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Cultivation of Lacrimal Gland Acinar Cells in a Microgravity Environment

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Abstract

Background: A Rotary Cell Culture System (RCCS) allows the creation of a microgravity environment of low shear force, high-mass transfer, and 3-dimensional cell culture of various cell types. Aim of this study was to evaluate the growth pattern and the secretory function of rabbit lacrimal gland acinar cells in a microgravity environment using a RCCS.

Methods: Lacrimal gland acinar cells from male New Zealand White rabbits were isolated and cultured in a RCCS up to 28 days. Cells were analysed by light and electron microscopy and apoptosis was assessed by the TUNEL-Assay at day 7, 14, 21 and 28. Secretory function was tested by measuring the β -hexosaminidase activity.

Results: After 7 days of culture, spheroidal aggregates were found inside the RCCS. The spheroids consisted of acinus-like cell conglomerates. Apoptotic centers inside the spheroids were observed at all time points by means of the TUNEL-Assay. Evaluation of the secretory function revealed β -hexosaminidase release after carbachol stimulation which decreased over the culture period.

Conclusion: A simulated microgravity environment promotes the development of three dimensional cell spheroids containing viable acinar cells up to 28 days. Due to the evolving central apoptosis, it is unlikely that such simple three dimensional cell communities can serve as tissue equivalents for clinical transplantation, but they promise opportunities for further applications in basic and applied cell research on lacrimal gland cells.

Keywords:

Lacrimal gland, tissue engineering, acinar cells, in vitro, bioreactor

Introduction

Aqueous tear deficiency due to lacrimal gland insufficiency is one of the major causes of dry eye. In severe cases such as Sjogren's syndrome, Stevens-Johnson-Syndrome or ocular mucous membrane pemphigoid the therapy with artificial tears can be insufficient to relieve severe discomfort. Transplantation of the lacrimal gland is hardly feasible due to the short ischaemia time of the tissue of less than 6 hours and the small dimension of the glands vascular supply. In the absence of a suitable lacrimal gland transplant, salivary glands have been used instead to treat patients with otherwise intractable severe dry eye.¹ However, the substantial differences between tears and saliva in their electrolyte and protein composition result in persistent ocular surface disease and can induce a microcystic epithelial oedema. Engineering a lacrimal gland construct may offer a suitable alternative transplant with a tear like secretion.

Several methods have been developed to maintain lacrimal gland cells in vitro,^{2 3 4 5 6} but preservation of the acinar phenotype and secretory function has proven to be difficult. Rotary Cell Culture Systems (RCCS) were originally designed to predict the impact of the microgravity environment in space for the culture of cells⁷. They have proven to support the development of three dimensional tissue structures and the formation of cell-cell contacts^{8 9} and to provide an environment of low shear force and high mass transfer of nutrients and metabolic wastes.¹⁰ Aim of this study was to evaluate the effect of a microgravity environment on the growth pattern and the secretory function of lacrimal gland acinar cells.

Materials and Methods

Animals

Six lacrimal glands from three male adult 2 kg New Zealand White rabbits (Charles River, Germany) were used for the study. The animals were treated in accordance with the ARVO statement for the use of animals in ophthalmic and vision research.

Cell preparation and Culturing

For preparation of lacrimal gland acinar cells a method described by Guo et al. was used.¹¹ After removal, the glands were cut into small pieces, washed and enzymatically digested by collagenase (350 U/ml, Sigma C 0130), DNase (40 U/ml, Sigma D 5025) and hyaluronidase (300 U/ml, Sigma H 3884). The cell digest was then centrifuged and washed twice, before filtering through a 70 µm mesh. The retained cell suspension was centrifuged through a ficoll gradient and the obtained cell subpopulation was washed, centrifuged twice and resuspended in Hepato Stim® culture medium complemented with EGF (5 ng/ml), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). A cell density of 9-10 x 10⁶ acinar cells was seeded per 10 ml-vessel. Four vessels were mounted on the RCCS and rotated at approximately 18 rounds per minute (~0.01 G).

Light and Electron Microscopy

Cultured cells were examined by light and electron microscopy at day 7, 14, 21 and 28. For light microscopy, cells were washed with PBS, fixed with 4 % buffered paraformaldehyde and stained with haematoxylin-eosin and methylene blue. For electron microscopy, cells were immediately fixed by immersion in 0.1 M cacodylate buffer containing 2.5 % glutaraldehyde and 2 % paraformaldehyde at pH 7.4 for 24 h. The specimens were postfixed in 1 % OsO₄ and stained “en bloc” with 2 % uranylacetate. After dehydration in graded alcohols the specimen were embedded in Araldite. Semi-thin sections (0.5 µm) were stained with methylene blue and azure II to visualize the regions of interest. Ultrathin sections (70-90 nm) were cut and stained with lead citrate and examined under an electron microscope (Phillips, EM 400). The findings were recorded both by conventional films (Agfa) and a digital image system (analySIS, Soft Imaging System, Münster, Germany).

TUNEL-Assay

Apoptosis was assessed by staining the fragmented DNA with the terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end labelling (TUNEL) ¹² using the ApopTag[®] Peroxidase In Situ Apoptosis Detection Kit (S7100, Chemicon[®]). In brief, specimens were dewaxed and stripped from proteins by incubation with 20 µg/ml proteinase K for 15 minutes at room temperature and the slides were then washed with dH₂O for 2 minutes, 2 times. Endogenous peroxidase was inactivated by covering the sections with 3 % H₂O₂ for 5 minutes at room temperature. The sections were rinsed with PBS and immersed with equilibration buffer (75 µl/5 cm²) for 10 seconds. Soft plastic coverslips were always placed on the slides after application of the reagents to spread them evenly by capillary action over the area of the slide. Working Strength TdT Enzyme was then added to cover the sections (55 µl/5 cm²) and incubated at 37°C for 60 minutes. The reaction was terminated by transferring the slides into the Stop/Wash Buffer for 10 minutes at room temperature. Then the specimen were washed three times in PBS for one minute and then the Anti-Digoxigenin conjugate was applied on the slides (65 µl/5 cm²) and incubated in a humidified chamber at room temperature for 30 minutes. The specimen were then washed four times in PBS for 2 minutes and the peroxidase substrate was added to the surface of the specimens (75 µl/5 cm²) for three to six minutes. Then the slides were washed three times in dH₂O for one minute each wash and incubated for 5 minutes. Finally the specimens were counterstained with methyl green (0.5 %).

Measurement of spheroid diameters

The mean diameters of the spheroids and apoptotic centers were determined as the mean of the largest and the shortest diameter using a light microscope (Zeiss Standard 20, Magnification 10x) with a calibrated micrometer scale. Seventy eight spheroids were measured at day 7, 77 at day 14, 80 at day 21 and 60 at day 28. The apoptosis ratio was determined by division of the apoptotic area diameter through the diameter of the spheroid and multiplication by 100. Differences between the mean diameters of the spheroids with and without apoptotic centers and the apoptosis ratio were evaluated by the analysis of variance (One-Way ANOVA-Test) and the Student's t-test. Differences at the level of $p \leq 0.05$ were considered statistically significant.

β-hexosaminidase Assay

Secretory response to carbachol was analysed at day 7, 14, 21 and 28 and at least three wells were set per condition. Before stimulation, the lacrimal gland cell spheroids were removed from the vessel and carefully rinsed with DMEM three times, then resuspended in DMEM and distributed drop by drop in wells of a 96-well plate. The suspension was then incubated at 37° with 5 % CO₂ for two hours. After removing a baseline sample, carbachol was added to a final concentration of 100 μM and cells were incubated for 30 minutes. The samples were centrifuged for 5 minutes and the supernatant medium was stored frozen at -20° until use. For measurement of the β-hexosaminidase activity, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide was used as a substrate and fluorescence intensity was determined using a method described by Barrett and Heath¹³. The experiments were repeated three times with lacrimal gland cells from three different rabbits.

Results

Morphology

After digestion and filtration of the lacrimal gland tissue, a suspension of single cells and small cell clusters was placed in the RCCS.

After 7 days of culture, small spheroidal aggregates with a mean diameter of 384.6 ± 111.8 μm (mean ± std. deviation) were found inside the rotating vessels (Fig. 1A). The spheroids consisted of acinar cell conglomerates with fine granulation in their cytoplasm, typical for secreting cells (Fig. 1A, Insert). The cell communities showed organisation into acinus-like structures with a central lumen as discernible by electron microscopy (Fig. 2A, Arrowheads). Cells showed polarity with a basal nucleus and apical electron lucent and electron dense secretory granules. Microvilli were found on their apical surfaces and the apical cell parts were connected by desmosoms (Fig. 2B, Arrowhead). Not only in the acinus-like structures, but also on parts of the surface of the spheroids the cells showed polarisation and short microvilli (Fig. 2C). However, in the center of 24.4 % of the spheroids, groups of apoptotic cells with a condensed small nucleus were observed. In spheroids, where no apoptotic centers were visible by light microscopy, the TUNEL-Assay revealed sporadic apoptotic cells inside the spheroids (Fig. 3A).

On day 14 the mean diameter of the spheroids was 382.4 ± 92.2 μm. Acinus-like structures were frequently detected inside the spheroids. The central lumina were regularly filled with suggested secretory material (Fig. 1B Arrowheads, Fig. 2D), but the number of cytoplasmatic secretory granules was reduced (Fig. 2D). Apoptotic centers were noted in 48.1 % of the spheroids. Apoptotic cells became also more abundant in spheroids without apoptotic centers by means of the TUNEL-Assay (Fig. 3B)

On day 21 the mean diameter of the spheroids was 290.7 ± 62.8 μm. Viable cells with histotypic features of acinar cells were still found predominantly in the periphery of the spheroids. Also on parts of the spheroid surface, still polarized cells with microvilli were noted, but large centers of apoptotic cells were observed in 20 % (Fig. 1C, Arrowheads). Massive central apoptosis was confirmed by the TUNEL-Assay, which also revealed scattered smaller apoptotic areas in the periphery of the spheroids (Fig. 3C).

On day 28 the mean diameter of the spheroids was 388.9 ± 179.2 μm and 41.7 % of the spheroids contained large apoptotic areas (Fig. 1D, Arrowheads). Viable acinar cells were only rarely found between the apoptotic cells, mainly in the periphery of the spheroids. (Fig. 3D).

Spheroid diameters and apoptotic centers

On days 14, 21 and 28 the mean diameter of spheroids containing apoptotic centers was significantly higher compared to spheroids without apoptotic centers ($p \leq 0.05$). The mean ratio of apoptosis on the entire spheroid was 40 % on day 7, 38.9 % on day 14, 46.8 % on day 21 and 89.7 % on day 28. The analysis of variance showed a significant difference of the apoptosis ratio between the four time points and a significant increase of the apoptosis ratio was found between day 14 and 21 and day 21 and day 28 ($p \leq 0.05$) (Table 1).

Secretory Response to Carbachol

The lacrimal gland acinar cells showed a secretory response to carbachol stimulation which decreased over the culture period. The analysis of variance (One-Way ANOVA-Test) showed a significant difference of β -hexosaminidase secretion between the four time points ($p \leq 0.05$) (Fig. 4). A significant reduction of the β -hexosaminidase secretion after carbachol stimulation was found between day 7 and 28 in all three experiments.

Discussion

In this study we evaluated the growth pattern of lacrimal gland acinar cells in a microgravity environment. Microgravity bioreactors simulate a microgravity environment by adjusting the vessel rotation speed such that the cells are cultivated in a state of continuous free-fall. These conditions provide a relatively well-defined fluid dynamic environment, efficient mass transfer and low shear force.¹⁴

In our experiments simulated microgravity promoted the development of spheroidal aggregates with a mean diameter of $384.6 \pm 111.8 \mu\text{m}$ after 7 days. The spheroids consisted of organized lacrimal gland cells and acini-like structures were observed. The mean diameter of the spheroids did not increase substantially during the culture period of up to 28 days. In the center of the spheroids an area of apoptosis was noted at all time points. The development of apoptotic centers inside the spheroids correlated with their size, as on days 14, 21 and 28 the diameter of the spheroids containing apoptotic centers was significantly higher than in spheroids without apoptotic centers. Also the duration of the culture period seemed to play a role, as the ratio of apoptosis inside the spheroids increased significantly between day 14 and 21 and also between day 21 and 28. Similar observations were made in a study of Khaoustov et al., where spheroids of human liver cells were formed in microgravity bioreactors and central apoptosis was noted usually after one week in culture.¹⁵ The development of central apoptosis is also found in growing tumor populations without blood vessels in-vivo due to poor diffusion to the center of the cell aggregates.¹⁶ It is therefore likely, that the areas of apoptosis observed in our experiments are also a result of poor oxygen supply or reduced diffusion of nutrients into the center of the spheroids. This is supported by the short time lacrimal glands can tolerate ischemia and highlights the need to establish a vascular supply for voluminous constructs of lacrimal gland cells. The morphological results agree with the secretory response to stimulation with carbachol, as the secretory response of the cells decreased during the culture period. This was also observed by us and others when lacrimal gland cells were expanded under two-dimensional conditions. After 3 days of culture on amniotic membrane we found strong β -hexosaminidase secretion which was about 3-4-fold of the baseline. The secretory response decreased to approximately 2-fold of the baseline at day 7

and was substantially diminished beyond day 7.² Similar observations were made by Andersson et al. who reported optimal secretory response of acinar cells after 2-3 days in culture¹⁷ and Schechter et al. who noted a strong reduction of acinar cell secretion beyond day 7.⁵ The reason for the loss of secretory function during extended culture periods is uncertain. Schechter et al. discussed the longterm absence of a basal secretomotor stimulation in culture as one of potential explanation.⁵

In our experiments, apoptosis observed inside the spheroids may contribute to the functional compromise. However it is unknown, whether the inner part of the spheroids significantly contributes at all to the β -hexosaminidase-activity measured in the culture medium or whether due to a kind of diffusion barrier this only reflects activity of the periphery of the spheroid. In addition, a lack of an extracellular matrix in our experimental set up may also be involved, as it has been shown, that cells will tend to retain its differentiated phenotype in vitro only under conditions that resemble its natural in vivo environment. In particular, the combination of high cell density and an appropriate substratum have shown to be substantial to induce cell-cell and cell-matrix interactions^{18 19} and are likely to support differentiation and secretory activity of lacrimal acinar cells.

In conclusion, a RCCS promotes the development of three dimensional cell spheroids containing viable acinar cells up to 28 days. Due to the evolving apoptosis, it is unlikely that such simple three dimensional cell communities of lacrimal gland acinar cells can serve as tissue equivalents for clinical transplantation, but it shows that a simulated microgravity environment is capable to form three dimensional lacrimal gland cell communities of relatively high size, which is essential for a possible lacrimal gland substitute as high cell numbers will probably be needed for a sufficient lubrication of the ocular surface. As a variety of cell functions, including proliferation, migration and differentiation are regulated by cellular interactions with the extracellular matrix, the use of three dimensional scaffolds as an extracellular matrix may help to retain a differentiated phenotype and cell function in vitro.²⁰ It may also improve the mass transfer and oxygen supply in the center of the tissue and thus avoid central apoptosis.

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Tables

Table 1

	Day 7	Day 14	Day 21	Day 28
Measured spheroids	78	77	80	60
Mean Ø (µm)	384.6 ± 111.8	382.4 ± 92.2	290.7 ± 62.8	388.9 ± 179.2
Spheroids with apoptotic centers	19 (24.4 %)	37 (48.1 %)	16 (20 %)	25 (41.7 %)
Mean Ø of apoptotic centers (µm)	151.7 ± 48.8	173.1 ± 46.6	157 ± 61.2	513.3 ± 206.9
Apoptosis ratio (%)	40 %	38.9 %	46.8 %	89.7 %
Mean Ø of spheroids without apoptotic centers (µm)	385.4 ± 119.4	325.4 ± 51.1	280.5 ± 58.2	270.8 ± 35
Mean Ø of spheroids with apoptotic centers (µm)	382.6 ± 87.3	444.4 ± 87.1	332.2 ± 65.4	553.8 ± 168.8

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Legends

Figure 1: Light microscopy of haematoxylin-eosin and methylene blue stain. A: Spheroidal aggregates containing viable lacrimal gland acinar cells after 7 days of culture. The acinar cells show fine granulation in their cytoplasm, typical for secreting cells (Insert). B: Organized cell communities with acinus-like structures inside of spheroids after 14 days of culture. Some of the central lumina are filled with suggested secretory material (Insert, Arrowheads). C: Spheroid with vital acinar cells in the periphery and a central apoptotic area after 21 days of culture (Insert, Arrowheads). D: Section of a spheroid after 28 days of culture, presenting only few vital cells in the periphery, but a massive central apoptotic area (Arrowheads). The bars inside the images represent 100 μm .

Figure 2: Transmission electron micrographs. A: Acinus-like structures inside of a spheroid after 7 days of culture, forming central lumina (arrowheads). The cells show polarity with a basal nucleus and apical electron lucent and electron dense secretory granules. B: The apical cell parts are connected by desmosomes (arrowhead) and microvilli are found on the apical surface of the cells. C: Surface of a spheroid after 7 days of culture presenting polarised cells with a basal nucleus and short microvilli on the apical surface. D: Acinus-like structure after 14 days of culture, showing a central lumen and suggested secretion into the central cavity. The number of secretory granules in the cytoplasm is reduced. The bars inside the images represent 5 μm .

Figure 3: Staining of fragmented DNA with the terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end labelling (TUNEL) method A: Image of a spheroid after 7 days of culture. Sporadic apoptotic cells inside the spheroids are shown by the TUNEL-Assay. B: Spheroid without an apoptotic center, but distributed apoptotic cells after 14 days of culture. C: Central apoptosis, but also scattered smaller apoptotic areas in the periphery of a spheroid after 21 days of culture. D: Massive apoptotic area inside a spheroid after 28 days of

culture. Viable acinar cells are only rarely found between the apoptotic cells, mainly in the periphery. The bars inside the images represent 100 μm .

Figure 4: β -hexosaminidase release from acinar cells after 30 min. carbachol stimulation (100 μM). The cells showed a secretory response to carbachol stimulation which decreased over the culture period. The analysis of variance showed a significant difference of β -hexosaminidase secretion between the four time points ($p \leq 0.05$). A significant reduction of the β -hexosaminidase secretion after carbachol stimulation was found between day 7 and 28 in all three experiments.

Table 1: Mean diameters of spheroids and apoptotic centers at the four time points (mean \pm std. deviation). The apoptosis ratio was determined by division of the diameter of the apoptotic centers through the diameter of the spheroids and multiplication by 100.







