Optimization of in vitro bioassays to investigate fibroblast functions

Ph.D. thesis

Csenge Szász, MD

Károly Rácz Conservative Medicine Division
Semmelweis University

Supervisor: Apor Veres-Székely, Ph.D.

Official reviewers: Orsolya Cseprekál, MD, Ph.D.
Judit Erzsébet Pongrácz, Ph.D., Prof.

Head of the Complex Examination Committee:

Károly Liliom, Ph.D., Dr. Habil.

Members of the Final Examination Committee:

Marcell Cserhalmi, Ph.D.
Ágnes Varga, Ph.D.

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1. Introduction

Fibrosis, the universal process of dysregulated extracellular matrix accumulation occurs as a consequence of numerous inflammatory diseases, physical and chemical injuries, or oxidative stress. As such, it affects a large group of patients, aggravating the outcome of several conditions. The therapeutic options are highly limited currently, listing only two approved compounds, both of which provoke severe adverse effects.

Cancer is one of the leading causes of mortality in most developed countries. While some forms are well treatable, certain types lack effective therapy even in early stages or need constant change of medication due to therapy resistance caused by mutations.

Therefore, both pathologies require active research and efficient systems to screen, validate and involve new potential therapeutic compounds into clinical practice.

At first sight, these two conditions might appear to be very different, however, beyond the well-described role of fibroblasts in tumor progression, fibroblasts and cancer cells share similar functional characteristics as well, namely proliferation, migration and the production of extracellular matrix molecules.

After targeted drug discovery being in focus in the last decades, the advantages of phenotypic drug discovery (PDD) are getting increasingly recognized nowadays. Instead of examining direct molecular interactions, PDD provides information about the complex biological changes following the application of compounds.

2. Objectives

The aim of our study was to establish a near-high throughput screening system for the parallel investigation of the main fibroblast and cancer cell functions by optimizing existing protocols or developing new methods to overcome the disadvantages of the current assays. We were focusing on creating cost-effective, time-efficient, simple assays that are suitable for 96-well plates, allowing for the examination of multiple samples in small quantities. In details, our aims were as following:

Cell migration

1) To develop a damage-free agarose-based collective cell migration assay TAS assay designed for 96-well plate.
2) To compare the performance of the TAS assay to the current gold standard scratch assay.
3) To automate the detection and evaluation steps using a new generation microplate reader.
4) To demonstrate the main considerations in the selection of suitable labeling technique.

Collagen production

5) To optimize Sirius Red staining for the detection of total collagen production in vitro.
6) To assess the major influencing factors and adjust the experimental conditions to achieve maximal collagen production in vitro.

3. Methods

3.1. Cell lines and treatments

Numerous human fibroblast and cancer cell lines were cultured in their adequate media under standard cell culture conditions (37°C, humidified, 5% CO2). Cells were activated with recombinant human growth factors important in the pathogenesis of fibrosis and cancer to enhance proliferation, migration or collagen production. Fluorescent protein expressing cell lines were used to set the conditions of microplate reader-based detection and evaluation.

3.2. Transient Agarose Spot (TAS) assay

TAS assay was performed by placing droplets of heated agarose solution into each well of a 96-well plate. After the droplets polymerized, cells were seeded around them and incubated for 24 hours. Subsequently, flowing the removal of the droplets, cells were treated and stained according to the experimental setup.

3.3. Scratch assay

To perform scratch assay, cells were seeded into 12-well plates and incubated for 24 hours. Thereafter, the confluent cell layer was scraped with a pipette tip, wells were washed, and the relevant treatments were applied.

3.4. Data acquisition by microscopy for TAS assay

Brightfield or fluorescent images of each well were taken at specific time points during treatment. Cell-free gap areas were annotated manually by computer mouse, digitizer board without display and tablet or automatically using ImageJ 1.48v software applying macros.

3.5. Cell confluency

To evaluate the effect of cell confluency, fluorescent images of DiI stained cells were processed and analysed with ImageJ software. Cell confluency was calculated using an

equation based on differences in pixel density, with the gap area taken into consideration.

3.6. Cell staining and data acquisition by microplate reader

Various absorbance and fluorescence signal-based staining methods were applied targeting different cell components to create detectable difference between cell-free and cell-covered areas. Stained wells were screened using the well scanning function of a new generational microplate reader, then cell-free gap areas were defined using a manually set threshold.

3.7. MTT cell proliferation assay

MTT assay was performed to determine the mitochondrial dehydrogenase activity of attached viable cells. MRC-5 cells were seeded in 96-well plates and treated with activator and/or inhibitor agents. After incubation with MTT reagent, the intracellular crystals were solubilized, and the optical density (OD) of the solution was determined using CLARIOstar microplate reader.

3.8. LDH cytotoxicity assay

LDH assay was performed to determine the activity of lactate dehydrogenase in the supernatant released by injured cells. LDH reagent was mixed with equal volume of supernatant derived from the cells of the MTT experiment on a 96-well plate and incubated to develop colorimetric reaction, then the OD was measured.

3.9. Sirius Red histological staining (Picrosirius)

To visualize the collagen accumulation, slides with histological samples from fibrotic mouse lungs were deparaffinized and stained with dye solution containing Direct Red 80 and picric acid. Subsequently, following dehydration, slides were covered using DPX Mountant for Histology.

3.10. Sirius Red collagen detection assay for cells

Cells were cultured in 96-well plates in FBS containing medium until reaching full confluence, then FBS-free medium containing ascorbate was applied for the course of treatment. Thereafter, the supernatant was separated to perform fixation, Sirius Red staining, washing, and elution steps on the cells. Finally, the OD was determined.

3.11. Sirius Red collagen detection assay for cell culture medium

To conduct the optimization steps, a dilution series of rat tail-derived Collagen type I was prepared in distilled water or DMEM. Various 96-well microplates were compared for the washing and centrifugation including flat-bottom, V-bottom, or 0.45 μm filter plate. In the final assay, the supernatant was transferred into a V-bottom plate, stained with Sirius Red containing 3% acetic acid and centrifuged. The resulting collagen-dye precipitate was washed, centrifuged, and the dye was eluted to measure the OD.

3.12. Immunofluorescence staining

Cells were grown into 4-well chambers for 7 days. Following fixation, procollagen 1 alpha 2 and collagen type 1 alpha 1 were labeled with specific primary antibodies. Fluorescently labeled secondary antibodies were applied for visualization. Nuclei were stained with Hoechst DNA staining.

3.13. Data acquisition by microscopy for Sirius Red assay

Brightfield, fluorescent or polarized light illumination images were taken using Olympus IX81 microscope system with 20× or 100× objectives. Samples from the stained collagen solution or supernatant were resuspended in hydrochloric acid, dripped onto a slide and cover slipped. Images were processed in ImageJ 1.48v software.

3.14. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.01 or 9.1.2 software. Two-way ANOVA, Brown-Forsythe and Welch ANOVAs or ordinary two-way ANOVA with Dunnett's tests were applied for multiple comparisons, Pearson's correlation for correlation analyses and linear regression analyses for the dilution series. $p \leq 0.05$ was considered as statistically significant.

Descriptive statistics were performed to evaluate the performance of *Sirius Red collagen detection assay for cell culture medium*, characterized by linearity, limit of detection, accuracy, intra-assay variability and inter-assay variability. Non-linear asymmetric sigmoidal 5 parameter logistic curve was fitted on the collagen concentration-absorbance correlation graph.

4. Results

4.1. Optimization of TAS assay

4.1.1. Agarose spot stability and optimal cell density

The barrier function of the agarose spot was confirmed in a 7-day experiment observing cell migration via microscopy in the presence or absence of agarose spot. The assessment of the gap closure starting with different cell densities showed a correlation between cell confluence and the rate of gap closure.

4.1.2. Applicability of TAS assay to investigate fibroblast migration

The addition of FBS and EGF to the culture medium accelerated the migration of different fibroblast cell lines. The effect of FBS showed dose dependence.

4.1.3. Applicability of TAS assay to investigate cancer cell migration

The addition of FBS and EGF to the culture medium accelerated the migration of cancer cell lines, however, different cell types reacted unalike to the stimuli.

4.1.4. Comparison of different gap annotation methods

To examine the accuracy and reproducibility of different methods, gap sizes were annotated using different manual techniques (mouse, tablet, digitizer board) and automatic annotation using ImageJ software. Although automatic annotation provided a significantly quicker annotation, some cell types required preliminary fluorescent labeling.

4.1.5. Comparison of scratch and TAS migration assay

The two methods were conducted in parallel to compare sensitivity and reproducibility. When examining the initial gap sizes from numerous independent experiments, the confidence interval was significantly lower in TAS assay. Moreover, unlike scratch assay, the whole cell-free area did fit in one field of view in TAS assay. The intra-group variance of relative and absolute

gap size values determined by the coefficient of variation of group means was on average 3 times higher in scratch assay at the end of the experiment.

4.1.6. Principle and validation of micro plate reader-based data acquisition

Green fluorescent protein expressing cancer cells were applied to examine the well-scanning mode of the microplate reader. The software generated a signal intensity map, where threshold value was set manually to define the cell-free gap area, where signal intensity decreased strongly. Comparison of manual and microplate reader-based evaluation using green and red fluorescent protein expressing cells showed perfect correlation. To improve visualization, various colorimetric and fluorescent staining methods were tested for detection with the microplate reader.

4.1.7. Optimization of Hoechst staining

Hoechst DNA staining was chosen for the subsequent experiments. Gap sizes determined by microplate reader and microscopy showed very high correlation. The compatibility with living cells was tested on A549 and LCLC-103H cell lines. Hoechst provided stable fluorescent signal even at low concentrations during the whole 72-hour course of the experiment, meanwhile cytotoxic effect was not detectable.

4.2. Optimization of Sirius Red assay

4.2.1. Detection of cell-associated collagens by Sirius Red staining

Brightfield and polarized light illumination images of Sirius Red stained fibroblast cell cultures revealed that unlike tissue samples, cultures expressed minimal amount of cell-associated collagen fibers. Immunostaining of collagen and procollagen supported these findings as well. The supplementation of the culture medium with macromolecules to

achieve volume exclusion effect by macromolecular crowding did not enhance collagen production.

4.2.2. Detection of collagens in cell culture medium with Sirius Red staining

Rat tail collagen solution diluted in water and culture medium was used for the optimization steps. Experiment revealed that the cell culture medium required a staining solution with lower pH to counteract the buffer systems. Lowering the assay temperature to 4°C could increase the sensitivity further and maximal signal intensity was reached after 15 minutes of incubation. The optimal microplate type for the washing and centrifugation steps was the V-bottom plate providing firm attachment of the precipitate and minimal sample loss. The obtained data confirmed a wide range detection, high sensitivity, and very low intra-and inter-assay variability.

4.2.3. Optimization of in vitro experimental conditions to detect collagen production of fibroblasts

When investigating the effect of nutrient concentration, we experienced that FBS formed precipitates with Sirius Red leading to false signal elevation in the supernatant, therefore, FBS-free culture medium was used during the second phase of the experiment. Supplementation with ascorbic acid, an essential cofactor in collagen biosynthesis elevated collagen production in a dose-dependent manner.

4.2.4. Representative in vitro measurements to investigate the effect of kinase inhibitors on cell proliferation, migration and collagen production

The usability of the final assay system for the investigation of cell proliferation, cytotoxicity, migration and collagen production was demonstrated on fibroblast and cancer cell lines through the application of the antifibrotic agent nintedanib and the small molecular anticancer compounds sorafenib and gefitinib.

5. Conclusions

In conclusion, we obtained the following findings regarding the main objectives:

1) The execution of the agarose placement and removal during TAS assay on a 96-well plate did not damage the surface or the cells, therefore it did not interfere with the subsequent cell migration, ensuring thereby that the evaluation of factors influencing the migration rate remained unbiased.

2) The thorough and systematic comparison of the TAS assay to the current gold standard scratch assay prove the superiority of TAS assay in many aspects including a low variability in gap size leading to recognition of changes in migration at earlier time points.

3) Transferring the detection and evaluation in a new generation microplate reader for automation resulted in a significant decrease of assay time and manual work, maintaining the performance meanwhile.

4) The selection of the most suitable labelling technique and the optimization of Hoechst staining showed not only the applicability of Hoechst in a wide range of cell types and experimental setups, but it also presented a systematic method that may help the development of other staining protocols.

5) The optimization of Sirius Red staining for the detection of total collagen production in vitro resulted in a time-efficient protocol that could assess the cell-attached and soluble forms of collagen in parallel, overcoming the difficulties of modelling collagen biosynthesis in vitro and providing more information about the changes in collagen production.

6) Along the assessment of the major influencing factors, the elimination of fetal bovine serum from the cell culture before the experiments could prevent the high false positive signal due to nonspecific binding, while during the adjustment of the experimental conditions, the addition of ascorbic acid increased collagen production.

6. Bibliography of the candidate's publications

Publications related to the thesis:

1. Veres-Székely A, Szász C, Pap D, Bokrossy P, Lenzinger D, Visnovitz T, et al. Improvement in Transient Agarose Spot (TAS) Cell Migration Assay: Microplate-Based Detection and Evaluation. 2025;26(12):5584.
2. Szász C, Pap D, Szebeni B, Bokrossy P, Őrfi L, Szabó AJ, et al. Optimization of Sirius Red-Based Microplate Assay to Investigate Collagen Production In Vitro. 2023;24(24):17435.
3. Veres-Székely A, Szebeni B, Pap D, Bokrossy P, Lévai E, Szász C, et al. A fibroblastok funkcionális vizsgálata–IV. rész: Az ECM-termelés mérése. 2022.
4. Bokrossy P, Sepsi Z, Szász C, Pap D, Szebeni B, Szabó JA, et al. A fibroblastok funkcionális vizsgálata–III. rész: Sejtproliferációs mérések. 2022.
5. Sepsi Z, Bokrossy P, Szász C, Pap D, Szebeni B, Szabó JA, et al. A fibroblastok funkcionális vizsgálata–II. rész: Sejtmigrációs mérések. 2022.
6. Szász C, Sepsi Z, Bokrossy P, Pap D, Szebeni B, Szabó JA, et al. A fibroblastok funkcionális vizsgálata–I. rész: Bevezetés. 2022.
7. Veres-Székely A, Pap D, Szebeni B, Őrfi L, Szász C, Pajtók C, et al. Transient Agarose Spot (TAS) Assay: A New Method to Investigate Cell Migration. 2022;23(4):2119.

Publications not related to the thesis:

8. Zrufkó R, Pajtók C, Szebeni B, Veres-Székely A, Bernáth M, Szász C, et al. The DJ-1-Binding Compound Exerts a Protective Effect in Both In Vitro and In Vivo Models of Sepsis-Induced Acute Kidney Injury. 2025;14(6):719.
9. Pap D, Veres-Székely A, Szebeni Bt, Réka Z, Bokrossy Pt, Szász C, et al. Role of PARK7/DJ-1 in Gastrointestinal Diseases. 2024;12(1):28-32.
10. Pap D, Pajtók C, Veres-Székely A, Szebeni B, Szász C, Bokrossy P, et al. High Salt Promotes Inflammatory and Fibrotic Response in Peritoneal Cells. 2023;24(18):13765.
11. Veres-Székely A, Szász C, Pap D, Szebeni B, Bokrossy P, Vannay ÁJIjoms. Zonulin as a potential therapeutic target in microbiota-gut-brain axis disorders: encouraging results and emerging questions. 2023;24(8):7548.

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