EXTENDED REPORT

Expression of haematopoietic stem cell markers, CD133 and CD34 on human corneal keratocytes

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Aim: To study the expression of CD133 and CD34 antigens on cultured human keratocytes over time.

Methods: Primary cultures of human corneal stromal cells were established from explants derived from cadaver eye donors. The cultures were sorted for CD133+ and CD34+ cells using magnetic beads. Both the primary cultures and secondary passages of sorted cells were further analysed by flow cytometry and western blot analysis for expression of the same antigens over time.

Results: Four different cell populations—namely, CD133+, CD133–, CD34+ and CD34–, were identified in the culture samples. Two further specific subgroups were identified by flow cytometry: CD133+/CD34– cells and CD133+/CD34+ cells. Expression of CD133 declines more than CD34 with time in cell cultures. Although most cells lost expression of these markers, small populations retained staining up to 5 weeks in culture.

Conclusion: Human keratocytes express the haematopoietic stem cell markers CD133 and CD34. This expression decreases with time in culture, with most but not all cells losing expression. On the basis of these markers, the corneal stroma shows a heterogeneous population of cells. Expression or down regulation of expression of these molecules could represent different stages of activation of these cells.

Keratocytes are the predominant cell type in corneal stroma. They are located within the corneal stromal lamellae in a three-dimensional network.^{1–3} CD34 serves as a unique marker of keratocytes in the human corneal stroma.^{4,5} Focal changes in CD34 expression have been observed in corneal pathology.⁶

CD34 and CD133 are widely recognised as haematopoietic stem cell markers.^{7–12} CD34 is a glycosylated type-I transmembrane protein expressed on early lymphohaematopoietic stem and progenitor cells, small-vessel endothelial cells, embryonic fibroblasts and fibroblast-like dendritic cells in connective tissue.¹² Its presence on keratocytes suggests that it belongs to a primitive phenotype, similar to embryonic fibroblasts. CD133 is a glycoprotein first described on CD34 bright haematopoietic stem cells.¹⁰ It is also expressed on haemangioblasts, retinoblastoma, neural stem cells and developing epithelium. The CD133 antigen has not yet been shown on adult epithelial tissue. It is rapidly down regulated during cell differentiation. Yu *et al*¹¹ have shown that CD133-2 is coexpressed with β -1 integrins on 10% of neonatal foreskin epidermis, with loss of expression as the cells differentiated in culture. No information is available on the expression of CD133 on human keratocytes.

We undertook a study to evaluate the expression of CD133 and CD34 in cultured human keratocytes representing the active (proliferation/migration) state of the cells.

MATERIAL AND METHODS

Primary cultures

Primary cultures of corneal cells were established from the peripheral cornea of 16 cadaver donors as reported previously.¹³ Briefly, fragments of human peripheral corneal tissue were digested with collagenase (CLSPA, Worthington, Freehold, New Jersey, USA, 20 U/ml), trypsin (Gibco Laboratories, Grand Island, New York, USA, 1:300 at 0.75 mg/ml) and 2% heat-inactivated chicken serum in Ca²⁺ and Mg²⁺ free Hank's balanced salt solution (Collagenase-Tripsin-Chicken Serum Solution, University of Udine, Italy)¹⁴ for 35 min at 37°C. The supernatant was centrifuged and cell pellets re-suspended

in medium and seeded in plastic culture dishes (Falcon, Becton-Dickinson, Oxford, UK). Cultures were established in hamster embryo culture medium composed of 60% TC199 (Sigma, St Louis, Michigan, USA) and 40% HUMED base (Gibco; 11 mM glucose, 1.1 mM CaCl₂, 0.5 mM Mg²⁺) with 8% fetal calf serum (Gibco).^{13–15} These cultures were termed primary heterogeneous cultures (PHC) as they contained the different immunophenotypes of corneal stromal cells. Time to confluence was recorded for each sample.

Of the 16 samples, eight were used for western blot analysis, nine for flow cytometry experiments and one for growth curve analysis.

Isolation of cell population subsets

The mini-Macs Direct-CD133 and CD34 positive kits (containing CD133 and CD34 antibodies directly conjugated to microbeads, for magnetic sorting^{16–20}) were used to isolate subpopulations of CD133 and CD34 cells. This technique has been shown to specifically isolate haematopoietic stem cells.¹⁶ When the PHC reached near confluence, the supernatant was discarded and the cells incubated in CTC solution for 15 min at 37°C. The resultant cell suspension was centrifuged and pellets used to sort subpopulations. Cells were resuspended in a final volume of 300 μ l phosphate-buffered saline (PBS; cell count $\leq 10^8$ cells) with 100 μ l FcR blocking reagent. In all, 100 μ l of CD133 microbeads (cell labels) was added, incubated for 30 min at 4°C and washed with PBS. The same steps were repeated with CD34 microbeads and the final pellet resuspended in PBS before magnetic separation. After magnetic separation two cell populations were obtained: (1) CD133+ and/or CD34+ and (2) eluate CD133– and CD34–.

Flow cytometry

Cytofluorimetric analysis was performed on PHC samples using antibodies to CD34 and CD133 both individually and in

Abbreviations: APS, after positive selection; BPS, before positive selection; EGF, epidermal growth factor; PBS, phosphate-buffered saline; PHC, primary heterogeneous cultures

combination. A time course study of a period of time course of 13–74 days, on PHC using anti-CD34 antibody, was also performed on six different cultures. Cytofluorimetric analysis of secondary cultures, obtained after positive selection of PHC, was also carried out to ascertain expression of CD34 and CD133 over time. Irrelevant thyroid cells were used as negative controls.

Briefly, the cells were suspended in CTC solution, counted, washed, harvested and suspended in 100 µl of PBS and incubated with 10 µl fluorochrome-conjugated antibodies (CD133–allophycocyanin; CD34–phycoerythrin; CD45–fluorescein isothiocyanate; Miltenyi Biotec) and incubated in the dark at 4°C for 10 min. Cell pellets were re-suspended in PBS. The following analyses were carried out:

- in PHC,
- in PHC before and after positive selection, using only CD34 antibody,
- in PHC before positive selection using both CD133 and CD34 antibodies and
- in PHC before and after positive selection using both CD133 and CD34 antibodies over a time course.

On average, at confluence there were 2×10^6 cells/plate. For fluorescence-activated cell sorting, the samples contained 250 000 cells, from which 10 000 events were recorded and counts expressed as a percentage.

Immunohistochemical studies

Samples of primary cultured keratocytes were stained with anti-platelet-derived growth factor receptor (Oncogene, Nottingham, UK), anti-smooth muscle actin (clone 1A4) and anti-vimentin (clone V9; DAKO, Ely, UK) antibodies using the alkaline-phosphatase/anti-alkaline-phosphatase (APAAP) method.

Growth curves

Cells from PHC samples (number 20868) were seeded (12.5×10^5 cells/60 mm tissue) in triplicate. Cells were grown in media with and without 40 ng/ml of epidermal growth factor (EGF; cat # 100–15 Pepro-Tech). Three sets of dishes for each medium were counted at days 0, 1, 3, 5 and 7.

Western blot analysis

Western blot analysis was performed on extracts of positive and negative subpopulations obtained after magnetic separation with CD34 microbeads. Samples were extracted with lysis BW-buffer (wash buffer; 10 mM NaPi, pH 7.6; 100 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.5% Na-deoxycholate; 0.1% sodium dodecyl sulphate (SDS)) including a protease inhibitor cocktail (sigma cat # 8340) and 50 µg/ml of phenylmethylsulphonylfluoride. Samples were standardised to 30 µg of protein and subjected to electrophoresis on 7.5% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride filter (Immobilon-P; Millipore, Massachusetts, USA) and immunostained with anti-proliferating cell nuclear antigen (sc56)²¹ antibody.

RESULTS

Primary cultures

All nine donor explants yielded PHC, which reached confluence at different time points, with a mean (standard deviation (SD)) of 45 (44) days (table 1).

Secondary cultures

Secondary cultures of CD34+ cells obtained after magnetic sorting also reached confluence at different time points. The

Table 1 Time to confluence of the primary heterogeneous and secondary cultures from the samples used for microbead magnetic sorting for CD34

Sample number	Age/sex	Before positive selection (days)	After positive selection (days) for CD34+
1 (12006)	66-M	48	58
2 (11880)	44-F	125	55
3 (11807)	74-F	113	53
4 (13214)	72-F	21	41
5 (13273)	33-M	14	39
6 (13953)	74-M	55	39
7 (13273)	33-M	14	32
8 (13751)	78-M	12	26
9 (14129)	75-M	11	17
Mean (SD)		45 (44)	40 (14)

F, female; M, male.

mean (SD) time to confluence was 40 (14) days (table 1). Samples which took longer to reach confluence in primary cultures also took longer in secondary cultures ($p < 0.03$, analysis of variance on polynomial fit, degree 2).

Growth curves

The population doubling time was 32 h with EGF and 48 h without EGF (fig 1). The two curves were compared using multivariate analysis of variance for repeated measures, yielding a value of $p < 0.05$.

Cytofluorimetric analysis

Flow cytometry of PHC

Four main immunophenotypes with regard to CD133 and CD34 were identified in the PHC: CD133+, CD133–, CD34+ and CD34– cells. CD133+ keratocytes constituted 1% of the cells in PHC before positive selection (BPS) by magnetic sorting, and 16% of the population after positive selection (APS). CD34+ keratocytes constituted 4.94% of the population BPS and 96.6% of the population APS. Figure 2 presents the results of cytofluorimetric analysis of one representative sample (32905). The CD133+ population was made up of two subpopulations: CD133+/CD34– and CD133+/CD34+. The CD34+ population was made up of two subpopulations CD34+/CD133– and CD133+/CD34+.

Flow cytometry of PHC maintained for different periods of time using CD34 antibody

The percentage of CD34+ cells showed a decreasing trend with time in PHC. In all, 16% of cultures were positive for CD34 at 2 weeks and this decreased to 0.5% at around 10 weeks (table 2).

Figure 1 Growth curve for primary cell cultures with and without epidermal growth factors (EGFs). The cultures of sample 20868 were performed in triplicate. SDs were very tight and hence are not shown.

Figure 2 Cytofluorimetric analysis of CD34+ and CD133+ (32905) human corneal cell cultures before and after positive selection (before and after positive selection): a representative sample is illustrated. The average values for the different subtypes was (A) keratocytes (before positive selection) 1% CD133; (B) keratocytes (after positive selection) 16% CD133; (C) keratocytes (before positive selection) 4.94% CD34; (D) keratocytes (after positive selection) 96.6% CD34.

Flow cytometry of PHC BPS using anti-CD34 and anti-CD133 antibodies

Two samples (20677 and 20595) of PHC maintained for 27 and 33 days, respectively, underwent cytofluorimetric analysis BPS. The first sample contained: 2.40% CD34+; 0.28% CD133+, which was made up of 0.03% CD133+/CD34- and 0.25% CD133+/CD34+. The second sample contained: 3.20% CD34+; 0.31% CD133+, which was made up of 0.08% CD133+/CD34- and 0.23% CD133+/CD34+.

Flow cytometry of secondary cultures of CD34+ cells obtained APS from PHC

After 48 and 125 days in the PHC, two sets of samples were positive for CD34+ and CD34- cells. These were retained in culture for a further 58 and 55 days, respectively. At this time

point, both the samples that were positive for CD34+ cells had only 2.3% CD34+ cells, whereas both samples that had CD34- cells showed that all the CD34- cells remained negative—that is, the CD34- cells did not acquire CD34 expression over time (table 3).

A longitudinal time course study was conducted on one sample as follows: the cells were cultured for 13 days and an aliquot was used to estimate the percentage of CD34+ cells BPS and APS. The percentages were 16% and 98%, respectively. APS, the CD34+ cells were seeded into three dishes. The percentages of CD34+ cells evaluated after a further 9, 13 and 17 days were 24.5%, 43% and 20.6%, respectively (table 4).

Table 2 Percentage of CD34+ cells by flow cytometry (fluorescence-activated cell sorting) of primary heterogeneous cultures according to time to confluence

Sample number	Culture time (days) primary cultures	CD34+ (%)
14 630	13	16.0
20 024	20	7.2
20 595	33	3.2
20 677	27	2.4
14 307	45	0.8
14 129	74	0.5

CD34+ cells are represented as a percentage of the 10 000 events recorded.

Table 3 Percentage of CD34+ cells in CD34+ and CD34- selected secondary cultures with similar times to confluence

Sample number	Culture time (days)		CD34+ (%)
	Before positive selection	After positive selection	
12006 (CD34+)	48	+58	2.30
12006 (CD34-)	48	+58	0
11880 (CD34+)	125	+55	2.33
11880 (CD34-)	125	+55	0

Cells derived from samples with medium and long times to confluence on primary heterogeneous cultures (fluorescence-activated cell sorting). CD34+ cells are represented as percentages of the selected cell population.

Table 4 Time course analysis by flow cytometry (fluorescence-activated cell sorting) of sample 14630 showing the percentage of CD34+ cells in relation to time after positive selection

Sample	Culture time (days)	CD34 (%)
BPS	—	16
APS		
CD34+	0	98
CD34+	+9	24.50
CD34+	+13	43
CD34+	+17	20

APS, after positive selection; BPS, before positive selection. All percentages are of the 10000 events recorded.

Flow cytometry of secondary cultures APS of PHC using anti-CD34 and anti-CD133 antibodies

The cells were cultured in vitro for 26 days. An aliquot was used to calculate the percentage of cells of the following immunophenotypes: total CD133+ and its two subpopulations (CD133+/CD34- and CD133+/CD34+) and CD34+ cells (table 5). The

Table 5 Flow cytometry

Sample	Culture time (days)	Total CD133 (%)	Total CD34 (%)	CD133+ /CD34- (%)	CD133+ /CD34+ (%)
BPS	—	0.99	4.94	0.30	0.69
APS	0	16.00	96.60	0.32	15.68
APS, CD133	+7	0.11	1.48	0.04	0.07
APS, CD34	+7	0.13	2.79	0	0.13

APS, after positive selection; BPS, before positive selection. Fluorescence-activated cell sorting of one sample (32905), showing the percentage of CD34+ and CD133+ cells APS. Cells were sorted after 26 days in culture. All percentages are taken from the 10 000 events recorded.

percentages of cell phenotypes in the original culture (BPS) were 1%, 4.94%, 0.30% and 0.69%, respectively, whereas these were 16%, 96.60%, 0.32% and 15.68% APS. APS, the CD34+ and CD133+ cells were seeded into separate dishes and the percentages of the different phenotypes estimated after a further 7 days. Table 5 presents the flow cytometry data.

No cells among PHC or secondary cultures were positive for CD45. Cultured keratocyte samples showed considerable expression of smooth-muscle actin and anti-platelet-derived growth factor receptor on immunohistological studies (fig 3). This expression corresponded to the decline in staining for CD34.

Western blot analysis

Western blot analysis for proliferating cell nuclear antigen²¹ showed that the nuclear protein, which correlates with the cell's proliferative state, was synthesised in all samples tested in both CD34+ and CD34- cells (fig 4).

DISCUSSION

Both CD34 and CD133 are expressed on human haematopoietic stem cells,⁷⁻¹¹ and are used as blood and bone marrow stem cell markers. On human keratocytes, CD34 seems to serve as an adhesion molecule facilitating retention of keratocytes in the corneal stroma.⁴⁻⁵ In normal human corneas, most keratocytes are positive for CD34.⁶ Activated keratocytes, after injury or disease, assume the characteristics of fibroblasts and myofibroblasts, and lose CD34.^{6, 22-24} Like CD34, CD133 is also expressed on haematopoietic stem cells and is regarded as a marker of undifferentiated cells.¹⁰⁻¹²

In this study, we showed that keratocytes in culture express CD133 and CD34. Expression by resting keratocytes suggests that these cells are relatively undifferentiated, similar to embryonic fibroblasts,²⁵ and the loss of expression could be inferred to represent the state of differentiation of these cells.

Figure 3 Immunostaining of cultured keratocytes showing (A) negative control, (B) smooth-muscle actin, (C) platelet-derived growth factor receptor and (D) CD34.

Figure 4 Western blot analysis. Expression of proliferating cell nuclear antigen in the cultures of eight samples of human corneal cells after positive selection. (The numbers and the letters identify the samples: 5 (E, CD34+ and A, CD34-). Each number represents a sample; the first alphabet in brackets is for CD34+ cells and the second alphabet for CD34- cells of that sample. 3 (D, C); 2 (F, F1); 4 (G, H); 10 (I, L); 8 (M, N); 7 (O, P); 1 (Q, R).

This study showed distinct patterns of expression of CD34 and CD133 in keratocytes in primary and secondary cultures. EGF increased the proliferation of PHC cells. The interdonor variation in time taken to reach confluence is difficult to explain given the vast variability in premortem and postmortem factors. Intradonor variation, however, was not as marked and not as statistically significant as was ascertained during our preliminary validation studies (data not shown).

Although magnetic bead sorting yielded enriched samples of CD34 and CD133 cells, the respective percentages varied considerably (96.6% and 16%). Cultured keratocytes adhered to the plastic and required trypsin treatment to separate them from the plastic dishes and beads. CD133 seems to be more susceptible to enzyme degradation than CD34 and could account for the low yield of CD133+ cells APS.

Cytofluorimetric analysis of cells from the PHC and APS showed four immunophenotypic subpopulations with regard to expression of CD34 and CD133. These were CD133+, CD34+, CD133+/CD34- and CD133+/CD34+ populations. At the time cultures reached near confluence, CD34+ cells were the predominant subtype, CD133+ cells being the next, followed by CD34+/CD133+ cells and finally by CD133+/CD34- cells. The overall proportion of CD34+ cells in PHC was 4.94% and that of the CD133+ cells was 1%. To understand these relative proportions, it is important to consider the manner in which the cells were sorted. The 1% of CD133+ cells included those that were CD133+/CD34+. When further sorting of these cells was carried out with the CD34 antibody, 0.69% of cells were separated, which had to be CD133+/CD34+, leaving 0.3% by inference that were CD133+/CD34-. Similarly the 4.94% of CD34+ cells included those that were CD133+/CD34+.

In PHC, there was a steady decline in the percentage of cells expressing CD34 with time, from around 16% at 2 weeks to 0.5% at 10 weeks. This decline could represent a differentiation of keratocytes to fibroblasts or myofibroblasts, as evidenced by expression of anti-platelet-derived growth factor receptor and smooth-muscle actin.¹⁹⁻²⁵ Moreover, much of this differentiation probably occurs within the first 2 weeks, as in situ immunohistological staining showed that most corneal stromal cells (keratocytes) were positive for CD34,⁵ whereas only 16% were positive at 2 weeks. Differences in tissue samples (corneal section v single-cell suspension), antibody used and method of detection (immunohistological studies v flow cytometry) could also account in part for this disparity. Analysis of positively selected cells for these two molecules showed that cells selected for CD34 had a steady decline in proportion with increased

duration in culture, but none of the cells selected for absence of CD34 (CD34-) acquired CD34 expression over the same duration. This indicates that under the culture conditions it was not possible for keratocytes to revert to their original immunophenotype once they had lost expression of CD34. Cultures of positively selected CD34+ cells showed a fairly rapid drop in the percentage of CD34+ cells from 98% to around 20% within 3 weeks of culture, suggesting that transdifferentiation of cells associated with loss of CD34 expression is a rapid process, with the loss of CD34 being an early event in this change. It was also noted that despite prolonged culture duration totalling between 106 and 180 days (inclusive of BPS and APS) a small proportion of around 2.3% of cells continued to express CD34, suggesting a prevailing heterogeneity even among the CD34+ population of keratocytes. Like CD34 expression, CD133 expression also decreases with time in culture. A recent study by Sosnova *et al*²⁶ using rodent tissue showed that two thirds of stromal CD34+ keratocytes were also CD45+, indicating their bone marrow origin. However, we did not find any CD45+ cells in our cultures on human tissue. This might be an important difference between humans and rodents.

This study thus suggests that there is diversity among the keratocyte population of the cornea. Differences in expression of CD34 and CD133, singly or in combination, could reflect differences in keratocyte function, state of activation or stage of development/differentiation of the keratocytes at any given time. The presence or absence of CD34 expression did not seem to influence the proliferative capacity of keratocytes, as the proliferating cell nuclear antigen was expressed equally by CD34+ and CD34- cells. As we did not observe cells to re-express CD34 or CD133 after loss of such expression, it suggests that cells may be following a differentiation pathway that is not reversible. However, as all studies were carried out in vitro with culture on plastic and in the presence of serum, this may not accurately reflect what transpires in vivo.

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