DOI: https://doi.org/10.17816/gc631097

Использование атомно-силовой микроскопии для оценки биомеханических свойств 3D-моделей опухолевых клеток

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АННОТАЦИЯ

Многоклеточные сфероиды являются уникальным объектом-моделью для токсикологических исследований. Клетки в 3D-кластере имеют микроокружение, межклеточные коммуникации, что позволяет использовать сфероиды в качестве более реалистичной модели по сравнению с традиционными клеточными культурами. Известно, что микрорегионы опухоли состоят из гетерогенных популяций раковых клеток, в которых рост клеток и ответ на противоопухолевые препараты зависят от их 3D-архитектуры, межклеточных контактов и взаимодействия с микроокружением. Кроме того, на рост и прогрессию опухоли оказывают сильное влияние механические сигналы. В настоящее время 3D-модели клеточных культур являются мощным инструментом для изучения токсичности лекарственных соединений и наноматериалов разного состава и морфологии.

В обзоре представлены данные об использовании различных методик, в частности атомно-силовой микроскопии, для исследования изменений механических свойств клеток в сфероидах. В частности, рассматривается использование атомно-силовой микроскопии как инструмента для выявления физико-химических параметров клеток при патофизиологических процессах или воздействии лекарственных препаратов. Актуальность данного обзора связана с возрастающим интересом к роли биомеханических свойств тканей, клеток и субклеточных структур как маркёров патофизиологических состояний.

Ключевые слова: сфероиды; атомно-силовая микросокпия; наномеханические свойства; нанотоксикология; раковые клетки.

Как цитировать:
Пучкова К.С., Лопарева В.Р., Шепелева Е.В., Магомедова О.А., Иванова Д.В., Замская Ю.В. Использование атомно-силовой микроскопии для оценки биомеханических свойств 3D-моделей опухолевых клеток // Гены и клетки. 2024. Т. 19, № 3. С. 348–358. DOI: https://doi.org/10.17816/gc631097

Рукопись получена: 25.04.2024 **Рукопись одобрена:** 09.07.2024 **Опубликована online:** 19.08.2024

DOI: https://doi.org/10.17816/gc631097

Use of atomic force microscopy to assess the biomechanical properties of 3D tumor cell models

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ABSTRACT

Multicellular spheroids are a unique object model for toxicological studies. Cells in a three-dimensional (3D) cluster contain a microenvironment and intercellular communication, which allows spheroids to be used as a more realistic model than traditional cell cultures. Tumor microregions consist of heterogeneous populations of cancer cells, in which cell growth and response to antitumor drugs depend on their 3D architecture, intercellular contacts, and interaction with the microenvironment. Tumor growth and progression are also strongly influenced by mechanical cues. Currently, 3D cell culture models are powerful tools for studying the toxicity of drug compounds and nanomaterials of different compositions and morphologies.

This review presents data on the use of various techniques, particularly atomic force microscopy, to investigate changes in the mechanical properties of cells in spheroids. Specifically, the use of atomic force microscopy as a tool to reveal physicochemical parameters of cells during pathophysiological processes or drug exposure is considered. The relevance of this review is attributed to the increasing interest in the role of biomechanical properties of tissues, cells, and subcellular structures as markers of pathophysiological conditions.

Keywords: spheroids; atomic force microscopy; nanomechanical properties; nanotoxicology; cancer cells.

To cite this article:

Puchkova KS, Lopareva VR, Shepeleva EV, Magomedova OA, Ivanova DV, Zamskaya YV. Use of atomic force microscopy to assess the biomechanical properties of 3D tumor cell models. *Genes & cells.* 2024;19(3):348–358. DOI: https://doi.org/10.17816/gc631097

Received: 25.04.2024 **Accepted:** 09.07.2024 **Published online:** 19.08.2024

INTRODUCTION

A tumor is believed as not just a collection of cells capable of high proliferation but a separate tissue that is distinguished by features of development and structure of the circulatory system, particularly associated immune cells and fibroblasts. Tumor resistance to therapy was linked to the properties of the extracellular matrix (ECM) [1]. Tumor cells within a solid tumor have different physiological states because of different accesses to oxygen and nutrients [2]. A comprehensive assessment of the morphology and physicochemical parameters of the tumor parenchyma and stroma has application and fundamental importance [3]. Tumor microregions consist of heterogeneous populations of cancer cells, in which cell growth and response to antitumor drugs depend on their 3D architecture, intercellular contacts, and interaction with the microenvironment. Tumor growth and progression are also strongly influenced by mechanical cues [4]. Thus, the state of cells within the tumor tissue must be examined to predict the disease course and assess the efficacy of the applied antitumor therapy. Owing to the complexity of studying the mechanisms of tumor development and their response to drug therapy *in vivo*, several solid tumor models have been developed, which reproduce the properties of tumor tissue to varying degrees [5]. One such model is 3D spheroids, which are multicellular 3D aggregates of tumor cells that reproduce the cell–cell and cell–intracellular matrix interactions found in solid tumors. 3D spheroids represent the most adequate model for examining the physical and mechanical properties of cells and preclinical studies of therapeutic drugs [6]; thus, they are increasingly used as models for basic research and drug screening [7]. Multicellular tumor spheroids (MCOs) are emerging as a powerful *in vitro* model for preclinical experiments, closely resembling the three-dimensional (3D) structure of avascularized tumors *in vivo*. Multicellular spheroids are an important *in vitro* model for preclinical experiments because they mimic the 3D structure of avascularized tumors *in vivo* [8]. When the diameter of multicellular clusters is increased to >500 μm, they can reproduce various pathophysiological features of living human solid tumors [9] because they exhibit altered gene and protein expression profiles, representing an intermediate model between two-dimensional (2D) and *in vivo* models [10]. However, differences in drug sensitivity have been observed between cells in monolayer and multicellular spheroids [11]. Moreover, methodological approaches to characterize the complex 3D multicellular organization of spheroids are being actively developed [12]. This review will closely investigate the mechanical properties of cells within 3D spheroids, with particular emphasis on the use of atomic force microscopy (AFM).

For example, it is reported that 3D spheroids demonstrated stronger anticancer drug resistance than 2D analogs [3]. Tumor cells inside a solid tumor differ in their physiological state, which is associated with different accesses to oxygen and nutrients [4]. A comprehensive assessment of the morphology and physicochemical parameters of the tumor parenchyma and stroma has a special application and fundamental importance [5]. Tumor microregions consist of heterogeneous populations of cancer cells in which cell growth and response to antitumor drugs depend on their 3D architecture, intercellular contacts, and interaction with the microenvironment. Tumor growth and progression are also strongly influenced by mechanical signals [6].

The state of cells inside the tumor tissue must be examined to predict the disease course and evaluate the effectiveness of the antitumor therapy. Owing to the complexity of studying the mechanisms of tumor development and their response to drug therapy *in vivo*, several solid tumor models have been developed, which reproduce the properties of tumor tissue to varying degrees [7]. One such model is 3D spheroids, which are multicellular 3D aggregates of tumor cells that reproduce the cell–cell and cell–ECM interactions in solid tumors [8]. 3D spheroids represent the most adequate model for studying the physicomechanical properties of cells and preclinical studies of therapeutic drugs [8], and the use of 3D spheroids as models for basic research and screening of medicines has been growing recently [9–11]. MCOs are becoming powerful *in vitro* models for preclinical experiments [12]. MCOs have intercellular contacts and cell–ECM interactions, which are necessary for the regulation of cell behavior and function. If the diameter of the MCOs increased to >500 microns, MCOs can reproduce many pathophysiological features of living solid human tumors [13], and in comparison with conventional 2D cell culture models, they demonstrated altered gene and protein expression profiles, which allows them to be considered an intermediate model between a 2D monolayer and solid tumors *in vivo*. Given these properties, MCOs are currently used in a wide range of cell biology research, bridging the gap between preclinical and clinical results [14]. Moreover, methodological approaches are actively developing to characterize the complex 3D multicellular organization of spheroids [15].

The review provides the latest data on the changes in the biomechanical properties of cells in the composition of 3D structures under the action of medicinal compounds. The mechanical properties of cells in the composition of 3D spheroids were also examined using various methods, with special emphasis on the use of AFM.

DIFFERENCE BETWEEN THE BIOMECHANICAL PROPERTIES OF CANCER CELLS AND NONCANCER CELLS IN 2D AND 3D MODELS

Even at the initial stages of carcinogenesis, changes in the mechanical and morphological properties of cells occur, which can be used as disease markers [16]. Currently,

the role of biomechanical properties of tissues, cells, and subcellular structures is receiving research interest as markers of pathophysiological conditions [17, 18]. As known, different cells have different stiffness, for example, the Young modulus of embryonic stem cells is 500 Pa; osteoblasts, 1.6–2.6 kPa; and osteosarcoma cells, 1 kPa [19, 20]. Nontumor cells tend to be tougher than cancer cells [21, 22]. The reason for the observed biomechanical differences is not yet fully clear; however, during the transformation of healthy cells into cancerous ones, the structure of their cytoskeleton changes from an organized to an irregular network, which probably changes their mechanical properties [23]. Biomechanical properties such as cell adhesion and cytosolic viscosity also differ in tumor and normal cells [21]. Moreover, the tumor is generally tougher than the surrounding healthy tissues [17], and the rigidity of cancer cells increases with the induction of apoptosis [24].

Traditionally, cells growing as a 2D monolayer have been used to examine the effectiveness of antitumor drugs (including AFM). However, in the last decade, the development of methods for growing 3D cell cultures has significantly changed the way preclinical drug research is conducted. Monolayer cell cultures are believed to not reproduce the mechanisms of drug resistance of tumors associated with the tumor cell microenvironment [25–27], and the smallest functional units of tissues preserving the basic physiological characteristics are micro-sized tissues in the range of 100 microns to 1 mm [28]. In 2D cell cultures, cell–cell and cell–ECM interactions are reduced, and the cellular reactivity level is limited [29]. Moreover, the cell culture medium can influence the cell phenotype and, consequently, affect the cell response to added substances, such as drugs [30]. The phenotype and functions of each cell largely depend on carefully thought-out interactions with neighboring cells, ECM, and proteins [31]. These cell–cell and cell–ECM interactions differ in 2D and 3D cultures and between cell layers in spheroidal structures, and this may affect cytotoxicity [32]. Thus, testing the toxicity of materials and substances on 2D cell cultures does not allow for the accurate prediction of body changes. 3D cell cultures more accurately mimic the natural cellular microenvironment. The morphology and physiology of cells in 3D cultures differ from that of cells in 2D cultures, demonstrating reactions that in some ways more resemble *in vivo* behavior [33, 34]. For example, in 2D models of Alzheimer's disease, medium removal will mean that the secreted beta-amyloid (Aß) will be removed; therefore, the analysis of Aß aggregation will change, and the use of 3D cell culture may limit the diffusion of Aß into the culture medium [29]. In addition, the use of 3D spheroids as tumor models allows us to approach *in vivo* conditions because, as mentioned above, they retain the influence of the ECM, intercellular interactions, and tissue-like 3D organization [33].

Various methods of 3D modeling of tumors (cell culture on special hydrogels and substrates, in micro- and macro-bioreactors, production of spheroids and organoids, organs-on-a-chip, and computer and bioinformatic models) are available, which reproduce the properties of solid tumors to varying degrees [34, 35]. 3D cell cultures in the form of 3D cell spheroids are one of the most reliable and widely used tumor models because they reproduce numerous tumor properties, including oxygen gradient, intercellular interactions, and cell–ECM interactions observed in solid tumors [8, 12, 34, 36, 37]. In addition, spheroids can grow up to several hundred micrometers in diameter and exhibit a cell proliferation gradient similar to that observed in tumor microblasts [38]. Like tumors, the cellular organization inside the spheroid varies and depends on the stage of spheroid growth [39, 40]. Loosely located cells on the surface of the spheroid demonstrated the highest level of proliferation, and cells located in internal compact areas are in a state of hypoxia or anoxia (spheroids with a diameter of 250–300 microns) [41] or necrosis (spheroids with a diameter of >550 microns) [39]. Given the importance of spheroids as *in vitro* tumor models in preclinical studies, their physical and mechanical properties must be elucidated using several methodological approaches.

COMPARATIVE ANALYSIS OF DIFFERENT APPROACHES FOR THE DETECTION OF BIOMECHANICAL PROPERTIES OF CELLS

Optical coherent elastography has been used to measure the biomechanical properties of spheroids from breast cancer cell lines with and without multidrug resistance [42]. Spheroids from multidrug-resistant cells are tougher than spheroids from drug-sensitive cells. In addition, the stiffness of drug-sensitive spheroids increased after their 24-h co-cultivation with extracellular vesicles isolated from multidrug-resistant cells [42, 43]. 3D light microscopy in combination with mathematical modeling has recently been proposed to estimate the elasticity of multicellular spheroids [44]. Previously, Kelvin probe force microscopy was also used for the quantitative analysis of biomolecular interactions [45] by measuring the contact potential difference between the probe and the sample; however, the resolution of the AFM is much higher [46].

Recently, AFM has been considered a tool for diagnosing diseases and evaluating the effectiveness of treatment based on differences in the topography and mechanical properties of cells. Some serious human diseases indicate changes in the ultrastructure of blood cell membranes. Chen found that mononuclear cells of the peripheral blood of patients with uremia had a larger volume, height, and roughness of the membrane than cells of healthy people [47]. AFM has also been used to detect morphological changes in erythrocytes in type 2 diabetes [48], multiple myeloma [49],

and iron deficiency anemia [50]. The AFM also allowed for real-time examination of the dynamics of structural changes associated with neurodegeneration induced by the activation of N-methyl-D-aspartate receptors (a subgroup of glutamate receptors) in living SH-SY5Y cells [51].

The reaction of cancer cells to drug therapy is also being actively explored. Several studies have shown an increase in the roughness of cancer cells after the induction of apoptosis using drugs [52–56]. A time- and dose-dependent increase in the roughness of the membranes of HeLa, HepG2, and C6 cells after treatment with colchicine and citrabine was reported [57]. The rigidity of cells and tissues (which can be judged by Young's modulus) has been identified as a key factor in cell function, including their adhesion, mobility, and invasion abilities [58]. Cancer cells are on average softer (exhibit a lower Young's modulus) than noncancerous cells; thus, measuring stiffness can help distinguish between malignant and nonmalignant tissue [59] and evaluate the effectiveness of drug therapy. In turn, literature analysis allows us to consider AFM as a promising method for such studies, including the study of multicellular clusters.

ADVANTAGES OF USING ATOMIC FORCE MICROSCOPY IN ASSESSING CHANGES IN THE BIOMECHANICAL PROPERTIES OF CELLS IN 3D SPHEROIDS

The AFM, originally developed for imaging various surfaces, is currently actively used to examine the mechanical properties of biological objects, including tissues, cells, membranes, protein complexes, individual protein molecules and nucleic acids [60], as in sections of fixed tissues [61], and physiological conditions [62, 63]. The use of AFM for the integral assessment of the mechanical properties of an entire 3D spheroid has been described [64, 65]; however, the physiological state of cells on the surface and inside the spheroids and mechanical properties of cells on varying distances from the surface of the spheroid also differ. AFM is considered a fast and convenient method for detecting such changes. However, when studying cells in a monolayer, the use of various substrates (matrix proteins, gels, etc.) can strongly affect the measured parameters, and nonphysiological conditions cast doubt on the reliability of the results obtained. To assess the rheological properties of spheroids, methods such as centrifugation, compression with parallel plates, aspiration with a micropipette, and rupture of connected spheroids were also used [64, 66, 67]. Although these approaches provide some information about the mechanical properties of large biological systems, they have several limitations. For example, the pipette aspiration method requires optical correction of the focusing error of the micropipette and inner diameter and measurement and compensation of the taper of the micropipette [68], whereas microplate rheology does not have sufficient force sensitivity for a thorough description of biomechanical phenomena [69]. In contrast to these approaches, AFM allows for the direct application and measurement of forces in the range from pico- to micronewton in spatially strictly defined areas with precise vertical positioning control in the range from subnanometers to several tens of micrometers.

The AFM system is based on a flexible cantilever, and its movement is tracked by the reflection of a laser beam directed at the outer surface of the cantilever using a photodetector. The resolution of AFM images can reach 0.5–1.0 nm in the plane and 0.1–0.2 nm in height, and the recorded forces range from piconewtons to micronewtons. The fixation and dehydration of cell samples for AFM are significantly less destructive than sample preparation of samples for transmission and scanning electron microscopies [70]. Using AFM, the choice of cantilever geometry plays a special role when studying the mechanical properties of cells. For example, cantilevers with a sharp tip can be used to probe nanoscale regions of the cell surface, whereas flat cantilevers without a tip are more suitable for examining the mechanical properties of an entire cell [71]. In the AFM analysis of the mechanical properties of cells [72, 73] and intracellular structures [74], pressure or compression of the cell with standard or modified AFM probes is used. Such an application of AFM is similar to the method of compressing objects with parallel microplates [75], where a cantilever without a tip is used as an upper microplate for performing cell deformation and measuring forces [76, 77]. An obstacle to the widespread use of AFM in cell research is the standard cantilever tilt of 8–12, which does not allow for the application of a unidirectional load to the sample surface. To solve this problem, a modification of the cantilever without a tip was proposed to create an adhesive wedge-shaped plate on it, allowing for the application of a uniform load to the sample [78].

In the study of spheroids by AFM, in spheroids, as in solid tumors, gradual increases in pressure are induced by the intrinsic growth of the spheroid/tumor [68]. Growth-induced pressure affects tumor growth and its resistance to therapy. In addition, the physical properties of spheroids are influenced by the conditions of their formation: even if spheroids have the same size, the growth-induced pressure in spheroids grown for 2 days from 5,000 cells was lower than the pressure in spheroids grown for 6 days from 500 cells [68]. Young's modulus obtained in this study for spheroids from HCT116 cells was comparable to those previously found for spheroids from breast cancer cells and healthy cells using micro-tweezers [79]. In large cells or 3D multicellular aggregates, standard AFM probes may be unsuitable because their small size limits them to press only on a single cell or part of it. Although the size of AFM probes can be increased by gluing large beads with a diameter of up to 50 microns to the cantilever, this approach does not allow for achieving a uniform and stable contact area for homogeneous and reproducible compression of a large

biological system (>100 microns). In addition, the shape of the pressure device begins to play an important role in such measurements [64]. In this regard, a method has been proposed for creating flat AFM macro-probes consisting of a large flat plate connected to a chip using two flexible legs, which are used to apply/measure compression forces similar to standard AFM cantilevers. With the help of such macrobonds, the viscoelastic properties of small (up to 200 microns in diameter) and large (>200 microns in diameter) spheroids from T47D tumor cells were evaluated, and in large spheroids, the outer layer of cells was found to be mainly subjected to compression deformation, whereas small spheroids are exposed to much deeper deformations, which corresponds to data on the similar cellular organization of small spheroids and the outer layer of large spheroids [64].

CONCLUSION

Multicellular clusters (spheroids) are important *in vitro* models for preclinical experiments, and with increased diameter, they can reproduce many pathophysiological features of human solid tumors. 3D spheroids represent an intermediate model between 2D and *in vivo* models. Differences in drug sensitivity between monolayer cells and multicellular spheroids are observed, with important changes occurring at the level of nanomechanical properties of cells, such as roughness, stiffness, etc. In addition, methodological approaches for characterizing the complex 3D multicellular organization of spheroids are being actively developed. In this literature review, current data on methods for detecting changes in mechanical properties of cells in 3D spheroids are considered, and atomic force microscopy application is thoroughly discussed.

ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. Все авторы подтверждают соответствие своего авторства международным критериям ICMJE (все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией). Наибольший вклад распределён следующим образом: К.С. Пучкова — обзор литературы, написание текста и редактирование статьи; В.Р. Лопарева — обзор литературы, сбор и анализ литературных источников, написание введения статьи; Е.В. Шепелева — сбор и анализ литературных источников, подготовка и написание заключения; О.А. Магомедова — сбор литературных источников, оформление литературных ссылок; Д.В. Иванова — анализ литературных источников; Ю.В. Замская — написание текста и редактирование статьи.

ADDITIONAL INFORMATION

Funding source. This study was not supported by any external sources of funding.

Competing interests. The authors declare that they have no competing interests.

Authors' contribution. All authors confirm that their authorship meets the international ICMJE criteria (all authors have made a significant contribution to the development of the concept, research and preparation of the article, read and approved the final version before publication). The greatest contribution is distributed as follows: K.S. Puchkova — literature review, writing the text and editing the article; V.R. Lopareva — literature review, collection and analysis of literary sources, writing the introduction of the article; E.V. Shepeleva — collection and analysis of literary sources, preparation and writing the conclusion; O.A. Magomedova — collection of literary sources, design of literary references; D.V. Ivanova — analysis of literary sources; Y.V. Zamskaya — writing the text and editing the article.

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