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3D MODELS FOR THE ANALYSIS OF TUMOR INFILTRATION BY MONOCYTE-MACROPHAGES

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An analytical review of literature data of modern studies of monocyte migration (movement) in tumor microenvironment using various three-dimensional models of monocyte migration in tumor microenvironment is presented. The given literature data can serve as a characteristic of three-dimensional models as an optimal platform for studying the functions of both individual cell populations of immune cells and whole cell ensembles in the development and therapy of malignant neoplasms. One of the most important characteristics of a three-dimensional model, which has a significant impact on its prognostic ability, is the cellular composition used. The given data clearly demonstrate the importance of the diversity of cell types of the used models, including the introduction of stromal cells (in particular, fibroblasts). Various cell types form a complex system of interactions, thus forming three-dimensional models closest to native organismal conditions. Different types of tumor cells and their individual cell lines are of no less high importance for prognostic ability. For example, tumor spheroids that contain different types of cancer cells show a different secretory profile. As such, the pattern of monocyte infiltration and polarization may differ depending on the type of tumor cell line. In addition to the components themselves used to create the 3D model, the nature of the organization of the above components (different cell types and populations, as well as structural extracellular components) is important. Therefore, this analytical review contains a separate structural section including the analysis of the diversity of structural groups of three-dimensional tumor models. Among the variety of the mentioned structural groups the following should be mentioned: suspensions of multicomponent cancer spheroids, various variants of microfluidic systems and a separate group of organoids - miniature models of native organs and tissues. Thus, this analytical review demonstrates the importance of further optimization of three-dimensional tumor models in order to obtain even more effective means of reproducing the native structure of the tumor microenvironment.

Keywords: three-dimensional matrix, monocytes, tumor microenvironment.

Introduction: The study of the complex structure of the tumor microenvironment includes the analysis of interactions between cancer cells, stromal component and immune system cells. In turn, studies using three-dimensional models allow a more accurate analysis of the structure of the tumor microenvironment and the interactions of its individual elements with each other compared to classical two-dimensional models. Numerous studies convincingly demonstrate the relationship between the nature of intercellular interactions and the progression of malignant solid tumors [32]. In recent years, the complexity of the tumor microenvironment has been reproduced in advanced three-dimensional cell culture-based models. While *in vivo* models are expensive and time-consuming to develop, classical 2D *in vitro* models cannot sufficiently replicate the spatiotemporal dynamics characteristic of physiological conditions *in vivo* [3], 3D models have the ability to reproduce to a greater extent the architecture and metabolism of the original tissue [16]. Moreover, there is a continuous improvement and specialization of three-dimensional models depending on the initial data and ultimate goals of research, among which we can mention the inclusion of different extracellular matrix proteins or the addition of different types of supporting cells and the creation of angiogenic environment [19, 15, 23].

As a rule, existing three-dimensional models of tumor microenvironment focus on the interaction of cancer cells and their derivatives with stromal cells such as fibroblasts, but the accumulation of more and more new data shows the significant relevance of studies of the interaction between elements of the immune system and the tumor microenvironment [32]. Indeed, modern multicomponent three-dimensional cell culture models may be an optimal platform to study the role of the immune system in malignancies. A clear example of this is the study by K. Heungnam using three-dimensional models of mesothelioma, which reproduces changes in complex networks of biomarkers associated with immune response and apoptosis [21].

The role of different cell populations for the prognostic ability of three-dimensional tumor models. The development of malignant neoplasm promotes qualitative changes in cells of innate and adaptive immunity. It is known that monocytes can differentiate into two functionally distinct subtypes of macrophages: classical anti-inflammatory macrophages known as M1-like and M2-like macrophages, which are known to be associated with tumor progression through remodeling of the extracellular matrix and stimulation of angiogenesis [32]. In the context of the present study, the results of using existing three-dimensional cellular models to analyze the complex system of interactions between monocytes, macrophages, cancer cells and their microenvironment are of considerable interest.

Currently, the classical type of three-dimensional models is spherical

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cellular structures (spheroids). According to the origin of cells, the following types of spheroid models are distinguished: multicellular tumor model using cell lines, oncospheroids from cells of dissociated solid tumor, tissue tumor spheres formed by partial dissociation of solid tumor, and organotypic multicellular spheroid models, which differ from the previous model by the absence of tissue dissociation. The latter two models involve a blurring between the terms "spheroid" and "organoid". Nevertheless, the term "spheroid" is the most used term for three-dimensional cell cultures including multicomponent suspensions of primary cells or cell lines [8].

Analysis of three-dimensional spheroid models of human colon cancer cell culture (cell line HRT-18) formed in agarose wells showed association of macrophages with the spheroid surface within 24 hours, with their subsequent infiltration and disintegration in the absence of pronounced cytotoxicity after 5 days of co-culture. Also, cancer spheroids showed intensified migration in the presence of anti-inflammatory macrophages compared to anti-inflammatory and resident macrophages in collagen substrate [32]. Analysis of peripheral blood monocyte recruitment using three-dimensional spheroid models of fibroblasts in agarose wells showed that monocyte infiltration was characteristic of cancer spheroid models of fibroblasts, whereas normal spheroid models of fibroblasts showed a low degree of infiltration [35]. Equally, a higher rate of monocyte migration was shown for spheroid models of breast cancer cells containing fibroblasts relative to models without them [17], raising the question of cancer and stromal cells as bases for recruitment of immune cells. However, these results were not confirmed in studies of head and neck squamous cell carcinoma (hereinafter referred to as HNSCC), as no significant differences in the intensity of infiltration by peripheral blood mononuclear cells (hereinafter referred to as PBMCs) of fibroblast spheroidal models of HNSCC cells and spheroidal models of HNSCC cells without fibroblasts were recorded. At the same time, when the amount of MPCs increased, their concentration on the surface of spheroidal models increased without further infiltration. Nevertheless, experimental suppression of epithelial growth factor receptor expression resulted in increased infiltration [5].

Studies using three-dimensional spheroid models of breast cancer cells (cell line SUM159PT) showed a significant infiltrative capacity of macrophages mediated by the activity of matrix metalloproteinases. Breast cancer cell spheroids themselves were largely capable of infiltrating in type I fibrillar collagen medium, but they lost any pronounced infiltration capacity in the substrate of murine extracellular matrix extract (Matrigel1). Incorporation of macrophages both as part of cancer spheroids and directly into the substrate of murine extracellular matrix itself significantly improved the infiltrative ability of breast cancer cell spheroids, but did not increase the invasiveness in the medium of fibrillar collagen [33].

M2-type macrophages promote invasiveness of three-dimensional spheroidal models of breast cancer cells in Gultrex1 basal membrane extract through paracrine signaling as well as through direct intercellular interactions during co-culture in type I collagen substrate [14, 11]. Fibroblasts, which are part of the cancer microenvironment and cancer cells themselves, promote M2-differentiation of macrophages by secreting such factors as interleukin-6 (IL-6) and stromal growth factor-1 (SDF-1) [22].

Studies of three-dimensional spheroid models of cell cultures using the alginate porous platform AlgiMatrix™ showed that the cultivation of mouse breast cancer cells with fibroblasts alone had a negative effect on the formation of cancer spheroids, whereas co-culture with macrophages alone inhibited their growth. In turn, only co-culture of macrophages, fibroblasts and cancer cells resulted in an increase in the number of spheroids [31]. Addition of macrophages to a rat tail collagen substrate that included a layer of dermal murine fibroblasts on top of which murine squamous cell carcinoma cells were cultured resulted in the polarization of macrophages into an M2-like phenotype followed by active invasion of carcinoma cells into the collagen substrate by increasing the collagenolytic activity of metalloproteinases (MMP2 and MMP9). The researchers confirmed the obtained result by analyzing the co-culture of human squamous cell skin cancer cells, primary human dermal fibroblasts and macrophages derived from human monocytes [20].

These results indicate a significant influence of the composition of the three-dimensional matrix on the interaction between immune cells and cancer cells. In addition, the above confirms the significant influence of immune cells in the interaction between cancer cells and their microenvironment.

The significance of different tumor cell types and their individual cell lines for the prognostic ability of three-dimensional tumor models. Tumor spheroids including different types of cancer cells also show differences in secretory profile. Accordingly, the intensity of monocyte infiltration and the pattern of monocyte polarization may differ depending on the type of tumor cell line. In particular, a recent study showed the highest infiltrative activity of monocytes for spheroids created using MIA and PaCa-2 (pancreatic cancer) cell lines, while the lowest infiltrative activity was demonstrated by monocytes in MCF-7 breast cancer spheroid models [24]. Moreover, the above results are consistent with other studies [25, 17]. 1BR.3.G fibroblasts were also found to improve the stability of spheroids and enhance the overall physiological relevance of the models. When 1BR.3.G fibroblasts and MIA PaCa-2 cancer cells were co-cultured together, the formation of smaller and more compact spheroids was observed. However, their degree of stability often remained low [24], which seems to be a common problem for this cancer cell line [25, 6], indicating that active infiltration and polarization of monocytes are not associated with the fibroblast cell line.

The results of analyzing the effects of CSF1R inhibitors and antibodies administered simultaneously to monocytes in similar three-dimensional models of MCF-7, HT-29 (colorectal cancer), PANC-1 (pancreatic cancer), and MIA PaCa-2 cell lines should not be overlooked, as here, too, a heterogeneous pattern of reduction in monocyte infiltrative capacity was shown between the models in question in response to exposure to CSF1R antibodies and inhibitors. A visible decrease in monocyte infiltrative capacity was shown for models including MCF-7 and MIA PaCa-2 cell lines, while spheroids of HT-29 and PANC-1 cell lines show a slight decrease in infiltrative capacity [18].

As a result of cancer cell type-dependent polarization of monocyte precursors, researchers observe different phenotypic manifestations of tumor-associated macrophages, as clearly captured by analysis of 3D models. Specifically, MIA PaCa-2 spheroids polarize infiltrating monocytes into M2-like macrophages, whereas bone marrow-derived macrophages polarized by MCF-7 spheroid models exhibit an M1-like phenotype. Among other models generated using HT-29 and PANC-1 cell lines, a mixed type of polarization is observed with a predominance of macrophages of M2-like phenotype and expression of markers of M1-like phenotype. Noteworthy differences be-

tween macrophages determined by the tumor microenvironment and bone marrow-derived macrophages polarized by co-culture with tumor spheroid models are worth noting. The present results suggest a specific role for cell-cell interactions between cancer cells and monocytes [24], and also correlate well with data on a more pronounced development of a phenotype associated with tumor development for macrophages embedded directly in tumor spheroids compared to macrophages that are diffusely dispersed throughout the collagen substrate [7].

Analysis of the secretion profile of the considered tumor cell models showed marked differences already before co-culture with monocytes, confirming the ability of cancer cells to influence infiltrating monocytes depending on their individual secretion profile. Generally, MCF-7 spheroids exhibited an overall low level of soluble factor secretion. On the other hand, the microenvironment of MIA PaCa-2 spheroids exhibited a distinct pro-inflammatory profile combined with high secretion of the anti-inflammatory cytokine IL-10 and pro-angiogenic factors. However, secretion of most soluble factors was significantly increased when monocytes were added to the spheroid cultures. Common to all models was an increase in the levels of CCL2, CCL22, CCL24, and IL-10. CCL2, CCL22 and CCL24 were associated with M2-like polarization and significantly associated with poor prognosis in cancer patients. A concomitant decrease in the secretion of IL-12, a key factor in the antitumor immune response, was observed. For models including HT-29, PANC-1 and MIA PaCa-2 cell lines, high levels of VEGF secretion have been shown to be associated with the expression of CCL2 and IL-4 factors necessary for monocyte recruitment, in addition to transforming the immune microenvironment into an immunosuppressive state [24]. On the other hand, monocytes infiltrating HT-29 spheroids may also possess antitumor activity, since their characteristic secretion of CXCL10 and CXCL11 has been associated with tumor suppression [24, 13].

Histological analysis of the models showed the following structural features: fibroblasts in the three-dimensional model of HT-29 cell line are structurally connected with necrotic nucleus, for spheroids of MCF-7 cell line direct interaction with MCF-7 cells with subsequent formation of differentiated structures was shown.

Similar results were found already with different breast cancer cell lines: the highest intensity of monocytic infiltration was shown for three-dimensional spheroid models of cell line Hs578T (ER-negative carcinoma); spheroid models of cell line T47D showed a moderate degree of monocytic infiltration; the lowest degree of monocyte infiltration was shown for spheroid models of cell lines BT549 (ductal carcinoma), BT474 (ductal carcinoma) and MCF7 (ER-positive adenocarcinoma) [17], which indicates the ability of three-dimensional models of cell cultures to reliably reproduce the processes of tumor development down to differences at the molecular level. Based on the analysis of spheroid models of two different breast cancer cell lines (MCF-7 and MDA-MB-231) with different aggressiveness, fundamentally different effects of interaction between monocytes and cancer cells were shown. On the one hand, for spheroid models of the less aggressive MCF-7 cell line, the presence of monocytes reduced the expression of tumor malignancy markers such as metalloproteinase 9, urokinase plasminogen activator, cyclooxygenase-2, and osteopontin. On the other hand, co-culture of monocytes with three-dimensional spheroid models of the aggressive cell line MDA-MB-231 increased the expression of matrix metalloproteinase genes, as a result of which cancer cells and monocytes showed greater joint infiltrative capacity in the substrate of murine extracellular matrix extract (Matrigel1) [22].

Similar results were shown for dendritic cells derived from peripheral blood cells by exposure to IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) when co-cultured with spheroids of different tumor cell lines. The character of dendritic cell modulation differed depending on the tumor cell line [18].

There are studies that demonstrate a correlation between monocytic and neutrophil infiltration. For example, infiltration of spheroids with CD14+ monocytes resulted in a subsequent decrease in neutrophil infiltration. In contrast, prior infiltration with neutrophils did not affect subsequent monocyte infiltration, and tumors with macrophage depletion in mice showed higher neutrophil infiltration [28].

Thus, three-dimensional cell models are a feasible means to study the infiltrative capacity of monocytes and the polarization of tumor-associated macrophages down to the specificities of different tumor types and their individual cell lines, allowing its use as a susceptible screening tool for anticancer compounds *in vitro*.

Diversity of structural groups of three-dimensional tumor models. In order to create *in vitro* tumor models of non-small cell lung cancer (NSCLC) that include the myeloid compartment, Sofia P. Rebelo et al. prepared three-dimensional cell culture models encapsulated in alginate microcapsules with an average diameter of 652 ± 26 μm. This three-dimensional model included three cellular compartments: spheroids of NMSL cells (NCI-H157 cell line), cancer-associated fibroblasts (CAF), and the monocytic cell line THP-1. The microencapsulated models were maintained in suspension culture under constant agitation for three weeks. Each cell type exhibited high viability throughout the culturing time. Cell proliferation was uniform in all compartments and resulted in a tenfold increase in tumor cell concentration by week 3, which was comparable to the increase in tumor monocultures and double co-culture control groups (NSCLC cells were cultured with either tumor-associated fibroblasts or the THP-1 monocytic cell line). These results indicate that co-culture of stromal and monocytic cells had no significant effect on tumor proliferation [2].

During the culture period, the models composed of the three components contained numerous spheroids and cell clusters comprising all cell types (NSCLC, CAF, and THP-1 cells). In contrast, one to three large spheroids were found in the microcapsules of tumor monocultures, which had a loose shape and consisted of N-cadherin and vimentin positive cells (NCI-H157 retain their typical mesenchymal phenotype when microencapsulated). Moreover, in the observed multicomponent models there was an intense accumulation of extracellular matrix proteins: fibronectin, collagens of the first and fourth types, which, together with multiple cell clusters and single cells, formed a tissue-like model. At the periphery of alginate microcapsules, there was an intensive accumulation of extracellular matrix proteins, in which cells demonstrating a migratory phenotype were located, indicating active cell movement within microcapsules and the formation of a more invasive phenotype [2].

Single CD45+ positive cells and their small clusters were found around larger tumor spheroids both in three-dimensional models with three compartments and in immunocyte-tumor models. Such a distribution of CD45+ cells in the models considered indicates an enhancing activity of the tumor microenvironment with respect to the infiltration of myeloid cells into tumor spheroids. A similar distribution is shown for CD68. High expression of CD68 indicates active differentiation

of monocytes into macrophages. Moreover, high levels of CD163+ cells were recorded in three-compartment models and paired control models (THP-1/CAF and THP-1/NMRL), while CD163+ cells were recorded at residual levels in monocytic monocultures. This indicates the ability to stimulate monocytic cell differentiation towards an M2-like phenotype for both tumor cells and CAFs. The ratio of CD163+/CD68+ cells, which characterizes the proportion of M2-like macrophages, is approximately 15-20% for the three-compartment 3D model and the "THP-1/NMRL" control model and only 2% in the "CAF/THP1" culture. The high degree of polarization was confirmed using three-dimensional models with peripheral blood monocytes (PBM) of donor origin. On average, after 4 days of culturing, 70-80% of CD45+ cells expressed M2-like markers (CD206 and CD163) in complete models, whereas only 2-6% expressed M2-like markers (CD206 and CD163) in microencapsulated PBM monoculture.

The high proportion of CD163+ cells indicates that the presented model (three-dimensional spheroidal structure of three compartments encapsulated in an alginate shell) promotes cell migration similar to myeloid infiltration in human lung cancer under physiological conditions. At a significantly lower level, CD163+ cells were observed in control cultures with "THP-1/NMRL" and "THP-1/CAF". This is consistent with previous reports of high CAF activity in the context of monocyte recruitment associated with a marked change in secretory profile and high production of extracellular matrix proteins. Moreover, in models of co-culture of blood monocytes with breast cancer cell spheroids and tumor-associated fibroblast (CAF) spheroids, monocyte migration was higher towards CAF spheroids, for which CCL2 overexpression has been shown [17]. The high importance of CAF is confirmed in studies using classical cancer models. Within the tumor microenvironment, CAFs promote monocyte recruitment and differentiation into immunosuppressive M2 macrophages via interleukin-6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF) synthesis [10], and into myeloid-derived suppressor cells (MDSC) via signal transduction and activation of transcription 3 (STAT3) [26].

An important feature of the shown three-dimensional model is the use of an inert framework, since the introduction of physiologically relevant extracellular matrix proteins remains a fundamental problem for *in vitro* tumor analysis [30]. The considered alginate microcapsules allow to accumulate collagen types I and IV and fibronectin, forming collagen fibers with single cellular inclusions, which contributes to the formation of tissue-like structures [29].

The use of classical three-dimensional models allows obtaining relatively reliable results in the context of tumor microenvironment analysis. However, these models themselves have low predictive power with respect to the dynamic parameters of tumors *in vivo*. This problem has been addressed by combining standard 3D models with controlled dynamic environments. An interesting illustration of this approach is a three-dimensional multicomponent cell model (breast cancer cells, CAF, endothelial cells) cultured on a microfluidic chip. The architecture of the above model involves the flow of medium from peripheral chambers populated by breast cancer cells and PBMCs through a central chamber occupied by CAFs. The analysis of the presented model confirms the ability of CAF to modulate immune cells, reducing the time of their contact with tumor cells [8].

Microfluidic systems are three-dimensional models with high throughput and the possibility of automated processing of multiple samples, allowing the creation of mimetic media according to the properties of the studied organ and tissue [27]. Analysis using three-dimensional microfluidic models has shown the high importance of interferon regulatory factor (IRF-8) for the ability of immune cells to limit the invasiveness of cancer cells, which is supported by *in vivo* studies [4, 9]. A curious result was shown using three-dimensional microfluidic systems to reproduce and analyze the interaction between cancer cells and the tumor vasculature in the presence of immunocytes. Two channels populated with human breast carcinoma cells (MDA231) and endothelial cells (HUVEC) were connected by a three-dimensional hydrogel structure based on extracellular matrix, resulting in endothelial cells forming a layer on the surface of the extracellular matrix and tumor cells actively infiltrating it. As a result of tumor canal repopulation by murine macrophages (RAW264.7), a significantly higher rate of intravasation into the endothelial layer was observed [34].

The following study uses a three-dimensional model that represents a microfluidic system of channels filled with medium and collagen substrate that include hepatocellular carcinoma cells. Operation of this model involves the migration of fluorescent dye-labeled immune cells from the peripheral media-filled channels into the central channel filled with collagen substrate and hepatocellular carcinoma cells. In addition, the inclusion of so-called dead and live discriminatory dyes in the substrate composition allows to detect the dynamics of cell death [3]. The above three-dimensional tumor model was adapted by increasing the cellular diversity of the microfluidic system by including primary human monocytes. Monocytes were suspended together with aggregates of target cells in collagen gel, injected into the central hydrogel region of the microfluidic device and cultured overnight. The final arrangement of cells in the microfluidic platform mimics some features of the tumor microenvironment *in vivo*, which allows the analysis of numerous intercellular interactions [12]. In addition to increasing cellular diversity as a qualitative change in 3D models, some researchers suggest the importance of a proportional ratio of different cell groups, as this may contribute to the mimetic nature of the developed models [8].

A separate group of three-dimensional models, close in their properties to classical spheroidal models, includes so-called organoids, which are miniature models of organs embedded in extracellular matrix and formed from stem and poorly differentiated cells obtained by mechanical or enzymatic cleavage of primary donor tissue. Organoids reproduce the architecture as well as the diversity of cellular compartments and organization of the original tissue, allowing for a significant reproduction of physiological conditions. Patient-derived organoids allow for three-dimensional culture of cancer cells isolated from primary treated tissues, which can result in the loss of stromal and immune compartments. After formation of a research model of patient-derived organoids, peripheral blood mononuclear cells or other immune and stromal cell groups can be introduced as a co-culture [8].

Conclusion. The data presented above provide a clear picture of the importance of further optimization of 3D tumor models in order to obtain optimal means of reproducing the full variety of structures and conditions of the tumor stroma. The evaluation of therapeutic compounds in classical 2D models may allow a relatively reliable study of the diverse effects of antitumor agents on immune cells outside the context of the tumor microenvironment. However, the effects of antitumor agents on the infiltration and modulation of immune cells in conjunction with the tumor microenvironment cannot be adequately studied by classical 2D models. This probably

explains the relatively low success rate of anticancer immunotherapies preclinically tested on 2D models [1, 2]. Tumor 3D cellular models appear to be an optimal means to study the infiltrative capacity of monocytes and the subsequent polarization of tumor-associated macrophages. It is necessary to note the high level of sensitivity of three-dimensional models, allowing to capture a complex set of changes according to different cell populations and cell lines, which allows using it as a susceptible screening tool for anticancer compounds *in vitro*.

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