely negative cells were also observed.

Flow Cytometry Analysis: Epidermal cells labeled with KM48 McAb were analyzed by the computer interfaced with the cell-sorter. This analysis was made taking into account both cell size (FAS) and green fluorescence intensity (GREEN). Cell-size distribution was the same in the control and test suspensions, indicating that no fraction was lost due to application of the antibody directed against the keratinocyte cell surface. At the same time, distinct differences on green immunofluorescence between
obtained through the "IF negative" gate were mainly small keratinocytes and large flat corneocytes with some melanocytes and Langerhans cells. The "mildly IF positive" cells were generally bigger keratinocytes, which could also be found in small quantities in the "strongly IF positive" cell sample, the latter being mostly composed of characteristic SG keratinocytes. A closer examination of the samples that had been cell-sorted with a flow cytometer allowed us to rearrange slightly the sorting gates in order to obtain more uniform EC subpopulations. The KM48 McAb-labeled suspension was analyzed using the 2 chosen parameters (FAS and GREEN) simultaneously (Fig 4A). The "IF negative" gate (#1) was reduced in such a way as to eliminate the largest nonstained cells (corneocytes). The "strongly IF positive" gate (#3) was modified to sort out the largest KM48-positive cells only. The intermediate gate of "mildly IF positive" cells (#2) was situated in between, taking care to avoid overlap with the other gates.

Analysis of the Flow Cytometry-Separated Fractions of EC Suspensions Cell-subtype distribution in the samples obtained after cell sorting through the rearranged gates is shown in Fig 4B which presents pooled data from 3 different experiments. The determinations were based on cell counts of at least 200 cells per sorting gate and per experiment performed on EM following the morphology criteria described in Materials and Methods.

Quantitation of BPA-positive cells on smears of the same samples gave the following results: gate #1, 45.5% of cells exhibited red fluorescence (restricted to a part of the cell membrane); gate #2, 2% BPA-expressing cells; gate #3, 1.6% BPA-positive cells. Some of the contaminating BPA-positive cells in samples #2 and #3 were attached to undissociated clumps of more differentiated, strongly KM48-positive keratinocytes. Some small BPA-positive, KM48-positive cells could also be observed. All the BPA-positive cells from sample #1 were absolutely KM48-negative (as revealed by fluorescence microscopy) in comparison with the remaining slightly KM48-positive keratinocytes. A noteworthy feature was that most of the BPA-negative, slightly KM48-positive cells found in this sample were smaller than BPA-positive SB keratinocytes (Fig 5).

Cell-viability after sorting varied according to the time lapse between EC isolation and the end of the experiment: from 65% in short experiments to only 40% after the 30-h one.

**DISCUSSION**

Flow cytometry cell-sorting proves valuable for keratinocyte subpopulation separation. Combined with IF labeling of differentiation-related keratinocyte surface antigen (KM48) the method permits simultaneous isolation of at least 2 highly purified suspensions of keratinocytes originating from granular and spinable epidermal layers. Cell-sorted KM48-negative or weakly positive keratinocytes, which are relatively less differentiated cells [22], form a more heterogeneous sample. Ultrastructural morphology criteria, which are far better for SS and SG keratinocyte definition when compared with photonic microscopy [8,14,15], fail to provide decisive information regarding the basal-suprabasal origin of the cells sorted through the "IF negative" (#1) gate. Morphologic heterogeneity of the basal layer keratinocyte subpopulation has been reported [24]. Additionally, transition to a suprabasal compartment of the basal keratinocytes which are destined for terminal differentiation is accompanied by changes related to keratinocyte maturation [21,25]. This results in a series of cells presenting a transitional morphology, which cannot be clearly defined by EM criteria. Therefore, we have used anti-BPA immunoscerum with rhodamine IF labeling to facilitate analysis of cell-sorted samples. This secondary antibody should be used on fixed (air-dried) smears in order to detect all the BPA-expressing cells, i.e., cells originally in contact with the basement membrane [18]. Only 45.5% of the cells sorted through the "KM48-negative," (#1) gate are BPA-positive whereas 53.1% SB keratinocytes could be counted following the EM criteria. The difference
may be related to the fact that some morphologically low-differentiated keratinocytes which had already lost contact with the basement membrane were counted as SB keratinocytes under EM examination.

The parameters of cell-sorting applied in the present study do not permit us to obtain a purified subpopulation of basal layer keratinocytes. The KM48 antigen is expressed in continuously increasing quantities, proportionally to the keratinocyte differentiation [22]. In such a situation, an attempt to establish a demarcation between IF negativity and IF positivity results in the sorting of the least-stained cells along with the completely negative ones through the "IF negative" (#1) gate. When verified under fluorescence microscopy some of these cells actually show a green IF. Such weakly KM48-positive keratinocytes are generally smaller than completely green IF-negative basal layer keratinocytes (BPA +) as has been demonstrated in double-labeling studies. Therefore, a possibility exists that further reduction of the sorting gate #1 and its focusing on the region of KM48-negative, medium-sized cells could increase the percentage of cells collected in samples corresponding to the 3 sorting gates (#3, #2, #1). Bars = 1 μm. Note clear morphologic differences between the 3 subpopulations.

Figure 4. Flow cytometry sorting of ECs according to the KM48 antigen expression (GREEN) and the cell size (FAS). A, Two-dimensional distribution of analyzed EC suspensions with superposition of the 3 areas corresponding to the definitive cell-sorting gates. #1, small, KM48-negative cells; #2, medium-sized, mildly KM48-positive cells; #3, big, strongly KM48-positive cells. B, Cell-subtype composition (%) of samples collected through gates #1, #2, and #3, evaluated on EM examinations. LC = Langerhans cell; Me = melanocyte; SB = stratum basale; SSB = intermediate SB/SS morphology; SS = stratum spinosum; SG = stratum granulosum; SC = stratum corneum. C. Micrographs of cells collected in samples corresponding to the 3 sorting gates (#3, #2, #1). Bars = 1 μm.
Figure 5. Rhodamine IF labeling of BPA-positive cells collected through the "KM48-negative" gate #1. Most of the BPA-positive cells are smaller than rhodamine-labeled basal layer keratinocytes. × 500.

Percentage of relatively undifferentiated keratinocytes defined on EM and of BPA-positive cells are comparable. This confirms the high purity of the SS and SG keratinocyte subpopulations selected. A few BPA-positive cells found in the "differentiated" pool (gates #2 and #3) are actually KM48-negative and represent a true contamination due to occasional incomplete cell dissociation. Such undifferentiated cells are cell-sorted through the "IF positive" gates along with the SS and SG keratinocytes to which they are attached. They form small aggregates composed of 2 cells which do not differ sufficiently in size from the superbasal compartment. As a whole, the method presented offers a possibility of comparison of various cell-sorted keratinocyte subpopulations including corneocytes (large, KM48-negative cells). Subpopulations of keratinocytes originating from the same EC suspension and displaying various degrees of cell differentiation can be simultaneously analyzed and sorted by flow cytometry. The collected samples contain viable cells from living epidermal layers which may be used for studies on epidermal growth and differentiation. We are currently investigating keratin polypeptide composition in keratinocyte subpopulations gathered by the flow cytometry method presented in this report.

REFERENCES


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