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INHIBITION OF TGF β CELL SIGNALING IN RABBIT FOR LIMBAL EXPLANT CULTURE IN SERUMLESS, DEFINED XENO-FREE CONDITIONS

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Abstract

Outgrowths of limbal epithelium by explant culture are used to treat limbal stem cell deficiency (LSCD). The explant culture medium is always complemented with serum, a complex solution which includes TGF β . Since TGF β is a cytostatic effector for epithelial proliferation we examined its effect on these cultures. Limbal biopsies were set on explant culture in DMEM/F12 with 5 ng/ml EGF and cholera toxin (ChT), ITS, and 5 % FBS, henceforth SHEM or a) SHEMSB = SHEM plus SB431542 an inhibitor of TGF β signaling; b) sfSHEM = SHEM with FBS replaced by 0.05 % Albumax II; and c) sfSHEMSB and sfSHEMA83 = sfSHEM plus, respectively, SB431542 or A-83-01, another TGF β inhibitor. After the initial outgrowths reached 2-3 cm in diameter, the limbal biopsies were serially transferred up to six times onto new inserts. Biopsy explant outgrowths were trypsinized and cell yield, morphology and stem-cell related JC-1 exclusion (IOVS, 52:4330) were determined by flow cytometry. Cells were plated at low density seeding to compare relative clonal proliferative activity. The expression of three proteins whose levels are associated with growth and differentiation states, Krt3, connexin 43 and p63 were determined by immunohistology and/or Western blot. Cell yield in rabbit, relative to SHEM (in %) were, SHEMSB, 104 ± 13 ($p > 0.95$); sfSHEM: 5 ± 3 ; and sfSHEMSB, 94 ± 18 ($p > 0.95$). Cell size and morphology, JC1 dye exclusion, Krt3, p63 and connexin43 content, proliferation efficiency and the preservation of extended proliferative potential of the serially cultured biopsies were similar for SHEM, SHEMSB and sfSHEMSB. The only differences observed were reduced expression of Krt3 and increased preservation of p63 in the FBS-free medium. Removal of EGF from sfSHEMSB reduced yield by 92 ± 6 % ($p < 0.05$). Removal of Albumax and ChT to establish a xeno-free medium caused a small, non-statistical decrease in growth rates. Equivalent results were observed in a preliminary experiment in human. These results suggest that in the absence serum endogenously generated TGF β act as an autocrine cytostatic agent and that TGF β inhibitors allow

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explant culture in xeno-free, chemically defined medium. Furthermore, the pro-growth effect of serum in limbal explant cultures may result exclusively from neutralization of the TGF β cytostatic effect.

Keywords

limbal epithelium; explant culture; TGF β ; SB431542; A83-01; xeno-free explants culture

1. INTRODUCTION

Outgrowths from explants of the contralateral limbus are a preferred method to generate autologous corneal epithelial sheets for the regeneration of a corneal surface in the treatment of limbal stem cell deficiency (Tsai et al, 1990; Ramesh and Dhillon, 2003; Shahdadfar et al, 2012; Basu et al, 2012; Satake et al, 2014). These cultures require the presence of serum. To overcome clinical regulatory concerns about exposure to exogenous biologicals, clinical treatments have resorted to the use of autologous serum. Nevertheless the use of any natural complex fluid hinder investigations and full control over the conditions that determine cell fate in terms of the preservation or augmentation of regenerative capacity within the outgrown cell population.

TGF β has long been recognized as a major negative effector for cell proliferation of normal epithelial cells from various tissues (Masui et al, 1986; Reiss et al, 1987; Siegel et al, 2003). Its cytostatic effects may allow accelerated cellular differentiation and thereby diminish the preservation of cells with regenerative capacity within primary cultures such as epithelial outgrowths. Given these adverse effects, it may seem paradoxical that TGF β is produced by many epithelia, including that of the cornea (Wilson et al, 1992). However, in vivo, the TGF β protein and its activity are controlled by complex local and systemic regulatory systems. TGF β is secreted in an inactive state in complex with latency-associated proteins (Walton et al, 2010). The latent TGF β complexes are retained within the extracellular matrix. They are released into active form by cell-secreted or circulating proteolytic enzymes. (Yu and Stamenkovic, 2000; Ge and Greenspan, 2006; Horiguchi et al 2012; Nistala et al 2010; Zilberberg et al, 2012). TGF β is also found in serum, where the great majority of it is in an inactive protein-bound state, mostly to alpha-2-macroglobulin (O'Connor-McCourt et al, 1987). This major protein component, as well as lipoproteins (Grainger et al, 1997), may sequester cell secreted TGF β . Serum components may also inhibit a variety of locally secreted proteases involved in release of free TGF β from latent complexes (Enghild et al, 1989; Sottrup-Jensen, 1989).

Given the factors outlined above we considered the possibility that, in the highly compact cultures generated from limbal explants, cell secreted TGF β may play a auto/paracrine significant role in the culture behavior. Thus, to evaluate the role played by medium FBS in the regulation of TGF β activity, we examined the effect of complete inhibition of the canonical TGF β signaling pathways by two structurally distinct TGF β receptor specific inhibitors, SB431542 (SB; Inman et al, 2002) and A-83-01 (A83; Tojo et al, 2005), on

outgrowth from rabbit and human limbal explanted biopsies. SB has been recently used to facilitate culture of human corneal endothelial cells (Okumura et al, 2013).

Our results demonstrate that, in these high compaction outgrowth cultures, the inhibitors fully abrogate the dependence of cell proliferation on FBS without modifying cell yields and with minimal or no effect on multiple phenotypic parameters. This finding allows culture and expansion of the limbal epithelium using fully defined, known, non-proprietary xeno-free medium and opens the door for the identification of conditions and reagents that can optimize the regenerative capacity of limbal epithelial cultures without resorting to reduction of $[Ca^{2+}]$ to non-physiological levels (Tsao et al, 1982).

2. METHODS

2.1 Tissue procurement and cell culture procedures

Rabbit tissue was obtained from sacrificed animals at local abattoirs. An anonymized human limbal rim was obtained as a discard from a corneal transplantation performed at St Mary's Hospital, Seoul, Korea. The study protocol was approved by the Institutional Review Board of the Ethics Committee of the College of Medicine of the Catholic University of Korea. Rabbit and human limbal segments equaling between one quarter to one eighth of the total limbal tissue, were soaked for 1 h in culture media and set for explant culture in either, 0.4 μ M-pore Costar inserts for six well plates, or 100 mm cell culture Falcon plastic dishes. For the first 2-4 days, fluid was maintained at the minimum level needed to ensure firm tissue contact with the substratum. After a 1-2 mm outgrowth of epithelium has developed around the explant the volume was increased so as to barely cover the explant surface. The reference culture media was a supplemented human epithelial culture medium (SHEM; Loureiro et al, Mol Vis. 2013). It consisted of a 1:1 mix of Dulbecco's modified minimal essential medium and HamF12 (DMEM/F12) plus 5 % FBS complemented per liter with 5 μ g EGF, 5 μ g cholera toxin (ChT), ITS mix (100 mg insulin, 55 mg transferrin and 2 μ g sodium selenite), 14 mg phosphoethanolamine, 5 mg ethanolamine and 5 ml DMSO. The alternative media used were, a) SHEMSB = SHEM plus SB431542 an inhibitor of TGF β signaling; b) sfSHEM = SHEM with FBS replaced by 0.05 % Albumax II and c) sfSHEMSB, sfSHEM plus SB431542; and d) sfSHEMA83, sfSHEM plus A83-01, another TGF β inhibitor. In some experiments media was complemented by TGF β 1. Unless stated otherwise below reagents were obtained from Sigma-Aldrich (St. Louis, MO). DMEM/F12, ITS and Albumax II, a lipid-rich bovine serum albumin fraction were from Invitrogen (Temecula, CA). FBS was from Atlanta Biologicals (Atlanta, GA). SB431542 and A-83-01 were purchased from Tocris (Bristol, UK). Recombinant TGF β 1 was purchased from EMD Millipore (Billerica, MA). Six growth factors used in human culture as cocktail, KGF, HGF, Epregrulin, IGF-1, IGF-2 and IGFBP7 were purchased from Peprotech (Rocky Hill, NJ).

After 7-13 days, just before reaching confluence in inserts, or 2-3 cm diameter in the plastic dishes, rabbit explants with a narrow rim of the outgrown cells were excised with a small scalpel from the surrounding outgrowths and transferred to a new culture insert or dish to continue explant outgrowth (Selver et al, 2011). The explanted biopsies were excised and replated for continuous outgrowth culture for up to five times to yield six sequential outgrowths or generations. Live cultures were photographed under transverse fiber optic

illumination to determine the outgrowth area and cells were then harvested by trypsinization. After harvest and pelleting, cells were resuspended in medium, an aliquot was diluted 10-fold in FACS buffer consisting of phenol red-free (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-buffered DMEM/F12 complemented with 5 % FBS and 1 µg/ml propidium iodide (PI) and the cell suspensions were analyzed in an Accuri6 flow cytometer to obtain a) exact counting of total and live cell numbers and b) forward (FCS; proportional to cell size) and side (SSC; proportional to cell granularity/intracellular complexity) light scatter features. Excitation intensity and detection gains in the Accuri 6 flow cytometer are fixed at the factory and unchangeable. Instead of variable gain, the Accuri 6 relies on a wide, six orders of magnitude, detection sensitivity range. Hence it delivers scatter and fluorescent emission values that are independent of the operator set up and are fully reproducible from day to day. Live cultures were photographed under phase contrast illumination. In some experiments cultures were fixed-stained by immersion in Commaassie blue R (0.45 %) dissolved in 10 % acetic acid-45 % methanol.

2.2 ABCG2-efflux activity

Twenty thousand cells were seeded overnight in SHEM in 6 well plates, incubated for 45 or 60 min with 250 nM JC1, released by a 2-4 min trypsinization and diluted 3-fold in FACS buffer. The % of dye excluding cells (JC1^{low}) was determined in an Accuri6 flow cytometer in bivariate 531 and 585 nm emission plots as described (Selver et al, 2011).

2.3 Immunocytochemistry and immunohistology

A rabbit cornea was fixed in formalin, embedded in paraffin and sectioned at 4 µm thickness. Sections were deparaffinized, rehydrated in PBS, permeabilized with 1 % Triton for 10 min, blockade with 1% bovine serum albumin (BSA) for 30 min, incubated overnight at 4° C with 20 µg/ml moAb anti-p63 in 0.1 % BSA (Clone A4A; Biocare, Walnut Creek, CA) and after three 5-min washes, incubated for 1 h in 10 µg/ml HRP-conjugated goat anti-mouse IgG and washed again. HRP activity was revealed with DAB reagent (Vector Labs, Burlingame, CA). Specimens were counterstained with hematoxylin and mounted in Vectamount (Vector Labs). Explant outgrowths were fixed for 10 min in 4 % paraformaldehyde, cut into ~ 1 cm-long angular segments and set in 24 well plates. After cell permeabilization and blockade the segments were incubated with the anti-p63 moAb or with anti-Krt3 moAb (clone AE5, 2 µg/ml in 0.1 % BSA). After washes the segments were reacted for 1 h with 2 µg/ml Alexa 488-conjugated anti-mouse IgG, counterstained with DAPI and mounted in Vectashield (Vector Labs). Specimens were observed and photographed in an IMT2 microscope equipped with a computer controlled Nikon D-90 camera.

2.4 Western blotting

Equal numbers of cells from a single experiment were spun down. Protein was fully dissolved by incubation in PAGE sample buffer. Equal protein amounts were electrophoresed in polyacrylamide gels and the protein was electro-transferred to nitrocellulose membranes or the gels were stained with Commaassie blue R250. Nitrocellulose membranes were blocked with fat free milk and incubated overnight with monoclonal antibodies against Krt3 (clone AE5), connexin 43 (clone CX-1B1; Invitrogen)

or two distinct anti-p63 rabbit polyclonal antibodies from Biolegend (San Diego, Ca) and Abgent (San Diego, Ca). The two antibodies were raised against distinct short peptides sequences of that are identical in human and rabbit. Antibodies against other proteins frequently used to characterize limbal-corneal epithelium growth and differentiation features are not functional on the equivalent rabbit proteins. After washings membranes were reacted with the appropriate HRP conjugated goat anti-mouse or anti-rabbit IgG antibodies (Santa Cruz) for 45 min. HRP activity was detected by chemiluminescence. Due to its very high concentration in the corneal epithelial cells, rabbit GAPDH could not be used as loading control for chemiluminescence. Instead, we used ERK1/2 (Santa Cruz). The equivalence to GAPDH as a loading control was confirmed in Western blots using a 10-fold lower protein load (not shown).

2.5. Real time PCR

Total RNA was extracted with RNeasy^R, cDNA was generated from 2 µg RNA using Omniscript^R. PCR reactions were carried out in triplicate in 384 wells plates using Quanti Tech Sybr green^R, 10 mM primers, 10 ng cDNA and 40 annealing cycles of 30 sec at 59° C. All three reagents above were from Qiagen (Boston, MA). For increased reproducibility wells were filled with the help of an EpiMotion 5070 automatic dispenser. The forward and reverse primer sequences for rabbit GAPDH and TFGβ1, synthesized by Bioneer (Alameda, CA) were, respectively, AAGGCCATCACCATCTTCCA and GGATGCGTTGCTGACAATCT, and AAGGGCTACCACGCCAACTT and CCGGGTTGTGCTGGTTGTAC. Correct amplicon products were confirmed by melting curves showing a single peak in the pre-validated temperatures. Relative concentrations of TFGβ1 were calculated using the Ct method and GAPDH as the normalization gene. Reactions missing the reverse transcriptase enzyme were used to control for the potential contribution of genomic DNA contamination to the PCR product.

2.6 Clonogenic proliferation

Identical numbers of live cells, as measured in the flow cytometer, were plated in Cnt20 (Zen Bio, Research Triangle Park, NC) on collagen I-coated 25 cm² flasks on six well plates, cultured for 7-11 days and stained with Commassie blue R. Cnt20 an advanced commercial formulation of the typical low calcium epithelial cell culture media (Tsao et al, 1982) had been previously shown to preserve proliferative capacity, colony forming efficiency and stem cell-like phenotype of human corneal epithelial cell (Lu et al, 2010). Colonies were manually counted and recorded as % of the SHEM count which was set at 100 %. Data from four independent experiments was averaged. A preliminary pilot experiments determined that CFEs were constant for seeding densities of between 25 and 200 cells per cm², provided the count was made before colonies fuse to each other.

3. RESULTS

3.1 Effects of FBS, TGFβ1 and SB on explant to substratum cell transference and cell proliferation in outgrowths

Figure 1, A-D provides a pictorial account of the principal observation underpinning this report. Rabbit limbal segments cultured on 100 mm plastic petri dishes in SHEM yielded

outgrowths of near identical size (Figure 1 A). Replacement of FBS with Albumax II to generate sfSHEM resulted in very little growth (Figure 1 B). The addition of 10 μ M SB to this latter medium restored the growth to a level comparable to that attained with FBS (Figure 1 C and see below section on culture compaction). Adding 0.2 ng/ml TGF β 1 (Figure 1, D) to the sfSHEM medium did not seem to cause any apparent further reduction in growth rate over that caused by the removal of FBS.

Daily observation of size and the features of the edge of these outgrowths suggested that the stunted growth in the FBS-free conditions did not merely reflect a proportional slower growth rate. Growth was comparable for the first 4-5 days in all four conditions described (not shown). However, from there on, area growth in the FBS and SB-free conditions stalled for the subsequent 10-12 days culture period. Phase contrast microscopy observations showed that while the leading edge of growth were extremely compact in both SHEM and sfSHEMSB (Figure 1, E and F and F insert), in sfSHEM the cells continuously rounded up and shed away from the culture (Figure 1, G). The phase contrast micrographs shown were obtained by lightly skewing the phase contrast ring of the microscope. The images obtained were particularly useful to ascertain that the cultures were of pure epithelial cells without admixing of stromal fibroblasts. A light trypan blue stain showed that in the sfSHEM medium (Figure 1, G insert), beyond a 1-2 mm distance from the explant, the culture cultures transitioned to a low cell density zone. Shedding edge features were very similar whether we used sfSHEM alone or complemented with 0.2 ng/ml TGF β 1 (not shown). The cell morphology of outgrowth made in SHEM, SHEMSB or sfSHEMSB (Figure 1, H-J) or in the cell yields achieved in the three conditions (Figure 1 K-L) were remarkable similar.

The difference in outgrowth area between SHEM and sfSHEMSB depicted in Figure 1 A and C, reflected increased culture compaction when FBS was replaced by SB in cultures made on plastic dishes; on those dishes, SHEM/sfSHEMSB density (outgrowth area/total cell yield) ratio was 1.52 ± 0.21 (n = 4, p < 0.05). Compaction differences were much smaller or nil for growths on permeable inserts (mean $\pm$ SD densities of 0.49 ± 0.12 ; 0.56 ± 0.18 and 0.55 ± 0.1 million cells /cm² for SHEM, sfSHEMSB and SHEMSB, respectively; n =4).

Since SB rescues growth in the FBS-free condition it was to be expected that TGF β 1 should have the opposite effect. Thus, the lack of increase in growth inhibition by 0.2 ng/ml TGF β 1 over the inhibition caused by the removal of FBS, was puzzling. To further examine the impact of exogenous TGF β on this issue, we initiated multiple explant cultures in permeable inserts in SHEM and after 5 days we selected cultures with comparable outgrowths, removed the explant biopsies to isolate effects related to cell proliferation in the outgrowth from potential contributions from cell migration from explant to the growth surface, and extended the culture of the cells already migrated there for another 6 days in either sfSHEM alone (Figure 2 A), or complemented with either SB, 0.2 ng/ml TGF β 1 or 1 ng/ml TGF β 1 (Figure 2 B-D, respectively). In all four conditions final outgrowth areas were similar, but cell densities were markedly different. In sfSHEM substantial zones of the cultures became sparse (Figure 2, A, see arrow in insert). The SB complemented samples remained homogeneously compact and cells displayed a substantially smaller surface area than without it (Figure 2, B). Adding 0.2 ng/ml TGF β 1 instead of SB resulted in a cell

distribution and cell surface area that was essentially indistinguishable from that seen with sfSHEM (Figure 2C, note arrow in insert); only when 1 ng/ml TGF β 1 was included there was a strong additional inhibitory effect that resulted in visible cell size growth and apparent cellular differentiation (Figure 2, D). Cell counts from two other duplicates of each condition showed that SB caused a 3.3-fold increase in cell yield compared with the sfSHEM medium. Addition of 0.2 ng/ml TGF β 1 caused only a small decrease (13 %) in yield compare with the yield in sfSHEM. The higher 1 ng/ml concentration, though, caused a 78 % decrease of the yield in sfSHEM.

Flow cytometry was used to collect data on the FSC and SSC distribution of the outgrown cell populations. The majority of the cells are included within an arbitrarily defined lower left quadrant (LLQ) of the bivariate plot representing small cells of low intracellular complexity (Supplementary Figure 1, A-C). Cells that fall outside this arbitrary quadrant are either substantially larger or have a substantially higher granularity (i.e., intracellular complexity). SB induced substantial reductions in the percent of cells present outside the LLQ. In six independent experiments (one including only SHEM and SHEMSB, the other five all three media compared), the % was decreased by 43 ± 11 % by the addition of SB to SHEM ($n = 6$, $p < 0.05$) and by 37 ± 10 % when SHEM was replaced by sfSHEMSB ($n = 5$; $p < 0.05$). The difference between the two SB complemented media was not statistically significant.

Flow cytometry was also used to determine differences in the % of cells that can exclude the mitochondrial dye JC1 (Supplementary Figure 1, D-F). The exclusion reflects the action of the stem cell related xenobiotic efflux transporter ABCG2/BCRP, generating a JC1^{low} cohort (Selver et al, 2011). Mean $\pm$ SD ($n = 4$) JC1^{low} for SHEM, SHEMSB and sfSHEMSB were 26.4 ± 14.3 , 27.3 ± 19.1 and 23.8 ± 11.4 percent, respectively and were non statistically different.

Immunohistology was used to examine the spatial expression of p63, a critical determinant of epithelial extended proliferative potential. p63 nuclear level may correlate with limbal epithelial stem/precursor status (Pellegrini et al, 2001; Epstein et al, 2005; Kawakita et al, 2009). In the adult human cornea p63 is present only in a subset of basal limbal epithelial cells. In order to interpret results in rabbit we first examined the distribution of the epitope in the native cornea. In our 6-8 month old rabbit, p63 was copiously present in both limbus and central cornea (Figure 3, A and B). In the explants outgrowths, most cells were p63-positive whether the culture was performed in SHEM F or sfSHEMSB (Figure 3, C-G) and between 1/3rd and half of these cells, depending of the area observed exhibited a high fluorescent level (Figure 3, I and J), commonly referred as p63^{bright}.

We also examined the distribution of Krt3; a differentiation marker expressed when basal cells transition from the limbus to the cornea or when they stratify within the limbus (Schermer et al, 1986; Rodrigues et al, 1987). The appearance of the stain showed a great variability in different areas of each culture whether in SHEM (Figure 3, K-M) or in sfSHEMSB (Fig 3, N-T), but overall most cells had minimal or nil Krt3 expression in either medium.

Commassie blue R stain of total electrophoresed protein from cells cultured in SHEM, sfSHEMSB or SHEMSB and Western blots were used to compare the expression of the major tissue proteins and of the two growth and differentiation-related proteins that were characterized above by immunohistology (Figure 4). Connexin 43, a protein whose accumulation correlates with the change from limbal to corneal phenotype (Matic et al, 1997; Wolosin et al, 2002; Chen et al, 2006) was also used. There were no visible difference between cells grown in SHEM, SHEMSB or sfSHEMSB (Figure 4, left panel, columns B, C and D, respectively) in the amounts of the major visible (about 20) polypeptides. The right panel of Figure 4 depicts Western blots for, a) p63 (labeled by the # 1; results for two different antibodies, as described in Methods); b) Krt3; and c). Neither p63, nor connexin 43, showed any remarkable difference in expression between the three media; the average signal intensities ($n=3$) for these two proteins in SHEMSB were 103 ± 8 and 107 ± 11 percent of the SHEM signal, respectively. The corresponding values for sfSHEMSB were 96 ± 11 and 116 ± 4 percent. Krt3, though, showed a statistically significant reduction in the sfSHEMSB medium (58 ± 16 %, $n=4$; $p < 0.05$) when compared with the expression in SHEM cultures.

Real time PCR was used to examine whether endogenous transcription of TGF β 1 was affected by the differences in culture media. Explants were cultured in SHEM, SHEMSB or sfSHEMSB. After outgrowths reached more than 2 cm in diameter, some of the sfSHEMSB cultures were transferred to sfSHEM for the last 72 h culture. Total RNA was then prepared and used to complete the real time PCR measurements as described in Methods. Two independent experiments, each using a different rabbit, were performed (details in Supplementary Figure 2) The C_t analysis of the duplicate experiments yielded mean $\pm$ SD TGF β 1 mRNA values relative to of the expression level in SHEM of 83 ± 2 %, 200 ± 50 % and 113 ± 42 % for SHEMSB, sfSHEMSB and sfSHEM, respectively.

Finally we compared relative proliferative capacities of outgrown cells under clonogenic conditions (Figure 5, A and B). Based on side by side comparisons for each rabbit studied the average $\pm$ SD SHEM/sfSHEMSB CFE ratio for 4 independent experiments was $100:92 \pm 16$. Fibroblasts, whether as single cells or cell colonies were very rarely observed in these clonogenic cultures, indicating that few and rarely fibroblasts infiltrated the epithelial outgrowth.

3.2 Xeno-free, defined medium

To define the minimal formulation that can support viable growth rates, explant cultures were performed in sfSHEMSB deficient in selected components in permeable inserts coated with collagen type I (Figure 6). In each experiment the corneal pair from one rabbit was cut into 12 to 16 similar limbal segments. These segments were then used to compare the two or three different deficient conditions against complete sfSHEMSB or sfSHEMA83. We confirmed in preliminary experiments that the two TGF β receptor inhibitors have indistinguishable effect on limbal outgrowth (not shown). Results for each independent experiment were normalized to the average yield for the complete sfSHEMSB/A83 media. All individual culture yields for each condition from the different experiments were then used to calculate mean $\pm$ SD for each group and student's t-test p values. Removal of EGF

did not prevent the explant-to-insert membrane cell migration, but it drastically inhibited proliferation of the transferred cells. In contrast, the subtraction of Albumax II (-Albmx) or cholera toxin (-ChT) in the presence of SB or the combined removal of Albumax and ChT (-A&C) when using A83, did not cause statistical changes in cell yields. In regard to potential effects of the subtraction of these factors from the culture medium on cell phenotype, neither a clonal growth test (Figure 5, C and D), nor the percent of cells in the lower left quadrant (LLQ) of SSC/FSC plots or of JC1 excluding cells (Table 1) suggested any substantial change in the outgrowth cells as a result of these subtractions.

3.3 Sequential rabbit explant culture

While the results for first generation of rabbit outgrowths demonstrated the independence of the outgrowth features on FBS, we considered the possibility that FBS may turn out to be necessary for the *in vitro* survival of limbal epithelial precursor cell within the explant niche. To investigate this possibility, limbal explants were subjected to a serial explant culture protocol (Selver et al, 2011). The limbus of a pair of rabbit corneas was divided in 12 very similar sections and used to carry 4 replicates in each of the 3 growth media for up to six generations, using culture intervals of 8 to 11 days for each generation. At various stages, to allow simultaneous comparative analyses of clonal proliferation, JC1 dye exclusion and protein expression, harvested cells were frozen using the same freezing protocol. In a few instances, after the transference of a limbal biopsy to the next culture step, the new outgrowths included fibroblasts, easily identifiable by their spindle shape. These specimens were discarded.

Cell yield results of these studies are summarized in Figure 7. There were no significant differences in the total numbers for the three conditions in each of the first three serial explant generations and numerical differences within each generation evened out when total yields over these three generations were added up. Clonogenic growth capacity was measured in the 3rd outgrowth generation (Figure 5, E-J). The SHEM: sfSHEMSB CFE ratio average from four independent experiments was 100:105 ± 21. The epithelial nature of colonies was generally ascertained by transmitted light microscopy (Figure 5 K and L).

By the sixth generation, after two months of continuous explant culture, when each of the 4 limbal quarters have yielded about 15 million outgrowth cells absolute yields where somewhat diminished with respect to the earlier generation yields but where not different between all three culture media compared. Expression of the major cell proteins (Figure 4, left panel, columns E and F) remained unchanged through the multiple culture rounds. The p63 immunoblots, though, suggested that p63 was better preserved in the FBS-free sfSHEMSB medium (Figure 4, right panel, sixth generation rows).

3.4 Human explants cultures

An experiment was performed on permeable inserts with human limbal tissue comparing SHEM, sfSHEM, SHEMSB and Albumax-free sfSHEMSB, with 3 limbal segments used for each condition. For the first seven days outgrowths proceeded similarly in SHEM and the two SB-complemented media but all three sfSHEM did not generated outgrowths. Average yields were 66, 93 and 73 thousands cells for SHEM, SHEMSB and sfSHEMSB. The only

visible difference in outgrowth appearance was a more contracted edge in the protein-free sfSHEMSB medium (Figure 8, A-C). The cell size distributions (Figure 8, D-F) and JC1^{low} cell content Figure (8, G-I) were also similar. The addition of SB, thought may have a positive effect on the preservation of clonal growth capacity (Figure 8, J-L). After this seven days the outgrowth in sfSHEMSB slowed. The reduced proliferation seems to be related to additional growth factor requirement; complementing shSHEMSB with a six growth factors cocktail containing KGF, HGF, epiregulin, EGF-1, EFG2 and EFGBP7, each at μM , we were able to maintain similar rates of growth in SHEM and sfSHEMSB for at least the first three generations (unpublished).

4. DISCUSSION

Expanded population of limbal epithelial precursor cells are used to treat limbal stem cell deficiency. Explant outgrowth is the most common approach to generate such expanded populations. These cultures are typically made in SHEM formulations similar to those used for the growth of keratinocyte stem/precursor cells in clonogenic conditions under 3T3 feeder cell support (Rheinwald JG and Green H, 1975, 1977; Stanley and Dahlenburg, 1984). Much like this clonogenic modality, limbal cell explant culture showed a strong dependence on serum complementation; FBS removal resulted in an arrest in the size of the outgrowth cell (Figure 1 B). Careful temporal observation of these patches indicated that the arrest reflects a dynamic state involving continuous cell exfoliation at the edge of the outgrowths which is numerically matched by continuous transference of cells from the explant to the outgrowth surface. The fact that the addition of 0.2 ng/ml TGF β 1 did not have any significant effect on the FBS-free outgrowths size or appearance, coupled to the known proliferation- arresting effect of TGF β on epithelial cells, made the TGF β pathway a likely candidate for the observed phenomenon. This hypothesis was confirmed by the observation that two structurally unrelated inhibitors, TGF β R/ALK5 inhibitors SB431542 and A-83-01, readily restore the growth rates achieved in the presence of FBS. By virtue of the defined nature of the sfSHEMSB/A83 media, this result indicates the endogenous (autocrine) origin of the TGF β agent. Finally, the seemingly identical rates of growth in sfSHEMSB and SHEMSB, suggested that under EGF support, the impact of FBS on cell proliferation in the explant cultures might be solely due to neutralization of the endogenously derived TGF β agent or agonist. The only difference between FSBS and non-FBS based media was a decrease in the rate of Krt3 generation. Such mild pro-differentiation effect is consistent with the concept that sera incorporate pro-differentiation factors. Preliminary experiments indicated that the effect of replacement of FBS by pathway inhibitors may also be effective for the human.

Given the known presence of TGF β in serum, the effect of FBS would seem paradoxical. However, most of the hormone occurs in latent form or bound to serum proteins and lipids. The concentration of free total TGF β in human serum has been measured to be between 0.04-0.1 ng/ml (Kropf et al, 1997; Grainger et al, 1995). Thus, assuming similar levels for FBS, SHEM (5 % FBS) may contain as little as 2-5 pg/ml free, intrinsic TGF β . Thus, rather than contributing to the overall local free TGF β levels, the serum may be buffering TGF β that emerges from endogenously-secreted latent forms, in particular within the tight inter-cellular spaces. This possibility is supported by the observation that α_2 -macroglobulin at

physiological concentrations is able to mask over 90 % of free TGF β 1 (Kropfa et al, 1997). Another possibility is that FBS induces cellular signal transduction states that cause cells to be irresponsive to TGF β (e.g., by reduction of surface receptor levels). Since removal of FBS does not cause a reduction in TGF β 1 mRNA (Results), a mechanism involving inhibition endogenous production of TGF β by FBS appears unlikely.

Our studies also addressed the possibility that FBS may still play specific roles in the stem/precursor cell survival within the limbal niche in the explanted biopsies. The fact that replacement of FBS by SB had no effect on the capacity of the rabbit limbal explants to continuously yield cells at equal rates for at least two months, suggests that, at least for this substantial time period *in vitro* limbal stem cell function does not depend on exogenous serum components. The only effect of the removal of FBS we observed was the putative enhanced preservation of p63 levels. However, this result has not been statistically confirmed.

Finally, our experiments show that neither (bovine-derived) Albumax II and/or (bacterial) ChT are essential for explant outgrowth in serum free medium, at least for the initial two week explant culture. The medium resulting from the subtraction of these two components is fully defined and of known chemical composition and can easily be made xeno-free by modifying the provenance of ITS components. Thus, shSHEMSB or sfSHEMA83 represent excellent platforms for research on cell-cell interactions in the compact outgrowth cultures and identification of chemical effectors that could increase outgrowth cell yield and/or the density of regeneration-able cells within these outgrowths. Our preliminary experiment on human limbal explants suggests the potential applicability of these results to human cultures. Control of TGF β signaling may also find application in the novel *in vivo* outgrowth approach to autologous treatment for unilateral stem cell deficiency (Sangwan et al, 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Removal of FBS from limbal biopsy explant cultures inhibits explant outgrowth proliferation.
- Complementation of the serum-free medium with an inhibitor of TGF β signaling fully restores explant outgrowths to its FBS-complemented condition, including preservation of multiple cellular phenotypes.
- Addition of the inhibitor to the FBS-complemented medium does not affect growth and differentiation
- The results suggest that the only role of serum in limbal epithelial explant outgrowth cultures is the neutralization of an autocrine cytostatic TGF β signaling.
- Results open the door for the production of limbal epithelial sheets for regenerative medicine using defined media of known composition.

Figure 1. Selected features of rabbit limbal explant outgrowths in various media

Left frame. Stained 14 day explant cultures on 100 mm petri dishes in SHEM (A), sfSHEM (B), shSHEMSB (C) or sfSHEM plus 0.2 ng/ml TGF β 1. **Center frame.** The edge of the explant outgrowths in SHEM (E), sfSHEMSB (F) and sfSHEM (G). Main images are phase contrast micrographs of live cells. Inserts are low magnification transmitted light micrographs of cultures lightly stained with Commassie blue. **Top right frame.** Mean $\pm$ SD cell yields for 6 independent experiments. Each experiment involved the culture of between 2 and 4 biopsies. H) SHEM; I) SHEMSB or J) sfSHEMSB. **Bottom right frame.** Phase contrast micrographs from locations equidistant from explant and edge culture in K) SHEM; L) SHEMSB or M) sfSHEMSB. Bar = 50 μ m.

Figure 2. Effect of FBS, SB and TGF β 1 on proliferation of cells outgrown from limbal explants
Biopsies were first explanted in permeable inserts for 5 days in SHEM, the biopsies were then removed and the outgrowths were cultured were for 6 additional days. Main frames are phase contrast micrographs of outgrowths cultured in A) sfSHEM; B) SHEMSB; B) sfSHEM plus 0.2 ng/ml; or D) 1 ng/ml TGF β 1. Inserts are low magnification light micrographs of the stained cultures.

Figure 3. Rabbit cornea and explant outgrowths stained with p63 and Krt3

A. Limbus. **B.** Central cornea **C-D** and **F-H.** p63 stain (**C** and **G**), DAPI counterstain (**D** and **F**) and merged images (**E** and **G**) of outgrowths in SHEMA and sfSHEMA, respectively. **I** and **J.** Lower and higher magnifications of **C.** **K-M** and **N-P,** Representative micrographs of outgrowths after staining for Krt3 when cultured in SHEMA or sfSHEMA , respectively. **Q-T.** Krt 3 stain, DAPI counterstain (**P**) and merged image (**Q**) of an outgrowth in sfSHEMA.

Figure 4. Commassie blue staining and Western blots of cells grown in three different media
Right panel. Commassie blue. A. Ladder. B-D, first outgrowth. E and F, sixth outgrowth. B and E, SHEM. C and F, sfSHEMSB. D, SHEMSB. **Left panel.** Western blots. The antibody targets are indicated. For p63 results are shown for the two different polyclonal antibodies described in Methods. Results for the first (1) and sixth (6) outgrowth generation from the same explants are shown. The false positive reaction of the secondary goat anti-rabbit antibody with an endogenous rabbit antigen (IgG) was used as a loading control for p63. For the monoclonal antibodies against connexin 43 (Cx43) and Krt3 (Krt3), we used monoclonal anti-ERK 2 as loading control. The presence of SB in the cultures reduces expression of Krt3 in the first outgrowth generation. The absence of FBS helps preserve p63 expression after 6 sequential outgrowths.

Figure 5. Paired comparisons of clonal proliferation in Cnt20 of cells derived from explant cultured in different media or in different generations

A-D. First generation explant cultures. Pair 1: A) SHEMA and B) sfSHEMA; pair 2: C) sfSHEMA and D) sfSHEMA without Albumax and ChT. **E-J.** Third generation cultures. E and H) SHEMA; F and I) SHEMA; G and J) sfSHEMA. Each duplicate was made with cells obtained from pools of multiple outgrowths from two different rabbits K and L) Light micrographs of the stained colonies acquired with 4x and 20 x objectives, respectively.

Figure 6. Effect of removal of biological components from shSHEMSB or sfSHEMA83 media
Mean $\pm$ SD for three independent experiments each involving multiple explant biopsies in each medium. The number of individual explants involved in the comparison against the complete media is indicated (n).

Figure 7.
Cell yield as a function of serial explant culture stage in different media.

Figure 8. Features of cells harvested from 7 day human limbal explant cultures
Top left. Phase contrast micrographs of the edge of outgrowths in SHEM (A), SHEMSB (B) and sfSHEMSB (C) in Costar insert. Bottom left. FCS/SCC and (D-F), JC1 emission (G-I) plots for SHEM (D, G), SHEMSB (E, H) and sfSHEMSB (F, I). Right panel. CFE images from 2000 harvested outgrowth cells; the duplicates represent cells from independent outgrowths from the same donor tissue in SHEM (J), SHEMSB (K) and sfSHEMSB (L).

Table 1

Percentiles of cell included in the lower left quadrant of SSC/FCS plots (LLQ)and JC1^{low}

Mean $\pm$ SD for three independent experiments, each one including between 3 to 5 explants per media.

Medium	sfSHEMA83					
	Complete		no Albmx- ChT		no Albmx	
	Mean	SD	Mean	SD	Mean	SD
% LLQ	82.5	2.5	85.8	1.9	83.8	10.1
% JC1 ^{low}	19.1	3.9	15.8	5.4	10.1	7.3