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The presence of a feeder layer improves human corneal endothelial cell proliferation by altering the expression of the transcription factors Sp1 and NFI

Gaëtan Le-Bel[a,b,c,d], Claude J. Giasson[a,b,e], Alexandre Deschambeault[a,b,d], Patrick Carrier[a,b,d], Lucie Germain[a,b,c,d], Sylvain L. Guérin[a,b,c,*]

[a] CUO-Recherche, Médecine Régénératrice - Centre de recherche FRQS du CHU de Québec-Université Laval, Québec, Canada
[b] Centre de Recherche en Organogénèse expérimentale de l'Université Laval/LOEX, Québec, QC, Canada
[c] Département d'Ophtalmologie, Faculté de médecine, Université Laval, Québec, QC, Canada
[d] Département de Chirurgie, Faculté de médecine, Université Laval, Québec, QC, Canada and
[e] École d'Optométrie, Université de Montréal, Montréal, Québec, Canada

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ABSTRACT

Based on the use of tissue-cultured human corneal endothelial cells (HCECs), cell therapy is a very promising avenue in the treatment of corneal endothelial pathologies such as Fuchs' dystrophy, and post-surgical corneal edema. However, once in culture, HCECs rapidly lose their phenotypic and physiological characteristics, and are therefore unsuitable for the reconstruction of a functional endothelial monolayer. Expression of NFI, a transcription factor that can either function as an activator or a repressor of gene transcription, has never been examined in endothelial cells. The present study therefore aimed to determine the impact of a non-proliferating, lethally irradiated i3T3 feeder layer on the maintenance of HCEC's morphological characteristics, and both the expression and stability of Sp1 (a strong transcriptional activator) and NFI in such cells. The typical morphology of endothelial cells was best maintained when 8×10^3/cm^2 HCECs were co-cultured in the presence of 2×10^4 cells/cm^2 i3T3. HCECs were found to express both Sp1 and NFI *in vitro*. Also, the presence of i3T3 led to higher levels of Sp1 and NFI in HCECs, with a concomitant increase in their DNA binding levels (assessed by electrophoretic mobility shift assays (EMSA)). Specifically, i3T3 increased the expression of the NFIA, NFIB and NFIC isoforms, without a noticeable increase in their mRNAs (as revealed by gene profiling on microarray). Gene profiling analysis also identified a few feeder layer-dependent, differentially regulated genes whose protein products may contribute to improving the properties of HCECs in culture. Therefore, co-culturing HCECs with an i3T3 feeder layer clearly improves their morphological characteristics by maintaining stable levels of Sp1 and NFI in cell culture.

1. Introduction

Skin keratinocytes can form colonies when they are grown in the presence of a feeder layer of irradiated mouse fibroblasts (Rheinwald and Green, 1975a; b). Such a co-culture helps maintain the morphological characteristics of the cells, while appearing to delay terminal differentiation *in vitro* by preventing the extinction of transcription factors, such as Specificity protein-1 and -3 (Sp1 and Sp3, respectively), by what appears to be post-translational modifications of these proteins through glycosylation (Duval et al., 2012; Masson-Gadais et al., 2006). The beneficial influence of the feeder layer depends on its ability to preserve telomerase activity through a sustained expression of the gene encoding the catalytic subunit of this enzyme (hTERT), and whose transcription has been reported to be under the regulatory influence of Sp1 (Bisson et al., 2015). Besides Sp1, co-culturing keratinocytes along with a feeder layer also improves DNA binding and expression of Nuclear factor I (NFI) family members. The feeder layer seems to prevent the proteolytic degradation of both Sp1 and NFI, thereby contributing to delay terminal differentiation by inhibiting the activity of the proteasome (Duval et al., 2012). In vertebrates, the NFI family includes four genes: NFIA, -B, -C and –X (Gronostajski, 2000; Qian et al., 1995), whereas Sp1 is the founding member of a family that comprises 8 other

* Corresponding author. CUO-Recherche/LOEX, Centre de recherche du CHU de Québec – Université Laval, 1050, chemin Sainte-Foy, G1S 4L8, Québec, QC, Canada.
E-mail address: Sylvain.Guerin@fmed.ulaval.ca (S.L. Guérin).

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related transcription factors (Sp2 to Sp9) (Pearson et al., 2008).

Cultured human corneal epithelial cells also benefit from the presence of irradiated fibroblasts (Lindberg et al., 1993). As for skin keratinocytes, they also express Sp1, and NFI and both were found to respond to components from the extracellular matrix, such as collagen type I (Lake et al., 2013, 2015). Human corneal epithelial cells isolated from the eyes of different donors express very inconsistent levels of Sp1/Sp3, even between passages of a same donor. In these cells, Sp1 is required for the expression of genes whose encoded protein products drive the process of differentiation, leading stem cells to become transient-amplifying and migrating superficial cells (Adhikary et al., 2005). Indeed, Sp1/Sp3 levels are elevated in less differentiated corneal epithelial cells at low-passage, whereas they both disappear as corneal epithelial cells reach terminal differentiation at high cell passages (Gaudreault et al., 2003). Based on these analyses, it was proposed that monitoring the levels of Sp1/Sp3 and NFI might prove to be a good predictor of which epithelial cells (either from the skin or the cornea) are likely to proliferate, stratify, and differentiate the best on tissue-engineered skins and corneas (Gaudreault et al., 2003, 2013).

Corneal endothelial cells, very important for corneal hydration, line the posterior layer of the cornea. Because they are arrested in the G1-phase of the cell cycle (Joyce et al., 1996b), corneal endothelial cells do not divide *in vivo*. They can however proliferate *in vitro,* under conditions that lift the block on the G1-phase inhibition. This block is the consequence of a negative regulation by cyclin kinase inhibitors 1B (p27kip1), 1A (p21Cip1) and 2A (p16INK4a), which essentially prevent the activation of the E2 transcription factor (E2F) (Joyce and Harris, 2010). When human corneal endothelial cells are treated to silence both p21Cip1 and p16INK4a mRNA, the number of dividing cells increase, as do the protein levels of p21Cip1 and p16INK4a (Joyce and Harris, 2010). Treating corneas *ex vivo* with a mitogen and either a mechanical wound or a calcium chelator (to break cell-cell contact) induces cell division of the endothelial cells, suggesting that contact inhibition plays a role in inducing the mitotic arrest (Senoo and Joyce, 2000; Senoo et al., 2000). In rat corneal endothelial cells, contact-induced inhibition of cell arrest is mediated, at least in part, by p27kip1, in both the developing neonatal and mature endothelial monolayer (Joyce et al., 2002). Other factors, such as insufficient intra-cameral paracrine or endothelial autocrine growth factor stimulation or inhibition of cell cycle by Transforming Growth Factor β2 (TGFβ2) could account at least in part for this effect (Joyce et al., 2002).

Cultured human corneal endothelial cells (HCECs) from older individuals proliferate less than those from young individuals. This is related to the age-dependent increase in negative cell cycle regulation by p16INK4a and p21WAF1/Cip1 (Enomoto et al., 2006). In turn, the expression of p21 has been shown to be modulated both by Sp1 (Yan and Ziff, 1997) and NFI (Ouellet et al., 2006). Although the expression of Sp1 has been reported in HCEC primary cultures (Bisson et al., 2015), no study has yet examined the expression of NFI proteins in these cells. As with skin keratinocytes and corneal epithelial cells, the demonstration that co-culturing HCECs along with i3T3 could also result in an increase in the expression of both Sp1 and NFI is particularly interesting, in that both these transcription factors are known to be intricately linked to growth/cell cycle progression. Indeed, Sp1 is known to regulate the expression of both the dihydrofolate reductase and thymidine kinase genes, two proteins associated with DNA synthesis (Black et al., 1999; Rotheneder et al., 1999). It also participates in cell division arrest by upregulating the expression of the cell cycle negative regulator p21 in p53-dependent growth control (Torgeman et al., 2001) or in the p53-independent pathway of terminal differentiation (Gartel et al., 2000). In a study by Chen and collaborators, the ectopic expression of a truncated variant of Sp1 that competes with the endogenous Sp1 protein was shown to prolong the S phase and reduce the growth rate, thereby supporting an important participation of Sp1 in cell cycle progression (Chen et al., 2000). Similarly, NFI was shown to act as a critical repressor of p21 gene expression during cell cycle and senescence (Ouellet et al., 2006).

The present study examined the expression of both Sp1 and NFI in HCECs, and explored whether co-culturing these cells with a feeder layer (irradiated 3T3 (i3T3) murine fibroblasts) also alters their expression and/or DNA binding properties.

2. Material and methods

2.1. Cell culture

Endothelial cells from the corneas of 11-month (HCEC-11), and 18, 19 and 24-year old donors (HCEC-18, HCEC-19 and HCEC-24, respectively) at passages 4 (P4) and 7 (P7), respectively, were harvested according to the method of Joyce and Zhu (2004). Medium was changed every other day. Cells were trypsinized when they reached near-confluence (approximately 90% coverage of the culture plate) and frozen in FBS with 10% DMSO at their third passage. Cells were kept in liquid nitrogen until use. Prior to each experiment, frozen cells were thawed and seeded with irradiated 3T3 mouse fibroblasts (i3T3), as described (Proulx et al., 2007).

2.2. Indirect immunofluorescence

Indirect immunofluorescence assays were performed on tissue cultured endothelial cells (HCEC-11, HCEC-18, HCEC-19 and HCEC-24) grown on μ-Slide chambered coverslip (Ibidi cell in focus, Fitchburg, WI, United States). Slides were incubated for 90 min with primary antibodies directed against either the ZO-1 (1:100 dilution, Life Technologies, Waltham, MA USA) or N-cadherin (1:50 dilution; Dako, New Bond St Bath, United Kingdom) adherens junction proteins. All antibodies were diluted in PBS containing 1% bovine serum albumin. Samples were washed with PBS before adding Alexa Fluor 594 conjugated AffiniPure Goat secondary antibody against either mouse IgG (1:800, Thermo Fisher Scientific Inc., Rockford, IL, USA) and further incubated for 30 min. Cell nuclei were also labeled with Hoechst reagent 33258 (1:100; Sigma) following immunofluorescence staining. The cells-containing slides were then observed with a confocal microscope (Zeiss Imager. Z2 LSM 800; Zeiss Canada Ltd., North York, ON, Canada). Negligible background was observed for controls (primary antibodies omitted).

2.3. Nuclear extracts and EMSA (electrophoretic mobility-shift assay)

Endothelial cells (at P7) were seeded at low, medium or high density (2×10^3, 8×10^3 and 1.6×10^4 cells/cm^2, respectively) in the presence of either a low or regular (1 or 2×10^4 cells/cm^2, respectively) concentration of i3T3 in 75 cm^2 tissue-culture flasks and grown for 2 days at 37 °C until they reached near-confluence or were maintained at confluence for one week (Fig. 1). As negative controls, i3T3 and endothelial cells from the same passages were also seeded alone. Cells were photographed using a Nikon Eclipse TS100 (Nikon Canada, Mississauga, ON, Canada) equipped with a numeric CCD camera (AxioCam 105 Color; Zeiss). Nuclear extracts were prepared from all cultured cells, dialyzed, and kept frozen in small aliquots at −80 °C as previously described (Roy et al., 1991).

Electrophoretic mobility shift assays (EMSA) were conducted with double-stranded oligonucleotides bearing the DNA binding site for either Sp1 or NFI (Supplementary table 1) as previously described (Zaniolo et al., 2007). Supershift analyses in EMSA were performed using antibodies against the proteins Sp1 (ab13370 (monoclonal), 1:8; Abcam), Sp3 (Sc-644 (polyclonal), 1:8; Santa Cruz Biotechnology) and total NFI (sc-5567 (polyclonal), 1:8; Santa Cruz Biotechnology).

2.4. Western blots

Western blot analyses were conducted as described (Larouche et al.,