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FORMATION OF MULTICELLULAR AGGREGATES UNDER DIFFERENT CONDITIONS OF MICROENVIRONMENT

Multicellular aggregates (spheroids) represent an intermittent level between monolayer growing cells and tissue culture. Spheroids are rather objective model of the three-dimensional growth and organization, the cell-to-cell interactions and influence of microenvironmental conditions on tumour microaggregates. In our work formation and growth of spheroids depends on concentration of CMC and FCS. Conditions of microenvironment influence on intensiveness of proliferation as well as on cells adhesiveness and formation of microaggregates.

Introduction. During the last three decades multicellular spheroids of both normal and malignant cells have been used as model systems for studying three-dimensional growth and differentiation *in vitro*. Spheroids can maintain several biochemical and morphological features similar to the corresponding tissues *in vivo*. Complex cell-to-cell interactions in microenvironments that simulate intervascular microregions *in vivo* can be studied with help of spheroid system under controlled condition *in vitro*.

During development, as well as in the differentiated organism, cell behaviour and histotypic integrity is determined to great extent by impulses presented to the cell membrane from the surrounding environment which consists of contiguous cells, endocrine substances, extra-cellular matrix. This three-dimensional cell arrangement is also important in malignant tumours where irregular distribution of oxygen and nutrients is reflected by cellular complexity observed in neoplastic tissues. Therefore, tumour micro-region is cellular subpopulation which consists of proliferating, quiescent, and necrotic cells that differ in their sensitivity to anticancer therapy [1]. In the spheroid model cell proliferation and apoptosis take place, and detachment of cells from the spheroid surface (cell shedding) is also observed. Cells allocated between the proliferative outer layer and the inner necrotic layer of the spheroid moved to either of the two compartments. The non-dividing but viable cells located at a distance from the capillaries may represent a particular therapeutic problem, since drug penetration here may be insufficient and since non-dividing cells are generally more resistant to drugs and irradiation than proliferating cells.

In spheroids cells are organized in a three-dimensional network displaying cell-to-cell and cell-to-matrix contacts [2, 3]. In contrast to the limited morphological and functional differentiation of the cells grown in monolayer cultures, the three-dimensional spheroid culture systems allow cells to grow and to develop very similar in many aspects to situation *in vivo*. Cells obtain significant information from cell-to-cell contacts and cell recognition and adhesion constitute important mechanisms of tissue growth and differentiation *in vivo*. Additionally, the interactions with the extracellular matrix and humoral factors provide information required for cellular development and function. Spheroids are therefore composed of heterogeneous groups consisting of proliferating,

quiescent, and necrotic cells and, as such, represent *in vitro* model for studies of the biology of normal as well as malignant cells [1, 4].

The establishment of spheroids from a single-cells suspension depends upon diverse cellular properties, such as cell adhesion molecules, cell-matrix interactions, cell surface changes, and the formation of junctional complex. In general, the ability to form spheroids is a characteristic trait of malignant cells derived from solid tumours, although cells from normal tissues may also form spheroids and differentiate in vitro. In this review we survey the adapted methods for generation, culturing and visualization multicellular tumour spheroids.

Spheroid formation in spinner flasks. Cell culture in spinner flasks has been the most widely used method for culturing spheroids, originally introduced by Moscona. The main advantage of this method is that a very large number of spheroids may be generated in large-volume cultures, the spheroids reaching a considerable size (diameter 1–2 mm), due to continuous vortex that enhances the oxygen tension in the medium. Monolayer cell culture is tripsinized and seeded in growth medium in 250 ml siliconized glass spinner flasks. Rotation (180 rpm) is obtained by placing the spinner flasks containing a stir bar on a magnetic stirrer inside an incubator. Culture medium has changed twice a week. In such culture spheroid can be cultivated during 1–3 weeks [5].

Spheroid formation on agar overlay. Growth of spheroids in medium-agar overlay culture was first described by Yuhas. For agar medium preparing – 1 g agar noble is dissolved in 26.6 ml of distilled water, boiled thoroughly over an open flame until the agar is melted, and then allowed to cool during a brief period. 20 ml of liquid agar solution is mixed with 80 ml warm (37 °C) culture medium by gentle shaking. The medium agar is transferred to the culture vessels under 40 °C. The agar will solidify in about 5 min at room temperature, and the vessels can be stored at 4 °C for 1 to 2 weeks [5].

The main problem in spheroid culturing is standardization in a size, number of cells in one aggregate and growth kinetic of cell population. In our investigation we have changed conditions of culture (percent of FCS, CMC and time) for generation of standard spheroids.

Materials and methods. Adhesion line of Human Caucasian of breast adenocarcinoma (MCF-7) was used as experimental model of tumour micro-aggregates. The line was established from the pleural effusion from a 69-years-old Caucasian woman suffering from a breast adenocarcinoma. Cells were epithelial-like and exhibited some features of differentiated mammary epithelium including oestradiol synthesis and formation of domes. Cells can carry B or C type retrovirus and are considered to represent a category 2 pathogen (P2 containment). Cells express the wild-type and variant oestrogen receptors as well as progesterone receptor.

The cells were handled in standard tissue culture conditions (100 % humidity, 5 % CO_2 in air; 37 °C) under laboratory containment level 2.

For generation of spheroids we adapted the methods of Yuhas and Kelm [6, 7]. Cell confluent was trypsinized and single-cell and suspension were seeded on low-adhesive substrate at a density $5.0 \cdot 10^4$ cells/ml in the medium with 0.24 % of carboxy-methyl-cellulose (CMC). We generated spheroids in 6-well-plates and Petry's dishes. Dishes with cells were placed on shaker with low rotation (150 rpm) for one hour. Spheroid formation depends on the type of the cells used, cell density at seeding, the speed of rotation, the type of culture medium, concentration of the FTS and the incubation time [8]. Spheroids were transferred to new flasks and separated by size with gentle replacement of spheroid-containing medium in conical tubes. When the tubes were placed vertically, the spheroids were rapidly sunk to the bottom, leaving single cells and debris in the supernatant which was removed. New portion of growth medium was then added to the tubes and the whole sample was transferred to new dishes.

For counting of proliferation and number of dead cells in spheroids cell suspension was transferred to the tubes and centrifuged under 200 g 2–3 min for separating single cells and aggregates. Supernatant was removed, the aggregates were resuspended and the quantity of live/dead cells in the aliquot of suspension was calculated. Cell proliferation in spheroids was measured regularly every 4–8–16–24–32–48 hours.

Light microscopic observations was made in fixed by ethanol : formalin (1 : 9) cell samples. Cells were stained with hematoxylin using the standard methods [9].

Staining by MTT was used to study cell proliferation by colorimetric assay [10] in culture with different concentrations of FTS. Cells were cultured in the standard conditions with 0,5 mg/ml of 3-[4,5-dimetltiazol-2]-2,5-dipheniltetratetrazolium (MTT) during 4 hours. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The resulting purple solution was spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and in an increase in absorbance. Crystals of formasan form sharp needles after incorporating in cells (Fig. 1). Formasan crystals were incorporated in alive cells, after that the samples were centrifuged under 1500 g during 5 min. For development of staining100 μl of DMSO («Sigma») and 25 μl of glycine («Sigma») were added in all wells. Optical absorption was detected using multi-well spectroscopy reader Multyscan («Labsystem», Finland) (OP540 nm).

Results. We generated spheroids using 0.24 % of carboxy-methyl-cellulose (CMC). CMC has high viscosity, is not toxic and stimulates formation of cell microaggregates to prevent adhesion of cells to the bottom and to each other. On Fig. 2 we fixed MCF-7 culture at the same stages (24 hours) with CMC (*b*) and without (*a*).

For comparing influence of FCS concentration on growth of MTS we hold tumor microaggregates in 0, 2, 5, 10 % of FCS. As we can see from the Fig. 3 growth of spheroids has an exponent kinetics in concentrations of Fetal Calf Serum (FCS) from 0 to 10 %. In 24-well test plates in experiments aggregates, single cells in suspension, adhesive cells on the bottom of wells were calculated. After two hours of shaking number of spheroids in field of view was different in different concentration of FCS (2 % – 2 ± 0.3, 5 % – 4 ± 0.7, 10 % – 6 ± 0.5).

Interesting that we observed increasing quantity of MTS in field of view during first 8 hours without FCS. After that numbers of microaggregates decreased ($p < 0.05$) and didn't had exponent characteristics (Fig 3). At the same time, under other concentrations of FCS spheroids growth achieved his maximum in different time period: the lower concentration of FCS, the earlier growth maximum is detected. For 2 % it was after 16 hours, for 5 and 10 % after 48 hours after the start of incubation. Intensiveness of proliferation had proportional dependence from increasing concentration of FCS from 0 to 10 % and was higher in

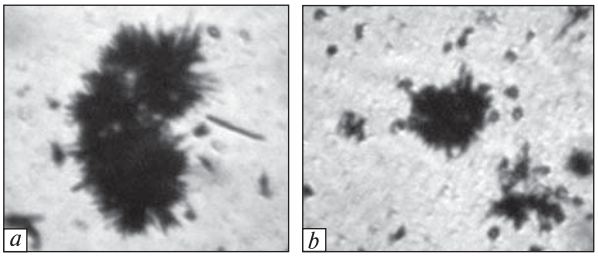

Fig. 1. MTS in 10 % (*a*) and 2 % (*b*) of FCS (accordingly) in 48 hour culture after coincubation with MTT

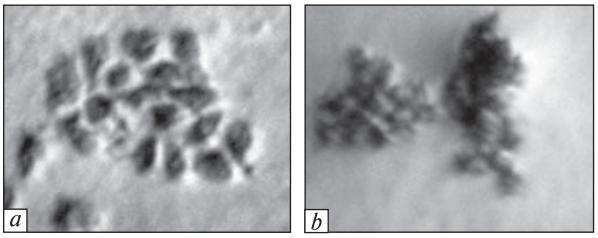

Fig. 2. Monolayer (*a*) and spheroid (*b*) culture, 24 hours

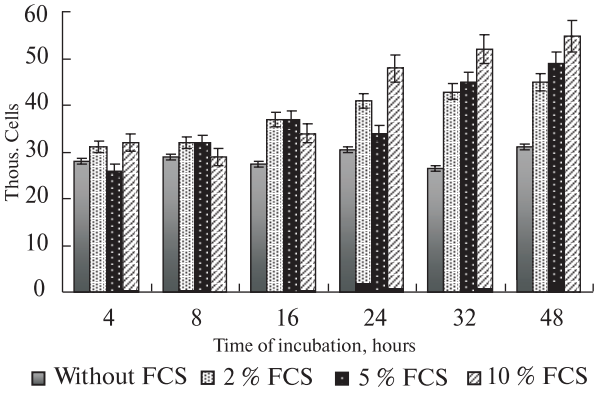

Fig. 3. Dependence of cell proliferation on concentration of FCS

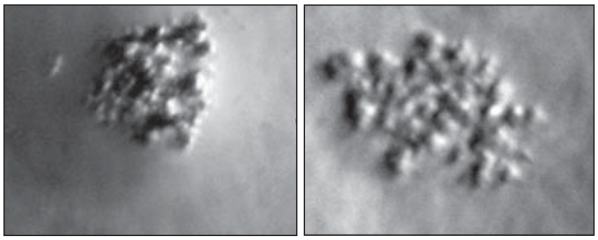

Fig. 4. MTS in 2 % FCS culture 48 hour

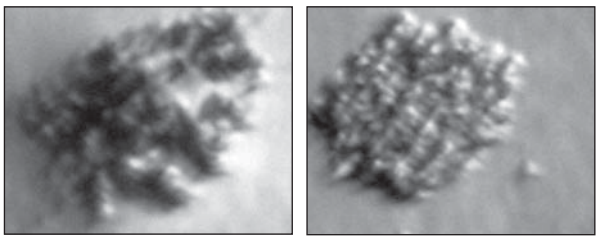

Fig. 5. MTS in 10 % FCS culture 48 hour

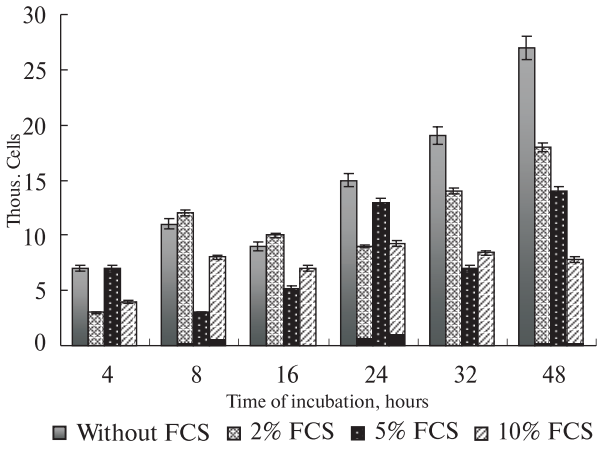

Fig. 6. Dependence of cell death on concentrations of FCS and time of culturing

culture with 10 % of FCS. However, number of MTS decreased from 0 to 10 % of FCS despite cell proliferation took place. Obviously, number of cells included not only aggregated cells but single cells too. Since at the start of experiment we separated MTS for incubation, we can conclude that lack of the FCS decreased adhesive characteristics of cells and led to disintegration of MTS. More than that, in different concentrations of FCS sizes of MTS were different (Fig. 1 and 4). In 10 % of FCS cell aggregates were bigger (650–750 μm in diameter) than in 2 % culture (250–350 μm in diameter). In 5 % percent of FCS MTS were smaller but had low dispersion in sizes (near 460 μm in diameter). In addition – in 10 % FCS culture MTS had sphere form and in 2 % FCS – ellipsoid (Fig. 5).

Number and quality of tumour microaggregates were determined by viability cells in culture in different conditions of culturing. That's why we counted alive/died cells in every well for all time points. As a result, we determined that number and percentage of died cells depends on concentration of FCS in culture and time of incubation (Fig. 6).

As we can see from Fig. 6 number of dead cells was the biggest in culture without FCS and increased with the time of incubation. In that time in culture with 10 % of FCS during 48 hours of investigation the number of dead cells wasnrt more than 10 % and wasnrt increasing during the whole period of incubation. For 2 % of FCS – it was determined low level of cell death in 4 hours and rapid increasing of this characteristic in 8 times for the period of 24 hours of incubation. For culture with 5 % of FCS was fixed middle level of cell death, despite the fact that in 8, 16 and 32 hours of incubation it was lower than for 10 % of FCS.

Conclusion. The observation has demonstrated that CMC and FCS are important agents for generation and holding multi-cellular tumour spheroids in culture. Although MCF-7 can form micro-aggregates without CMC, it happens on 3–4 days of culturing after appearance of confluent monolayer, when culture exhausts medium and potential for exponent growth. CMC stimulates generation of MTS in first hours and gives a preference for experiments development. Our investigation proves essential necessity of FCS for spheroids culture. The optimal concentration of FCS for cell proliferation in spheroid culture is 10 %. Under this concentration cells form micro-aggregates with high proliferative activity and low cell death. In additional it has been found that concentration of FCS influences on intensiveness of proliferation as well as on cell adhesiveness to each other and formation of microaggregates.

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ФОРМИРОВАНИЕ МНОГОКЛЕТОЧНЫХ АГРЕГАТОВ В РАЗНЫХ УСЛОВИЯХ МИКРООКРУЖЕНИЯ

Многоклеточные агрегаты (сфероиды) по сложности структуры занимают промежуточное положение между монослойным ростом клеток и организованной тканью. Сфероиды являются адекватной моделью трехмерного клеточного роста и организации, межклеточных контактов и влияния микроокружения на опухолевый микроагрегат. В нашей работе продемонстрировано, что формирование и рост сфероидов зависит от концентрации карбокси-метил-целлюлозы и фетальной телячьей сыворотки. Условия микроокружения влияют не только на интенсивность пролиферации, но и на адгезивность клеток и формирование микроагрегатов.

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ФОРМУВАННЯ БАГАТОКЛІТИННИХ АГРЕГАТІВ ПРИ РІЗНИХ УМОВАХ МІКРООТОЧЕННЯ

Багатоклітинні агрегати (сфероїди) за складністю структури займають проміжне місце між моношаровим ростом клітин та організованою тканиною. Сфероїди є адекватною моделлю трьохвимірного клітинного росту і організації, міжклітинних контактів та впливу мікрооточення на пухлинний мікроагрегат. У роботі продемонстровано, що формування та ріст сфероїдів залежить від концентрації карбокси-метилцелюлози та фетальної сироватки теляти. Умови мікрооточення впливають не тільки на інтенсивність проліферації, але й на адгезивність клітин та формування мікроагрегатів.

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