SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Long-term *in vitro* video microscopy and digital image analysis in the examination of ophthalmological tissue regeneration and tumor growth

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The Examination will be held 11 am, July 8, 2020

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Live online access will be provided. If you wish to take part in the discussion, please send an e-mail to varoczy@internal.med.unideb.hu not later than 12 pm on the day before the discussion (July 7, 2020). After the deadline, for technical reasons, it is no longer possible to join in to the defense.

1. INTRODUCTION AND THEORETICAL BACKGROUND

2. Introduction and identification of the problem

The issue of continuous snapshot sampling: If we subject a process to more in-depth and thorough analysis, results will be falsified by the artificial products created as a result of invasive, prolonged and frequent samplings. Long-term, real-time and non-invasive examinations with high temporal resolution using video microscopy can be used for the examination of cultured cell populations in which the importance of one or a couple of cells increases during the culture cycle.

Anatomical location, functions and features of the examined cell types

Uveal tract, uveal melanomas

The uvea is the heavily pigmented middle layer of the ball of the eye with a dense vein system between the retina and the corneoscleral shell. The uvea, which contains several anatomical regions of the eye, comprises the iris, the ciliary body, and the choroid layer.

Uveal melanomas belong to the most common malignant intraocular tumors. According to the data of the National Cancer Institute its incidence rate in the USA is 5,4 new cases/million men and women. 85% of ocular melanomas are of uveal origin, and, based on their location, can be anterior (iris, ciliary body) or, more frequently, in 80-90% of the cases, posterior (choroid).

Cell types of uveal melanoma; tumor growth

In terms of cytology, uveal melanomas are diverse: based on the cell type they can be: spindle cells A or B, epithelioid cells, and mixed cells. In the epithelioid cell type, a considerable percentage of tumors, monosomy of chromosome 3, and loss of heterozygosity can also be observed in chromosome 3 and more

rarely in chromosomes 9, 13, and 17. During the growth and spread of tumors vascularization has an important, limiting role. Primary metastasis through the circulatory system most frequently occurs in the liver. An explanation can be the chromosome disorder of the cells of epithelial and mixed cell tumors that possess a strong metastasizing ability.

The OCM-1 cell line (ocular choroidal melanoma-1) was established from the choroidal melanoma of a female patient diagnosed with uveal melanoma in 1985. The cell line was described as aneuploid, near tetraploid, linked to absence of chromosome 6.

Role of corneal and limbal cells in corneal degeneration

The transparent front part of the eye is the cornea. Its structure, from front to back is as follows: the corneal epithelium, Bowman's layer, the corneal stroma, the Dua layer or pre-Descemet's membrane, Descemet's membrane and the corneal endothelium.

The limbus is the transitional zone between the transparent cornea and the opaque sclera. Its functions include: nourishing and maintaining formation of the peripheral cornea and containing the pathways of aqueous humour outflow. Further, it plays a role in the healthy functioning of the corneal epithelium, ensuring that the epithelial stem cells stay in the limbus and by providing the limbal niche for them, and its function as limbal barrier. If conditions for these functions are not met, limbal stem cell deficiency accompanied by visual impairment may ensue.

Limbal epithelial stem cells play a key role in corneal epithelium regeneration. These cells have properties similar to those possessed by adult somatic stem cells; they are small and have a high cell nucleus to cytoplasm ratio. Although

their cell cycle is slow, they are capable of rapid proliferation in the case of superficial corneal erosion. During the healing process the cells have a wave-like action moving in a centripetal fashion in the corneoscleral region from the intact corneal epithelium towards the direction of the corneoscleral limbus. Migration of the epithelial cells can be described using the X Y Z hypothesis, where Z represents desquamation of superficial cells, which equals the sum of proliferating basal epithelial cells (X) and centripetally migrating cells (Y).

The scratch model for cell migration

The *in vitro* scratch model is a simple and advanced model for measuring cell migration. It can also be used to quantify migration rate, and can partially be used to study appropriate cell-matrix and cell-cell interactions during the examination of *in vitro* wound healing.

Materials tested during practical use

Antibiotics used in ophthalmology.

Corneal regeneration might take months to complete. During this time prevention of infections is of paramount importance. Success of the local therapy depends on whether epithelial growth can be sustained in the presence of antibiotics.

1, Chloramphenicol

Due to its bactericide and bacteriostatic effects chloramphenicol is widely used in medicine. However, in addition to its advantageous use in several therapies, it also has adverse effects. Several pieces of research carried out on human cell types show that it inhibits cell proliferation and can even cause apoptosis.

2, Rifampicin

It is used as an antituberculotic agent in human application, but is also used during treatment of some ophthalmological infections. In the normal and

tumorous cell lines originating from different tissues exposition has showed cell death correlating with time, and its cumulative damaging effect has also been demonstrated. It has also been observed that its toxicity is affected by cell cycle and its sensitivity by the mitotic phase.

Biological use and effects of nanoparticles

Owing to their size, nanoparticles, having entered the organism, can cause cell-level changes that affect the entire body.

1, Carbon nanoparticles

Carbon nanotube (CNT) is a special form of carbon, in which carbon atoms, through their chemical bonds, form a tube-like structure. Two subtypes are distinguished: single walled (SWCNT) and multiwalled (MWCNT) nanocarbon tubes. Due to their special properties that eliminate obstacles in the case of other nanoparticles, their biomedical applicability has been widely investigated. However, carbon nanotubes are length to width ratio particles, thus, with regard to their structural properties it can be seen that their toxicity is similar to that of other tube-like nanoparticles such as asbestos. Synthetic carbon nanoparticles are chemically inert, do not dissolve in aqueous media, and are biologically insignificant. For cleaning purposes and to increase solubility, they are often oxidized or functionalized with strong acids, which in some cases has led to higher toxicity of the nanotubes compared to the original: cytotoxic, apoptosis and necrosis inducing effects have been found.

2, Gold nanoparticles

Literature data show that gold nanoparticles have no or only very little toxicity even in the case of particles with superficial coating. However, more recent studies have questioned the safety of gold nanoparticle use. They also suggest that intracellular toxicity can primarily be assessed on the basis of their physio-chemical properties. *In vivo* toxicity data are consistent in that they claim that

nanogold accumulates in certain organs, e.g., the brain, but, like with the issues related to cellular toxicity, further, long-term studies are necessary to understand its toxicity risks on the organism.

3, Silver nanoparticles

Like with nanogold, serious questions have emerged in connection with the safety of silver particles, too. In addition to the advantageous properties of silver nanoparticles literature data have also reported on their adverse effects. Considering the latter, this substance should only be used with the utmost care. Once in the cells, silver nanoparticles can cause serious damage as a function of particle size and concentration. Elevation in ROS, morphological and mitochondrial damage and occurrence of characteristic forms of cell death have been detected.

Time-lapse microscopy and digital image analysis

The notion of time-lapse images represents a series of images in large temporal resolution, which, played as a film, give us an insight into processes that take place slowly relative to human scales. This technique can be used for observing microscopic-size events in a simple and reproducible manner using appropriate optic systems. The development of microscopes capable of making time-lapse images was warranted by questions that could only be investigated after eliminating temporal constraints. Data provided through continuous observation can cause a breakthrough in a large body of *in vitro* research.

3. OBJECTIVES

Using the combination of time-lapse imaging video microscopy and the properly used digital image analysis methods, we aim to create a versatile model system. This system allows a simple, versatile, easily reproducible method while preserving the temporal dynamics of the studied life processes in ophthalmic clinical speciments, in vitro, at the cellular levels of individuals or populations. Thus, our study can be divided into two groups: studies of individual cells e g., multipolar division of OCM-1 cells, and the monolayer regeneration scratch model of isolated human limbal cells (HuLi) at the level of cell populations.

The selected life processes in our studies were as follows.

1) Examination of single cells of uveal melanoma (OCM-1): a qualitative morphological description and quantitative dynamic analysis of multipolar cell division.
2) Establishment, maintenance, and description of a limbal cell population isolated from clinical samples.
3) Development of in vitro corneal wound-healing model: Human limbal monolayer regeneration scratch model.
4) Use of the scratch model in pharmacological studies: effect of antibiotics on corneal regeneration.
5) Application of a scratch model for cytotoxicity assays: Effect of multi-walled industrial-grade carbon nanotubes. Effect of inert metal nanoparticles on monolayer regeneration.

4. MATERIALS AND METHODS

Cell isolation, culture, and model systems

Culture conditions of ocular melanoma cells

Cells were cultured in RPMI 1640 and DMEM-Ham's F12 media, supplemented with 10% FBS (fetal bovine serum) and 1% PSN (Penicillin-Streptomycin-Neomycin Antibiotic Mixture).

Isolation and culture of limbal cells

Corneal epithelial limbus was obtained from a 56-year-old female patient. Before use the limbal ring was kept in Eusenol-C transplantation liquid at 4 °C. Before cell isolation the corneal collar was disinfected with 70% ethanol followed by Betadin. Next, the remaining parts of the cornea and sclera were removed and the limbus was mechanically cut into 2X2 mm pieces. Then the tissue pieces were incubated in DMEM-Ham's F12 +10% FBS +1% PSN containing 5 ml 800 U/ml collagenase IV for 2 hours. To gain cell suspension, the cells were passed through sterile gauze. Finally, a viability test was performed and the isolated cells were cultured in DMEM-Ham's F12 medium supplemented with 10% FBS and 1% PSN.

Limbal regeneration scratch model

Using cells isolated from the limbus we created an *in vitro* regeneration model. HuLi cells were cultured in glass bottom dishes. When confluence reached 90%, we scratched the layer of cells with a 20 G syringe needle. Then the culture was placed in a long-term scanning system.

<u>Examination of monolayer regeneration using time-lapse video microscopy</u>

By the monolayer regeneration scratch model we mean the technique of following the regeneration of confluent limbal monolayer of cells after it is scratched using a video microscopy with a one-minute imaging frequency and

the subsequent digital analysis of the obtained image sequences. In order to determine motility (relative spreading speed) independent of the starting place of the scratch, a figure quantitatively characterizing limbal monolayer regeneration a Region of Interest (ROI) was defined in 3-5 image details selected in the sequences. ROI image details are areas of 150x300 μm^2 that characterize the entire scratch edges, 50 % of which are located on the torn, damaged part with the other 50% located on the intact monolayer.

Practical application of the HuLi monolayer regeneration scratch model

Effect of antibiotics used in ophthalmology on monolayer regeneration:

- To examine this effect antibiotics and dosages also used in ophthalmology were used: 0.5 and 1 mg/ml chloramphenicol, and 0.1 and 0.2 mg/ml rifampicin solutions, respectively.

Examination of nano- particles in the regeneration model

- Industrial grade multiwalled carbon nanotubes: Purity <85%, outer diameter 10-30 nm, length: 10-30 µm. Examined concentrations: 5, 50, 100 and 500 µg/ml.
- Macroscopically inert metal nanoparticles:
    - Gold nanoparticles: 100 nm in diameter, spherical, 1.0 optical density (OD), stabilized suspension in citrate buffer. The examined concentrations were 80, 200, and 320 ppm, which correspond to 0.41, 0.81 and 1.63 µM, respectively.
    - Silver nanoparticles: spherical, 10 nm in diameter on average. Applied concentrations were: 140, 200, and 320 ppm, which corresponded to: 1.13, 1.84 and 2.94 µM, respectively.

Digital image analysis using Time-Lapse video microscopy

Settings of the self-developed time-lapse video microscopic system used during the examinations: incubator type: SANYO MCO18-AC CO_2 cell culture incubator (Wood Dale, IL, US) equipped with four video microscopes. The microscopes (Olympus Tokyo Japan) are inverted, equipped with appropriate modifications: a light source in the revolver tower and CCD cameras installed in the ocular. The objectives (x10:0.25, achromat Carl Zeiss, Jena) are located in front of the CCD camera (2-megapixel UVC USB 2 webcam). Light source: 960 nm, near-infrared- emitting LED (5mm diameter, 1.2V 50 mA).

Recording: during the observation we made a photo every minute 10 frame averaging. The images were 1600x1200 pixels, 24-bit RGB format.

Digital image-sequence analysis

For image analysis Fiji/ImageJ image analyzing software developed by NIH was used. The three stages of image analysis processing are the following: image restoration and noise reduction, segmentation, and measurements.

The aim of noise reduction is to increase the signal-to-noise ratio, keeping the examined image information. Information filtering and noise reduction were performed based on the following pattern: setting contrast and light conditions: using the Enhance contrast or Brightness/Contrast command. This was followed by artifact filtering of the entire sequence using fast Fourier transformation. Background subtraction was followed by histogram equalization of the sequence, for which 0.4% of the image sequence pixels were saturated for contrast enhancement.

The aim of segmentation is to divide image information along some parameter, in other words, to separate the information content of the foreground from that

of the background. Segmentation and threshold values were determined using the Threshold function. In the case of the segmented images the Remove outliers or Fill holes commands were used, if necessary, for noise reduction. Following determination of threshold value, our actual measurements were performed in the binary images: the number and area of the particles determined using image sequence threshold value. Finally, the obtained measurement results were plotted as a function.

Isolation of cell nuclei and chromatin structures

After removal of the cell culture medium the cells were washed with PBS solution, and prewarmed 0.25% trypsin-EDTA solution was added to the cell culture. When time of trypsin exposure was over, the enzyme was inactivated, the cells were centrifuged (5 minutes 500g) and the supernatant was removed. After determining viable cell number, the cells were incubated for 2 hours (metaphase block) using a 0.2 ng/ml colcemid-containing medium, and were finally washed twice with PBS. During osmotic swelling of the cells the quantity of Swelling buffer was a function of the number of live cells:10^6 cell/ml. After the prescribed 10-minute incubation the swelling solution was removed by centrifugation (5 minutes 500 g). During isolation of the nuclei, under continuous stirring, 1 ml freshly prepared, 3:1 ratio methanol: acetic acid was added to the cells of the pellet, then the cells were washed twice again with a fixative solution. To open the chromatin structure of nuclei the cell-containing fixative solution was dropped onto slides from a height of 30 cm with a Pasteur pipette. The slides were left to dry at room temperature for a night and the next day they were dehydrated in a series of alcohol solutions in ascending concentrations (70, 90, 95, 100%). The samples were dyed with 20µl DAPI-Antifade and examined under a fluorescent microscope (Karl Zeiss Compound Universal Microscope IIIRS fluorescence vertical illuminator).

5. SUMMARY OF NEW SCIENTIFIC RESULTS

The growth and division dynamics of the individual uveal melanoma cells that account for the great incidence of ophthalmological tumors can be examined using the time-lapse video microscopy method. On the other hand, regeneration of the superficial damage to epithelial cells of the cornea can be examined using the limbal monolayer scratch model. Applicability of the model extends to examination of the toxicity of the substances used and potentially usable in therapy. In order to support the obtained data high-sensitivity genotoxicity tests were also carried out.

Applicability of video microscopy

Video microscopy enables long-term dynamic observation while use of a near-infrared lighting system minimizes phototoxicity during examinations.

The developed time-lapse microscopy examination system can be simply adapted to diverse examination needs as it meets the following criteria:

- Broad temporal resolution: the time of observation can cover an hour or a week; temporal resolution can also be easily varied: seconds, minutes, or hours.
- Settings easily adjustable to examined organisms: the system can be used to observe cell cultures of human, animal, and plant origin, molds and yeasts.
- High-sensitivity system that can be adjusted to the purposes of investigation: allows continuous investigation of the life processes of cells, even the smallest physiological changes that occur on a single or population level can be easily distinguished compared with the control population of the same origin.

- Parameters that can be examined during digital image analysis: motility and proliferation dynamics of cells, recognition and measurement of the temporal course of cell death forms.

***In vitro* examination of uveal melanoma OCM-1 cells**

During the investigations we detected spontaneous „irregular" divisions into three during standard cell culturing conditions in aneuploid cells, including OCM-1 uveal melanoma (near tetraploid) as well as other cell types (HeLa (hypertriploid), HaCaT (hypertetraploid) CCL 209 (near-diploid)) cells. In the case of OCM-1 and He La cells the ratio of three-way divisions compared to normal divisions was high: HeLa 1:24, (0.4 ‰), OCM-1 1:37, (0.3 ‰). The phenomenon was given the name trivision. The process was examined from a qualitative and quantitative point of view on individual cells selected from cell cultures:

- *Qualitative changes:* In the case of trivisions, their typical morphological indicator is tripolarization, a three-way division of the cell nucleus following cell rounding, in other words, the creation of a three-pronged structure.

- *Quantitative changes:* Comparison of trivisions and normal divisions and their daughter cells in OCM-1 cells.

    - *Time of division:* Starting point is the rounding and detachment of the mother cell; the endpoint is the adhesion of the daughter cells. Normal division: 28.8±8 minutes, trivision: 67.7±50 minutes

    - *Cell size change:* During cell division the size of the mother cells is nearly double that of the two daughter cells: ~43%, compared with ~22%, after trivision.

- *Frequency of trivisions:* Frequency is determined in two ways: A) The number of trivisions is correlated to the total number of divisions: 0.27% (1/37), B) Relative frequency, which indicates the quotient of the imaging (number of examined image sequences) and the number of all trivisions found in the image sequences: 31/79 (0.39).

Further follow-up of the daughter cells that had undergone trivision showed that they remained viable even after their division.

Examination of limbal cells: monolayer regeneration scratch model

Selective elimination of fibroblast cells from limbal stem cell culture was performed through the addition of geneticin (G-418-sulphate) for two days in 50 μg/ml final concentration. Based on our examinations, HuLi cells can be regarded as morphologically identical even after the 20th passage: they are polygonal epithelial cells, small-sized, with large nuclei compared with the cytoplasm, which is not granulated. The positive response of HuLi cytokeratin to 19 markers suggests the stem cell nature of the cells.

Limbal regeneration scratch model

Using cells isolated from the limbus an *in vitro* regeneration model was created. The limbal origin of the cells enabled the regeneration model to be used to study superficial injuries of the cornea.

Examination of monolayer regeneration using time-lapse microscopy

Examination of the damaged monolayers revealed four phases of regeneration according to the slope of the curve:

- Phase I.: burst-open rim and reattachment phase, delayed phase lasted for ~90-110 minutes, showing initial steep increase caused by the repeated adhesion of viable cells.

- Phase II: exponential phase, during which cells grow over the damaged area again. In control cultures it lasted for 120-130 minutes.
- Phase III. transient phase between logarithmic and stationary phases, closure time of the damaged region: lasted for 35-45 minutes. Cell-free areas can still periodically occur at this point.
- Phase IV. stationary phase took 250-320 minutes.

Relative spreading speed characterizing the lawn on the glass surface and quantitatively describing limbal monolayer regeneration was 62.94± 8 μm^2/minutes.

Practical application of the HuLi monolayer regeneration model

Effect of antibiotics on HuLi monolayer regeneration

Monolayer regeneration in the presence of chloramphenicol

Regeneration of HuLi monolayer treated with 0,5 mg/ml chloramphenicol

In the presence of 0.5 mg/ml chloramphenicol, the time shift observed during cell adherence and regeneration is due to a decrease in the number of dividing cells and cell death, as evidenced by the presence of residual bodies suggesting apoptosis. Reattachment of cells started after 2.5 hours. Regeneration was complete although delayed, taking nearly 11 hours. The slope of the regeneration curve was protracted, less steep with especially the reattachment phase drawn-out. The inhibitory effect is supported by the relative spreading speed of the monolayer, independent of the starting place of the scratch: 33.238 μm^2/minute.

Regeneration of HuLi monolayer treated with 1.0 mg/ml chloramphenicol

Regeneration was considerably prolonged; no full confluence was reached in 31 hours. Initial large-scale detachment was followed by rapid reattachment; after that, a large proportion of cells died. The exponential phase of the regeneration

process was 3 hours late starting. After closure of wound edges confluence level did not increase any higher, as presence of chloramphenicol inhibited cell proliferation. The reason for the large-scale oscillation on the motility curve was probably more robust cell migration, which persisted until the end of this examination, proving absence of full confluence. Spreading speed of the monolayer was 18.080 μm^2/ minute.

Monolayer regeneration in the presence of rifampicin

Regeneration of HuLi monolayer treated with 0.1 mg/ml

Even at smaller concentrations, rifampicin increased monolayer regeneration time from 5 or 6 to 17 or 18 hours. The regeneration curve showed merging of the individual phases and the presence of dead cells at the edges of the scratches. The first phase of regeneration started late and lasted for about 100 minutes. The exponential phase was incomplete with the curve showing a slight steepness as a result of the inhibiting effect. Relative spreading speed of the monolayer was 15.069 μm^2 /minute.

Regeneration of the HuLi monolayer treated with 0,2 mg/ml rifampicin

The causes of the reduction of the monolayer area on the regeneration curve were paralysis of the monolayer, detachment during cell death and shrinking of the resultant tearing of the monolayer. This explains the negative spreading speed of wound edges: -4.4398 μm^2/minute.

Effect of nanoparticles on HuLi monolayer regeneration

Effect of industrial grade multiwalled carbon nanotubes on monolayer regeneration.

HuLi monolayer regeneration in the presence of 5 µg/ml MWCNT

Due to rapid cell reattachment the first phase only took a few minutes. The further phases of regeneration took 1-1.5 hours longer compared to control. The

exponential phase was slightly prolonged, followed by a short transient phase. Regeneration took about 580-620 minutes. Relative spreading speed was 40.21 μm^2/minute.

HuLi monolayer regeneration in the presence of 50 µg/ml MWCNT

The 50 µg/ml concentration made nano carbon tube aggregates appear as large black patches in the images. The reattachment phase was missing in this case, too, the exponential phase was slightly prolonged followed by a short transient phase. Closure of the scratch became complete at around 540-580 minutes.

Motility independent of the initial scratch zone was 40.21 µm2/minute.

At 50 and 100 µg/ml concentrations nanotube microaggregates indicated the location of the closure of scratches with a scar, which might suggest *in vivo* scratch formation.

HuLi monolayer regeneration in the presence of 100 µg/ml MWCNT

Phases of the regeneration curve were blurred: the short unfolding phase with rapid reattachment was followed by a slightly prolonged exponential regeneration growth phase and a longer transient phase. Migration impeded and disturbed by carbon nanotube aggregates was indicated by increased oscillations on the motility curve. Areas around the scratch edges showed lasting wrinkling of the monolayer. Closure of the scratch occurred at about 540-590 minutes; however, about 20% of the scratch zone was covered by carbon nanotube aggregates, suggesting that the scratch was probably closed by the cells moving under them. Monolayer relative spreading speed was 38.9 μm^2/minute.

HuLi monolayer regeneration in the presence of 500 µg/ml MWCNT

At 500 µg/ml concentration regeneration was prolonged, reaching exponential phase after 18 hours. On the other hand, due to the presence of large aggregates, exact measurement was not possible. The scratched edges of the monolayer

showed considerable wrinkling due to the aggregates stuck between the scratch edges as the regeneration process progressed. Presence of residual bodies was also observed.

Effect of gold nanoparticles on limbal monolayer regeneration

Monolayer regeneration following treatment with 80 ppm gold nanoparticles:

In the first phase of the regeneration curve, when the scratch burst open, there was an increase of nearly 60% compared to the initial size of the scratch. The burst-open phase of the scratch took 25-30 minutes. Regeneration time was 260-310 minutes; we found a process occurring at a speed similar to that of the control cultures (62.94 μm^2/minute). Spreading speed independent of the scratch zone of the scratched monolayer was 65.664 μm^2/minute. Slope and profile of the regeneration curve was similar to the one found in the case of treatment with 140 ppm silver particles.

Monolayer regeneration in the presence of 200 ppm gold nanoparticles

In the first phase of the regeneration curve the initial area increased by ~20% during the burst-open phase. The prolonged reattachment phase took about 180-220 minutes. The exponential growth and transient curves that followed were also protracted. After treatment the regeneration process took about 15.5 hours. Relative monolayer spreading speed was 40.201 μm^2/minute.

Monolayer regeneration in the presence of 320 ppm gold nanoparticles

During the nearly 90-100 minutes of the scratch bursting open, the damaged area increased from 100% to 140%. This led us to conclude that the presence of gold nanoparticles facilitates detachment of cells from the cell culturing surface. At the beginning of the regeneration process the area of the damaged monolayer took 4 hours to reach its original size. During the reattachment phase (~100 minutes) the area of the damaged surface decreased by only 30-40%. The prolonged exponential and transient phases lasted a total of 16 hours. The entire

process lasted 19 hours, which might be accounted for by impeded reattachment and prolonged cell proliferation. Scratch-zone independent spreading speed was 30.872 μm^2/minute.

Effect of silver nanoparticles on limbal monolayer regeneration

Monolayer regeneration in the presence of 140 ppm silver nanoparticles

Analysis of the regeneration curve and its comparison with that of the control culture led us to conclude that the first, burst-open phase of the scratch was slightly elongated and the extent of the initial opening 60-70 minutes, was more significant (~ 20%). Reattachment of cells lasted for about another 60-70 minutes after bursting. The exponential phase was longer (280 minutes) than with the control culture (120 minutes). The slope of transient phase III was relatively steep. Relative spreading speed of the monolayer scratch independent of its original area was 53.757 μm^2/minute.

Monolayer regeneration in the presence of 200 ppm silver nanoparticles

The burst-open period of the first regeneration phase lasted 300-360 minutes, meanwhile the scratch area grew to about double its size, ~40%. The exponential phase is characterized in this case, too, by a relatively steep slope, whereas the transient phase was prolonged, for about 150 minutes. The exponential phase was characterized by large-scale motility. The regeneration process took about 690-750 minutes to complete. Spreading speed of the monolayer independent of the original area of the scratch was 42.597 μm^2/minute.

Regeneration of HuLi monolayer in the presence of 320 ppm silver nanoparticles:

Compared with the control we found the following differences: in the first phase to peaks, two sub-phases were found: the first one was the burst-open phase, which lasted ~350 minutes and during this time~220% of the scratched surface

burst open. This was followed by a second, slow reattachment phase, which took 360 minutes to complete, during which the reduction of the damaged surface area reached 150% of the original area, followed by another, smaller burst. The reattachment phase then lasted 200 minutes. The steepness of the exponential curve was smaller than at lower silver concentrations. Closure of the wound edges occurred after 17-18 hours. Relative spreading speed was 19.588 μm^2/minute.

Process of chromatin condensation in limbal cells

Intermediates of chromatin condensation of HuLi cells

Steps of the process of chromatin condensation: the veil-like structures of decondensed chromatin occurring in the early S phase and their supercoiling and polarizing forms followed by the appearance of more condensed chromatin cluster with a veil-like structure forming 4 or 5 larger groups of chromatin. As the process progressed, we detected supercoiled chromatin ribbons of nuclei and the praechromosomes occurring on the ribbon structures. The characteristic spiral, U and V shaped praechromosomes occurring before the appearance of metaphase chromosomes were followed by metaphase chromosomes.

Effect of antibiotics in chromatin condensation

Chloramphenicol

In terms of the structure of chromatin forms a typical effect of chloramphenicol present in 0.5 mg/ml concentration manifests itself in the local polarization of the chromatin pool and the opening of the nucleus. A rarer structural change was the appearance of incomplete, wrinkled chromatin forms which did not reach the solid stage characteristic of metaphase. At higher, 1 mg/ml concentration the chromatin condensation process got blocked in the fibrillar structure and did not move any further to reach ribbon forms.

Rifampicin

In the presence of 0.1 mg/ml rifampicin we found that characteristic holes had been formed in the nuclei. The chromatin condensation process did not reach the level of the appearance of veil-like structures. It caused a characteristic change in chromatin structures, which manifested itself primarily in the appearance of supercoiled chromatin ribbons.

Effect of nanoparticles on chromatin condensation

Multiwalled carbon nanotubes

In the presence of carbon nanoparticles, the chromatin structures showed no significant differences compared to controls. The presence of metaphase chromosomes was detected also at increased MWCNT concentrations without the appearance of apoptotic bodies or necrotically enlarged cell nuclei. The only detectable difference in cells treated with MWCNT was the appearance of micronuclei. In addition, smaller delays were found in the formation of chromatin patterns, suggested by the incomplete condensation of metaphase chromosomes. The appearance of micronuclei induced by nano carbon tubes does not directly suggest cytotoxicity, instead, it stems from chromatin expulsion in the early phase of condensation. Micronucleus index: The highest one, ~8.9%, was provided by the lowest concentration, 5 μg/ml MWCNT, showing a decreasing tendency of 4.6 and 2.9% at 50 and 100 μg/ml MWCNT.

Gold nanoparticles

The condensation process was characterized by the polarization of the chromatin veil and the predominance of unwrapped, decondensed ribbon structures. In rarer cases long, comet flame-like and more solid structures appeared among the condensed nuclei. The long chromatin ribbons condensed further into chromatin bodies, there were rare occurrences of early elongated prae-chromosomes, and no metaphase chromosomes were found. In early

chromatin forms few and often distorted veil-like structures were found. The fibrillary chromatin structures, chromatin bodies, and praechromosomes were found to be smaller than normal. The condensation process only reached the level of the appearance of pre-chromosomes and elongated chromosomes.

At higher concentrations (200 and 320 ppm) supercoiling block manifested itself in the appearance of unusually thin chromatin ribbons, accompanied by the inhibition of the formation of chromatin bodies and further forms. At 320 ppm concentration distorted forms of chromatin bodies and praechromosomes were detected. The appearance of characteristically narrowing chromatin fibers is an indicator of the dose-dependent effect, and inhibition of chromatin condensation.

Silver nanoparticles

At 140 ppm concentration, in the early phase of chromatin condensation, silver nanoparticles did not cause drastic distortions in chromatin structures. Only rarely were rounded, veil-like structures found, but polarized and distorted veil structures were common. Early loose chromatin supercoiling is likely to have taken place with decreased activity, causing the delayed appearance of other structures; the chromatin remained in the early phase of condensation with the long, thin, ribbon structures. Due to the thinner ribbons, smaller chromatin bodies were formed and a considerable number of cell nuclei with veil-like structure were left over. The most condensed structures reached the level of pre-chromosomal stage.

At a high concentration, 320 ppm, chromatin structures were smaller than average. Lack of apoptotic bodies excluded the probability of cells dying by apoptosis. Due to nuclear shrinkage early structures were characterized by a decondensed veil around the nucleus and a comet flame-like expulsion of the cell nucleus, suggesting a decreasing efficiency of supercoiling. The bursting of

the nucleus allowed for the appearance of semicircular, decondensed chromatin forms and chromatin ribbons but these structures only rarely reached higher levels of condensation.

6. DISCUSSION

Use of a time-lapse video microscope system enabled us to develop different types of models. Examinations during our work have led us to conclude that the method can be suitable for the long-term and real-time observations of ophthalmological samples.

In vitro time-lapse examination of OCM1 cells

During the examination of image sequences, we observed the three-way multipolar division of uveal melanoma cells, a process we named trivision. Quantitative data showed that the time of multipolar divisions was significantly different from that of normal divisions; with the OCM-1 cell type it took 40 minutes longer on average. During cell divisions we compared the volumes of mother cells and of the daughter cells. During normal division of OCM-1 daughter cells produced 43% of the volume of the mother cells, after trivision this number was only 32%, and this difference in the volume of the daughter cells may be indicative and suggest small-cell carcinogenesis. Frequency of multipolar cell divisions in OCM-1 cells was 0.27%. The process is induced by a typical morphological appearance, which can be described as the three-way division, or tripolarization, of the entire cell. In some of our studies we have already assumed that tumor progression and ability to metastasize may be linked to tripolarization. Cell trivision-induced aneuploidy in higher ploidy states can be an aggravating factor as it increases the number of irregular divisions. This assumption is in accordance with the multiple-mutation theory of tumors.

In vitro observation of clinically isolated limbal cells

On the basis of their tissue region origin, their morphological appearance and their identification using stem cell markers cells isolated from the limbus proved to be limbal transient stem cells. Human Limbal (HuLi) cells are small with a large nucleus-to-cytoplasm ratio and a polygonal shape. The HuLi cell line still

had a homogenous, unchanged morphology even after 20 subcultures and could be used reproducibly in cytological examinations.

Limbal regeneration scratch model

To study corneal wound healing, we used the scratch model developed in limbal HuLi cells originating from ophthalmological practice. Using the monolayer regeneration model, we observed the appearance of four characteristic phases on the regeneration profile: I.: burst-open rim and reattachment (expansion and re-adhesion), or delayed phase; II.: exponential phase: early stages of the regeneration processes; III.: transient phase: the closing phase of the damaged region; V.: stationary phase: the scratch area becomes confluent again.

Regeneration following injury to the monolayer was characterized numerically using a variable independent of the initial part of the damaged area. This is relative spreading speed, which represents the average per minute of the shift of cells on the borders of the scratch of the monolayer of cells.

Application of the HuLi monolayer regeneration model

Impact assessment of antibiotics used in ophthalmological therapy

Chloramphenicol inhibited cell growth to a lesser extent. A significant difference was found between the control and treated cells in terms of regeneration dynamics. Control monolayer took 5 hours to regenerate; in contrast, in 0.5 mg/ml concentration regeneration took nearly 12 hours, and it happened 30 hours after scratch closure in 1 mg/ml concentration, and no confluence took place. Growth of limbal cells was greatly inhibited by rifampicin at even lower, 0.1 mg/ml doses: regeneration took over 15 hours and was incomplete, while higher concentration, e.g. 0.2 mg/ml, caused cell death.

The typical damage to chromatin condensation intermediate structures supported the damaging effect of antibiotics. In smaller doses, chloramphenicol

caused opening of the nucleus and in higher concentration it blocked the transformation of fibrillary structures into ribbon structures.

In lower concentrations rifampicin blocked the condensation process after the appearance of the ribbon structures. This is in agreement with our observations using a microscope: the delay at the beginning of the regeneration suggests a cell-synchronization effect. In high concentrations, rifampicin caused the appearance of diverse-looking nuclei and condensation forms, suggesting more severe damage to the cells.

Limbal monolayer regeneration in the presence of nanoparticles

During examination of the effect of multiwalled carbon nanotubes we tried to model real conditions that enable us to come into contact with these substances. The results showed that the effect of industrial MWCNTs on limbal cells is dose-dependent in the long term, however, no significant toxic effect was found in the same way as in immortalized and embryonic cells. During the examination of the *in vitro* monolayer regeneration model concentration proportional accumulation of particles was found in the damaged region, which might be related to or amplify wrinkling of the monolayer at the edges. The dose-proportional effect of nano carbon tubes manifested itself in physically impeding cell migration. Along with inhibited and disturbed motility, as well the remaining residual bodies, this might suggest *in vivo* scarring.

We considered gold and silver particles, too, as potential carriers for active agents in the process of the renewal of the corneal epithelium. We obtained controversial results with the smaller, and hence regarded as more toxic, silver nanoparticles: in their presence monolayer regeneration took shorter than in the presence of gold nanoparticles. Cellular-level damage that causes a prolonged regeneration process after treatment can be detected in terms of the

genotoxicity-specific chromatin changes as well. The effect of silver nanoparticles could be sensed through the condensation forms of the nuclei. Silver particles caused the following changes in the process of chromatin condensation: appearance of the decondensed chromatin veil around the nucleus, rejection of comet tail-like structures and appearance of reduced-size elongated and metaphase chromosomes. Viability of the cells was proved by the lack of structures suggesting apoptosis. This led us to assume that these particles tend to have cytostatic and reversible effects. The presence of gold nanoparticles allowed transformation of chromatin ribbons into chromatin bodies but we could only rarely detect elongated pre-chromosomes. We experienced the proliferation-inhibiting effect through prolongation of the regeneration time, which was also supported by the lack of appearance of metaphase chromosomes.

7. NEW RESULTS AND THEIR RELEVANCE

The applicability of time-lapse video microscopy in ophthalmic clinic specimens was presented at two levels, on single cells by characterizing the abnormal divisions of OCM-1 cells; at the cell population level was demonstrated using a monolayer scratch regeneration model of limbal cells.

1. Examination of single cells of uveal melanoma (OCM-1)

By examining the image sequences, we observed a three-way multipolar division of uveal melanoma cells and termed trivision. Multipolar divisions i.e., trivisions, of uveal melanoma (OCM-1) cells: The onset of the process is identified by a characteristic morphological change, a process described as tripolarization, which a three-way separation of the nucleus and the whole cell. Based on the quantitative data obtained by digital image analysis trivisions lasted on average 40 minutes longer than the divisions. The volume ratio of mother and daughter cells was 22% compared to an average of 43%. The frequency of multipolar cell division was relatively high for OCM-1 cells, it was 0.27%. Based on our studies, we hypothesized that trivision may contribute to aneuploidy and tumor progression.

2. Establishment, maintenance, and description of a limbal cell population isolated from clinical samples.

HuLi (Human Limbal) cells isolated from the corneoscleral limbal ring, are considered as stem cells based on their tissue origin, morphological features, and genetic markers. The cells have been maintained from isolation in a long term and considered as a continuous cell line (HuLi). HuLi cells are epithelial, polygonal, small, have a high nuclear-cytoplasmic ratio and have no granularity in the cytoplasm. The positive reaction of HuLi with cytokeratin 19 indicates the stem cell nature of cells.

3. Development of in vitro corneal wound-healing model: Human limbal monolayer regeneration scratch model.

The time-lapse microscopic examination of the HuLi monolayer regeneration scratch model in combination with use of expedient digital image analysis

methods proved to be an appropriate experimental design for studying the dynamics of corneal surface regeneration. We have described four phases of the in vitro regeneration process that may be consistent with the in vivo mechanism of corneal reepithelialization. In addition, to describe monolayer regeneration, we have introduced a quantitative feature independent of the original area of the scratch, which quantifies the migration of cells at the edges of the scratch of the monolayer to the scratch area.

4. Use of the scratch model in pharmacological studies: effect of antibiotics on corneal regeneration

By combining the cito toxicological findings from the regeneration scratch model, with the data of genotoxicity throughout the morphological study of chromatin condensation, we have developed a widely applicable toxicological screening system by studying the effects of test substances. The system is capable of high sensitivity dynamically monitoring various biological processes on clinical samples.

During regeneration of the HuLi monolayer, cellular growth in the presence of antibiotics demonstrated an inversely proportional relationship between cell proliferation and applied concentrations. Complementing above to the morphological results of the chromatin condensation intermediates, cells treated with chloramphenicol and rifampicin shoved characteristic changes compared to control samples, which may indicate the maximum concentration of antibiotics used in ophthalmic regeneration therapy.

5. Application of a scratch model for cytotoxicity assays:

- Effect of multi-walled industrial-grade carbon nanotubes.

Regarding the effect of multi-walled carbon nanotubes, regeneration slowed as a function of concentration, due to inhibited, disturbed migration of cells, which is characterized by delayed growth in the regeneration curve, merging the phases, and increased fluctuations in motility. At the highest concentrations of MWCNT (500 µg / ml), nanoparticle aggregates prevented the regeneration process. During monolayer regeneration, the aggregates were embedded in scratches in proportion to the concentration used (from 50 µg / ml), causing the monolayer to wrinkle at the edges of the scratches, from 100 µg / ml concentration. This

phenomenon may indicate scar formation in vivo. Aggregates remaining in the scar may cause severe impairment of vision.

- Effect of inert metal nanoparticles on monolayer regeneration:

In the limbal monolayer scratch model, both silver and gold nanoparticle treatment caused a delay in regeneration and inhibited the chromatin condensation process of the cells prior to proliferation. The moderate time lag of monolayer regeneration compared to control was similar in the presence of small 10 nm silver and large 100 nm gold nanoparticles. In the absence of apoptotic cells, it was concluded that the inhibitory effects observed in both nanoparticles may reversible; this suggests that the changes in chromatin condensation are not permanent either.

The time lapse video microscopy allows us to examine both the dynamics of regeneration and the process of tumor formation during examination of cell cultures from clinical tissue samples, using digital image analysis of image sequences recorded during tumor formation easier, and in a more cost-effective way even long-term.

- Qualitative and quantitative data of the dynamics of multipolar cell division obtained during image analysis have verified the validity of the method in long-term, high temporal resolution arrangement.

- The monolayer scratch model modelling reepithelization of the corneal epithelium in limbal cells reflects corneal surface conditions and describes the cell-level dynamics of the regeneration processes.

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Candidate: Melinda Turáni
Neptun ID: A71NUO
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List of publications related to the dissertation

1. **Turáni, M.**, Bánfalvi, G., Péter, Á., Kukoricza, K., Király, G., Tálas, L., Tánczos, B., Dezső, B., Szemán-Nagy, G., Kemény-Beke, Á.: Antibiotics delay in vitro human stem cell regrowth. *Toxicol. Vitro. 29* (2), 370-379, 2015.
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Total IF of journals (all publications): 14,461
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Turáni, M; Baksa, V; Kovács, F; Kiss, A; Nagy, G **Többfalú karbon nanocsövek (MWCNT) hossz-összefüggés vizsgálata, geno- és citotoxikus hatásainak megfigyelése a szaruhártya regenerációja során.** Tavaszi Szél Nemzetközi Multidiszciplináris Konferencia 2019 Debrecen

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