Characterization of Putative Stem Cell Populations in the Cornea Using Synchrotron Infrared Microspectroscopy

Matthew J. German,1,2,5 Hubert M. Pollock,2 Bojun Zhao,1 Mark J. Tobin,4 Azzedine Hammiche,2 Adam Bentley,1 Leanne J. Cooper,1 Francis L. Martin,1 and Nigel J. Fullwood

PURPOSE. High-resolution synchrotron radiation-based Fourier transform infrared (SR-FTIR) microspectroscopy coupled with multivariate analysis was used to investigate the characteristics of putative adult stem cell (SC), transiently amplified (TA) cell, and terminally differentiated (TD) cell populations of the corneal epithelium.

METHODS. Spectra of individual cells in situ in cryosections of bovine cornea were collected by using a synchrotron microspectroscopy facility at Daresbury Laboratory (United Kingdom). The resultant spectra were analyzed by multivariate analysis.

RESULTS. The median spectra of the three different cell populations showed marked differences, which correlated with their degree of differentiation and proliferative capacity. Multivariate (principal component) analysis (PCA) showed that the three cell populations could be segregated into discrete clusters, with only a slight overlap between the SC and TA cell populations. Further analysis (Mann-Whitney test) indicated that the most significant (P < 0.001) spectral differences between the SC and TA cell populations were chiefly associated with changes in nucleic acid conformation.

CONCLUSIONS. SR-FTIR microspectroscopy coupled with PCA appears to enable the identification of SC, TA cell, and TD cell populations. The results also suggest that a small subpopulation of cells in the corneal epithelial SC niche possess TA cell-like characteristics. The most significant spectral characteristics associated with the SCs appear to relate to differences in nucleic acid conformation. This finding is consistent with recent theories suggesting that the control of differentiation is related to major changes in chromatin structure. (Invest Ophtalmol Vis Sci. 2006;47:2417–2421) DOI:10.1167/iovs.05-1254

From 1Biomedical Sciences, Department of Biological Sciences, and the 2Department of Physics, Lancaster University, Lancaster, United Kingdom; and the 4Synchrotron Radiation Department, CCLRC Daresbury Laboratory, Warrington, United Kingdom.

*Supported by Engineering and Physical Sciences Research Council (EPSRC) Grant GR/S75918/01.

†Present affiliation: School of Dental Sciences, University of Newcastle upon Tyne, United Kingdom.

‡Disclosures: M.J. German, None; H.M. Pollock, None; B. Zhao, None; M.J. Tobin, None; A. Hammiche, None; A. Bentley, None; L.J. Cooper, None; F.L. Martin, None; N.J. Fullwood, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked †advertisement in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Nigel J. Fullwood, Biomedical Sciences, Department of Biological Sciences, Lancaster University, Lancaster LA1 4YQ, UK; n.fullwood@lancaster.ac.uk.

Adult stem cells (SCs) are responsible for the regeneration of cell populations in tissues that undergo continuous turnover. The prevailing view is that SCs are slow-cycling cells that possess the capacity for unlimited or prolonged self-renewal.1

The putative adult SC population of the corneal epithelium is one of the better-understood SC systems in the body. The corneal epithelial SC population is believed to be localized to a specific niche in the limbal region2–5 (Fig. 1A). Adult corneal epithelial SCs are slow-cycling and have a proliferative capacity that lasts a lifetime. It is generally believed that the SCs can divide either symmetrically to produce two daughter SCs or asymmetrically to produce one daughter SC and one progenitor or transiently amplifying (TA) cell. These TA cells, which have only limited proliferative capacity, have been observed to migrate inward from the limbal region to form the basal layer of the corneal epithelium (Fig. 1A). It is also generally accepted that TA cells eventually give rise to terminally differentiated (TD) cells, which are highly specialized and have no proliferative capacity.6 The apical layers of the corneal epithelium are made up of these TD cells. One important feature about corneal epithelial SCs is that their spatial location is well defined as are the locations of the TA and TD cell populations (Fig. 1A).

The limbal basal cells are still often described as putative SCs because of the absence of a definitive SC marker, although there are several molecules, which are differentially expressed in comparison with other cells.7 The most widely used include the presence of K5/K14, the absence of K3/K12, and the presence of p63. Ultrastructurally, SCs appear to possess a more cuboid shape and to have a more primitive appearance (Fig. 1B) compared with the more columnar cells of the basal epithelium.

That corneal epithelial SCs are relatively well-defined spatially and are accessible for both surgical intervention and observation has meant that studies of the corneal epithelium have contributed extensively to our basic understanding of the way adult SCs function. It has also facilitated the rapid development of ex vivo SC expansion and transplantation techniques for ocular surface disorders.8–10

Although we know a great deal about corneal adult SCs, there remain many questions to be answered and problems to be solved. One of the most pressing is to determine what characterizes an SC and the fundamental differences between an SC and a TA cell. An approach that could provide valuable new information on SCs, is high-resolution Fourier transform infrared (FTIR) microspectroscopy. This powerful technique is becoming an increasingly important tool in cell biology. It has been used to detect subtle intracellular changes associated with diseases such as Alzheimer’s disease and osteoporosis and to discriminate between malignant and nonmalignant cells in several different tissues.11–13 Recently, an FTIR spectroscopic
The technique has been used to demonstrate that it is possible to distinguish cells in different stages of the cell cycle.14

The technique of FTIR microspectroscopy exploits the fact that cellular biomolecules absorb the mid-IR (λ = 2–20 μm) via vibronic transitions that are derived from individual chemical bonds, yielding richly structured “fingerprint” spectra. Thus, a great deal of unique information can be obtained about changes to the structure and conformation of the molecules within the cell.15–18 Conventional benchtop FTIR spectrometers are equipped with a relatively dim thermal IR source. The spot cannot be smaller than approximately 0.5 mm without reducing the throughput, so the signal-to-noise (S/N) ratio degrades rapidly when you attempt to achieve the diffraction limit. This drawback precludes high spatial (single-cell) resolution measurements. However, synchrotrons provide a highly collimated beam of light that is orders of magnitudes brighter. When the IR portion of synchrotron radiation, at a spatial resolution of 10 μm, is used, the S/N is ~1000 times greater.11

Because the synchrotron radiation source is many times brighter and has more S/N than a conventional thermal source, synchrotron FTIR microspectroscopy holds great promise for the interrogation of intracellular dynamics and molecular changes (e.g., the transition of SCs to TA cells to TD cells). Because each individual spectra contains more than 1000 wavebands and a typical experiment would necessitate the acquisition of tens of spectra, a problem associated with the use of FTIR microspectroscopy is the difficulty of identifying important, often subtle, differences within the massive amounts of information generated. However, developments in the use of multivariate analysis and in particular principal component analysis (PCA) now allow for spectra to be analyzed more efficiently. In PCA, each spectrum becomes a single point, or score, in n-dimensional space and when selected principal components (PCs) are used as coordinates, the data may be analyzed for clustering when viewed in a particular direction. The PCs are eigenvectors of the correlation coefficient matrix of squared deviations. They comprise a new set of variables, retaining almost all the variation present in all the original spectral variables, with the first PC presenting the most variance, the second PC (orthogonal to PC1) presenting the maximum amount of the remaining variance, and so forth.19

The information obtained may be plotted in the form of a three-dimensional (3-D) plot of the spectral PCs. Briefly, nearness in multivariate distance implies pattern recognition, and the separation of sample clusters in the plots signifies structurally dissimilar groups.19

In this study, we used synchrotron radiation-based FTIR (SR-FTIR) microspectroscopy to obtain unique in situ information about the putative SC, TA cell, and TD cell populations in the bovine corneal epithelium.

**METHODS**

**Specimens**

This work was performed on bovine cornea. Bovine eyes were collected from the abattoir within 2 hours of death and transported on ice to the laboratory where the corneas were dissected and frozen. Cryosections (10 μm) were cut and collected onto BaF2 slides (Photox Optical Systems, Sheffield, UK). The sections were then stored in a desiccator until use.

**Data Collection**

Spectra were collected from the cryosections of bovine cornea at the Daresbury Synchrotron Radiation Source on Beamline 11.1 (Daresbury, Warrington, UK).13 Spectra were recorded with a microscope (Continuum; Thermo Electron Corp., Waltham, MA) and spectrometer (FTIR Nexus; Thermo Electron Corp.). Collection was made in transmission mode, and spectra were converted to absorbance with the software package (Omnic; Thermo Electron Corp.). A 32× objective (Relflachromat; Thermo Electron Corp.) was used, and the sampling area was 10 × 10 μm. The spectra were collected at 4 cm⁻¹ spectral resolution and co-added for 1024 scans. They were baseline corrected and normalized to the amide II (~1533 cm⁻¹) absorbance band, by using the software (Omnic; Thermo Electron Corp.).

The SC spectra were collected from the basal cell layer in the limbal region. The location and appearance of these was consistent with that of limbal SCs. They were small, primitive cells, poorly differentiated, and contained numerous pigment granules with a high nucleus-to-cytoplasm ratio. There was no underlying Bowman’s membrane; instead, they had a thin basement membrane that invaginated deeply into the cells. The TA spectra were collected from the basal cell layer in the cornea approximately 4 mm away from the limbus. Their location and appearance was consistent with their being TA cells. They were large columnar cells with a low nucleus-to-cytoplasm ratio, wider than the SCs, and had an underlying Bowman’s layer. The TD spectra were collected from the superficial layers of the cornea approximately 5 mm away from the limbus. The location and ultrastructural appearance of these cells was consistent with that of TD superficial cells. These cells were squamous in appearance and contained few organelles. However, because we could not perform ultrastructural analysis on the identical tissue sections that we used for SR-FTIR we describe our SC, TA cell, and TD cell populations as putative.

**Statistical Analysis**

PCA was conducted on spectra by computer (Pirouette software package; Infometrix Inc., Woodinville, WA). After baseline correction and normalization, the spectra were processed as first-derivative (15-point) spectra with the software. Six PCs were selected for analysis, and loading curves for each PC were plotted for each sample. These loading curves allowed the influence of specific spectral features on each PC to be identified. Score plots (two-dimensional [2-D]) of each
PC pair were then plotted for each sample; and, by combining the clustering evident in these figures with the analysis of the loading curves, the most appropriate three PCs were selected for the 3-D cluster analysis. The Mann-Whitney test (Minitab; Minitab Inc., State College, PA) was used to compare absorption spectra.

RESULTS

Figure 2 shows a cluster plot for the principal spectral components for the SC, TA cell, and TD cell populations. It is clear that the three different cell types of interest formed discrete clusters. The cluster for TD cells was the most densely aggregated and was also distinctly spatially separated from both SCs and TA cells. Although SC and TA cell populations also formed distinct clusters, there was some slight overlap (≈2%) between these two populations.

Figure 3A shows that the median spectra for the SC and TA cell populations exhibited differences, especially in the 1490 to 1000 cm$^{-1}$ region of the spectrum that chiefly relates to changes in nucleic acid conformation.14 Figure 3B is a difference spectrum (i.e., the difference between the median spectra of the SC and TA specimens), which enabled the identification of those regions that exhibited the greatest difference between the cell types. Regions that showed the greatest spectral differences (>0.05 absorption units) were subjected to the Mann-Whitney test, and spectral peaks with a significance level of $P < 0.001$ are indicated in Figure 3B. The positions of these peaks correlated with the differences in the particular molecular groups. Those peaks identified as significant were the peak at 1714 cm$^{-1}$, which is associated with C=O stretching vibrations of nucleic acids;16 the peak at ~1600 cm$^{-1}$, which is at the edge of the spectral region sensitive to changes in nucleic acid base pairing;18 the peaks at ~1450 to 1480 cm$^{-1}$, which are due to CH$_2$ scissoring and CH$_3$ asymmetric bending vibrations of lipids, proteins, and nucleic acids;16 the peaks at ~1380 cm$^{-1}$ and 1260 cm$^{-1}$, which occur in a region linked to cell cycle changes;14,15 and finally the peak at ~1225 cm$^{-1}$, which has been linked to PO$_2^-$ asymmetric stretching vibrations of nucleic acids.16

Figure 4A shows that the median spectrum for TA cells compared with that derived from TD cells also exhibit apparent differences. Figure 4B is a difference spectrum, which enabled the identification of those regions that exhibited the greatest differences between the cell types. Regions which showed the greatest spectral differences (>0.05 absorption units) were subjected to the Mann-Whitney test and spectral peaks with a significance level of $P < 0.001$ are highlighted in Figure 4. The positions of these peaks are correlated with differences in particular molecular groups. Those peaks identified as significant are listed as follows: the amide I peak at 1650 cm$^{-1}$, which is associated with proteins and changes in protein secondary structure (α-helical/β-pleated);16 the peak in the ~1120 cm$^{-1}$ region, which is associated with RNA expression;14 the peak centered around 1080 cm$^{-1}$, which is linked to absorption by protein and RNA;17 and the peak around 1030 cm$^{-1}$, which is associated with glycogen content.13,14
**DISCUSSION**

FTIR microspectroscopy has been used with great success in the identification and characterization of many cell types (e.g., distinguishing both malignant and premalignant changes in several different tissues). The purpose of this study was to determine whether FTIR microspectroscopy could be used to shed new information on putative SC, TA cell, and TD cell populations in the corneal epithelium, and in particular, to determine what distinguishes SCs from TA cells.

Although this study was performed on bovine cornea, the distribution of SCs, TA cells, and TD cells appears to be the same as for human cornea. Evidence of this includes provided in the study and localization of cell populations in bovine corneas by Sun et al. This group describes undifferentiated basal (stem) cells in the limbus, “biochemically more differentiated” basal cells on the cornea, and “terminally differentiated,” superficial cell populations. Also, some of the first evidence for the location of SCs was provided by the occurrence in cattle of corneal epithelial tumors (cancer eye) that are predominately associated with the limbus. Our detailed ultrastructural examination showed that the different cell populations in bovine cornea were easily distinguishable by their location and ultrastructure, which was consistent with that described for these cell populations in humans. We also observed (Fullwood NJ, unpublished data, 2005) that the basal cells in the bovine limbus were strongly positive for p63, and experiments using a culture model showed that after total debridement of the epithelium, new epithelial cells migrated from the limbus.

The results of our investigation show that SR-FTIR microscopy coupled with multivariate analysis segregated into discrete cluster spectra derived from SC, TA cell, and TD cell populations (Fig. 2). Thus, this technique appears to possess potential as a tool that discriminates between the different cell types within the same epithelium. Of note, some slight overlap between the SC cluster and the TA cluster was observed (Fig. 2). The results suggest that a small proportion (~2%) of the SC population possesses TA cell spectral characteristics. A possible explanation for this finding is that there is a small proportion of TA cells in the SC niche before their exit. When an SC divides asymmetrically, it produces one daughter SC and one daughter TA cell. Because it must require a finite amount of time for the TA cell to migrate from the SC niche, it is reasonable to expect that a small number of TA cells may be present in the SC niche at any one time. Our data put this proportion of cells that have TA cell-like characteristics at ~2% of the total cell population present in the SC niche.

Examination of the difference spectrum shown in Figure 3 reveals that most of the statistically significant differences between the SC and TA cell populations was associated with changes in spectral regions that were in turn associated with changes in nucleic acid conformation. This finding lends support to observations made on hematopoietic cells, which suggests that fundamental changes to chromatin structure are linked to cell differentiation. Thus, the regulated production of several TD cells in the process of hematopoeisis is linked to changes in chromatin structure, as reviewed by Georgopoulos. Other workers have suggested that the differentiation of hematopoietic cells is actually triggered by changes in chromatin structure, which produce favorable conditions for stable enhancer complexes that drive transcription. The results of this study of adult corneal SCs confirm that there are significant differences in the conformation of nucleic acids between SC and TA cells.

TD cells clearly formed a discrete homogeneous population that, when examined using PCA, exhibited clustering with no overlap with SCs or TA cells. One would expect that highly specialized TD cells that possess no proliferative capacity would exhibit a cellular profile or composition radically different from that of SCs or TA cells. However, although separated spatially from both the SC and TA populations, the TD cluster was closer to the TA cell cluster than to the SC cluster, indicating that the spectral characteristics of TD cells are more closely aligned with those of TA cells than with those of SCs.

With respect to the underlying reasons for the spectral differences identified between the TD and TA cell populations, analysis of the difference spectrum (Fig. 4B) points to the fact that most of the statistically significant differences appeared to be associated with changes in spectral regions that may designate changes in protein and RNA absorbance. This supports known characteristics associated with TD cells of the corneal epithelium. Terminal differentiation is associated with loss of proliferative ability and increasing functional specialization. This functional specialization involves the production of the range of integral proteins necessary to allow TD cells to manage their operational demands. Such protein types include the keratin pairs K3/K12, clusterin, aldehyde dehydrogenase 3, and various tight junction proteins.

To date, FTIR microspectroscopy has been an underexploited technique in cell biology. Recent developments mean that it is now possible to obtain spectra from individual cells or even intracellular compartments, and that such spectra can be very conveniently collected from cryosections of tissues. It is...
even possible to collect spectra from live cells although this technology is in its early stages. Until recently, a major problem was the sheer amount of information obtained even within a single spectrum. However, the availability of software that facilitates rapid multivariate analysis now means that a large number of spectra within tissues or from different tissues can be routinely compared and that the most significant differences can be pinpointed with a remarkable degree of sensitivity and discrimination.

In conclusion, this study is the first to show that SR-FTIR microspectroscopy coupled with PCA is capable of discriminating and segregating putative populations of SCs, TA cells, and TD cells. Analysis of the different populations suggests that a small subpopulation of cells within the corneal epithelial SC niche appeared to possess TA cell-like characteristics, suggesting that these are newly generated TA cells before their migration. Differential analysis also suggests that the most significant spectral characteristics in the SCs compared with TA cells relate to differences in nucleic acid conformation. This finding is consistent with recent theories suggesting that the control of differentiation is related to major changes in chromatin structure.

References