Phenotypic Characterization of Porcine Interfollicular Keratinocytes Separated by Elutriation: a Technical Note

(elutriation / epidermal stem cell / galectin / keratin / keratinocyte / nucleostemin)

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Abstract. Separation of epidermal stem cells from other populations in suspensions of epidermal cells by sorting is hampered by a present lack of specific surface markers of this cell type. To address this issue we applied CCE combined with immunocytochemical phenotyping. On the basis of expression profiles for keratins (10, 14, and 19), nucleostemin, galectin-1 and epitopes reactive for this adhesion/growth-regulatory tissue lectin we identified a fraction of very small cells originating from the basal layer. The results demonstrated that CCE has potential merit for separation of epidermal cells to yield a population likely enriched in stem cells.

Introduction

The stem cells resident in diverse mature tissue types are attaining increasing attention. Here, we focus on stem cells in the epidermis. They are located in the bulge region of the outer root sheath of the hair follicle and in the basal layer of the interfollicular epidermis. Although no specific marker has yet been discovered for these cells, they can be identified on the basis of co-expression of several markers. Because none of them is presented at the surface of epidermal stem cells (EpSC), the application of FACS for separation of cell suspensions to obtain enrichment in EpSC is not possible. Clearly, other technical approaches are required toward this aim.

Counterflow centrifugal elutriation (CCE) is used to separate cells of different sizes and sedimentation velocity in mixed or heterogeneous populations of eukaryotic cells (Bauer, 1999). CCE thus reliably fractionates cells into subpopulations on the basis of their individual sedimentation coefficients, this parameter being a function of cell volume, shape and density. In a cultured cell line, individual cells will vary only slightly in density so that CCE will separate such cells on the basis of size (Grabske et al., 1975; Bauer et al., 1999; Schwanke et al., 2006). This methodological approach had already been applied to obtain an EpSC-enriched suspension of keratinocytes (McEwen et al., 1968). Also, CCE had been used in keratinocyte research by Pohl and coworkers (1984), who were able to single out small cells of the basal cell layer by this procedure. To explore the possibility to introduce elutriation for obtaining EpSC-enriched keratinocytes we separated and phenotyped suspensions of porcine interfollicular keratinocytes. Keratin 19 as an established marker for EpSC was monitored together with adhesion/growth-regulatory galectin-1 (epidermal and mesenchymal stem cells) and the non-exclusive marker of stem cells nucleostemin. Cells of the basal layer were visualized by detection of keratin 14 and the terminally differentiated postproliferative keratinocytes of suprabasal layers by keratin 10. In addition, the binding sites for galectin-1, which interacts with glycan or protein ligands crucial for differentiation/growth regulation (Gabius 2001, 2006; Rotblat et al., 2004; Villalobo et al., 2006), were monitored (Dvořánková et al., 2005; Kadri et al., 2005; Lacina et al., 2006a,b; Smetana et al., 2006).

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Abbreviations: CCE – counterlow centrifugal elutriation, EpSC – epidermal stem cells.

Material and Methods

a. Cell preparation and elutriation

Porcine skin specimens from one animal were obtained from a local slaughterhouse. Samples from three different areas were cut to obtain thin slices. They were incubated with dispase-containing solution (10 mg/ml) in OptiMEM GlutaMAX medium (Invitrogen, Carlsbad, CA) for 15 min at 37°C. After incubation, the material was washed in OptiMEM GlutaMAX medium, and epidermal sheets were isolated by a forceps and a needle. The obtained epidermal sheets were then incubated in 0.05% trypsin/EDTA solution for 15 min at 37°C. The epidermal tissue was mechanically resuspended. After neutralizing proteolytic capacity with foetal bovine serum (FBS), cells were washed twice with culture medium, i.e. OptiMEM GlutaMAX medium supplemented with 10% FBS and gentamycin (50 μg/ml). Cells were finally resuspended in culture medium and stored overnight at 4°C as a pellet prior to elutriation.

A Beckman JE-6B elutriation rotor with a small chamber (nominally 5 ml capacity, suitable for fractionating between 2×10^7 and 1×10^9 cells) was assembled according to the manufacturer's instructions (Beckman, Fullerton, CA). Separation runs were performed at room temperature in elutriation medium composed of phosphate-buffered saline (PBS) at pH 7.2 with serum content being reduced to 1%. The initial rotor speed for keratinocyte fractionation was 2000 rpm and flow rate of medium at 3.0 ml/min. The FT (flow-through) fraction represents a media (suspension) that had flown through the chamber while cells were loaded and equilibrated in the elutriation chamber.

After equilibration, size fractionation was performed at constant rotor speed by increasing the counterflow stepwise in 0.3 ml/min increments as indicated by numbers up to 6.0 ml/min. Eleven fractions of 80–100 ml in volume were collected, all fractions were centrifuged, each sediment was resuspended in 200 μl of medium. An appropriate aliquot of the resulting volume was then used for cytospin to achieve optimal density of cells on the slide.

b. Phenotype of elutriated cells

Cells were fixed with paraformaldehyde and washed with PBS. The presence of keratins 10 and 19 was visualized immunocytochemically by monoclonal mouse antibodies from Dako-Cytomation (Brno, Czech Republic) and of keratin 14 by a mouse antibody from Sigma-Aldrich (Prague, Czech Republic). Nucleostemin was visualized by a commercial goat polyclonal antibody (Neuromics, Bloomington, MN). Galectin-1 was detected by a rabbit polyclonal antibody fraction not reactive with other galectins (Lahm et al., 2001; Nagy et al., 2003; André et al., 2004) and binding sites for galectin-1 using the biotinylated galectin (Purkrábková et al., 2003; André et al., 2005; Lacina, et al., 2006a,b; Saussez et al., 2006). Swine anti-mouse or anti-rabbit immunoglobulin labelled with FITC (AlSeVa, Prague, Czech Republic), donkey anti-goat immunoglobulins labelled with FITC (Jackson Laboratories, West Grove, PA) as well as TRITC-labelled ExtrAvidin (Sigma-Aldrich) were used as second-step reagents. All antibodies and reagents were diluted according to the recommendations of the suppliers. The specificity of the immunocytochemical reactions was ascertained by a series of control reactions, especially by exclusion of non-specific binding of antibody via its Fc fragment, by replacement of first-step antibody by an isotype-matched antibody against an antigen not present in the epidermis. The specificity of lectin binding was evaluated using the cognate sugar lactose as a competitive inhibitor to the reaction medium. Nuclei were counterstained by DAPI, all specimens were mounted using Vectashield (Vector Laboratories, Burlingame, CA) and inspected by means of a Nikon Eclipse 90i microscope (Nikon, Prague, Czech Republic) equipped with filterblocks for DAPI, FITC and TRITC, a Vosskühler CCD camera and a LUCIA 5.1 computer-assisted analysis system, both supplied by Laboratory Imaging (Prague, Czech Republic). The same system was also used for automatic measurements of the cell diameter that was performed in minimally 500 of cells per fraction and phenotype.

Results and Discussion

The fraction FT exclusively contained cells expressing keratin 19 and nucleostemin as nuclear marker as well as galectin-1. Cells positive for these markers were small, i.e. 7.5-8.5 μm in diameter. Interestingly, the diameters of cells negative for nucleostemin expression were larger than those of cells with positive nucleoli. Practically all cells of this fraction were also positive for keratin 14. In contrast, no cell of this fraction harboured keratin 10, and an intense signal for galectin-1 binding in the cytoplasm and nucleoplasm was also observed (Fig. 1). Based on these phenotypic characteristics cells of this fraction appear to originate from the basal cell layer (presence of keratin 14 and absence of keratin 10). Moreover, this fraction appears to be enriched in EpSC because of the following criteria: expression of keratin 19, nuclear presence of galectin-1 and, although this marker is not specific for EpSC, also for expression of nucleostemin (Michel et al., 1996; Purkrábková et al., 2003; Dvořánková et al., 2005; Lacina et al., 2006a). Comparing the diameter of the keratin 19- and nucleostemin-positive cells with cells of other phenotypes (keratin 19-negative and keratin 14-positive, keratin 19-negative and keratin 14-negative as well as keratin 19-negative and keratin 10-positive), these cells are the smallest (Table 1). The rather minute diameter of these cells also argues in favour of this interpretation, because it is known that small epidermal cells are highly clonogenic and, probably, identical with EpSC (Barrandon and Green, 1985; Dvořánková et al., 2005). In accord with this reasoning the very small cells of 2–4 μm diameter isolated from the bone marrow have properties

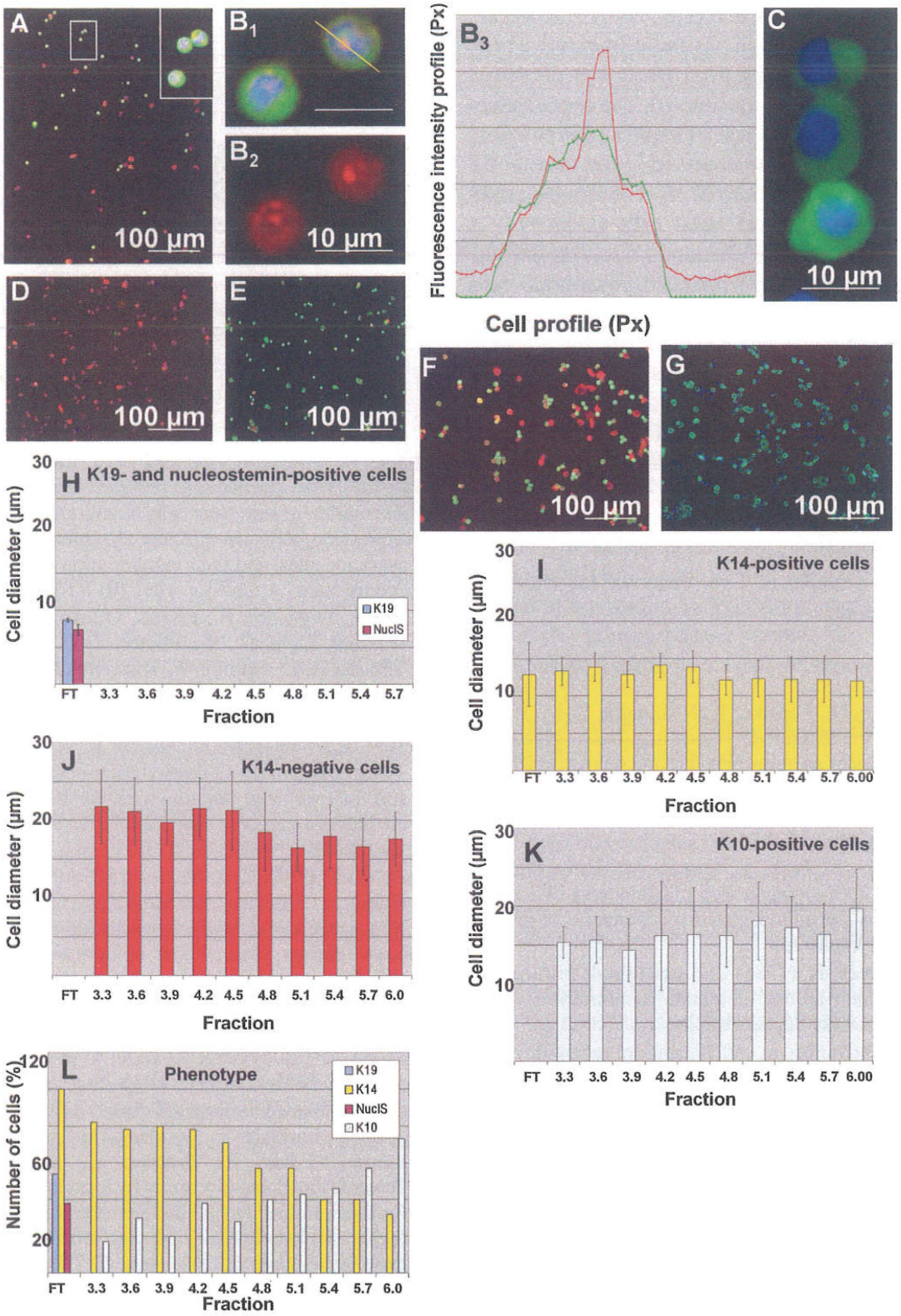

Fig. 1. Detection of keratin 19 (A, D, green signal), galectin-1-binding sites (A, D, F, red signal), galectin-1 (B1, C, green signal), nucleostemin (red signal, B1,2), keratin 14 (E, F, green signal) and keratin 10 (G, green signal) in cell fraction FT (A, B1, 2, C-E) and in cell fraction 5.1 (F, G). Figure B3 presents the cell fluorescence intensity profile in the section marked by a yellow line (B1). Cell diameter in relation to the nature of the fraction in cells positive for keratin 19 and nucleostemin (NuclS) (H), keratin 14 (I), in cells negative for keratin 14 (J) and in cells positive for keratin 10 (K) is also shown. Moreover, the relative frequency of cells of distinct phenotypes in relation to the nature of the fraction is illustrated (L)

Table 1. Mean diameter of keratinocytes (value ± SD)

Cell diameter ±SD (µm), for the phenotype:				
nucleo-stemin	**keratin 19**	**keratin 14**	**keratin 14-negative**	**keratin 10**
8.69±0.3	7.34±0.6	12.86±0.8	19.23±2	16.48±1.5

Cells positive for nucleostemin or for keratin 19 were significantly smaller ($P < 0.01$) than cells of other studied phenotypes evaluated by Student's non-paired t-tes

of embryonic stem cells (Vacanti et al., 2001; Kucia et al., 2006). Of note, the proportion of keratin 14-positive cells downstream of the fractions from FT to 6.0 decreased from 100 % to 30 % in the fraction 6.0. The opposite tendency was observed for the case of keratin 10 expression, where no positive cells were observed in the fraction FT and almost 80 % in the fraction 6.0. This observation indicated that the proportion of basal cells (keratin14) is decreasing downstream the elutriation fractions and the proportion of terminally differentiated postmitotic cells (keratin 10) is increasing in the same fractions. In summary, we herewith present evidence for cell fractionation by elutriation on the example of epidermal cells. The size of the cells and the immunocytochemical phenotyping are in line with the enrichment in EpSC. Further studies testing the indicated value of elutriation as a step for obtaining EpSC are warranted.

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