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Mimicking the Tumor Microenvironment to Regulate Macrophage Phenotype and Assessing Chemotherapeutic Efficacy in Embedded Cancer Cell/Macrophage Spheroid Models

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Abstract

Tumor associated macrophages (TAMs) are critical stromal components intimately involved with the progression, invasion, and metastasis of cancer cells. To address the need for an *in vitro* system that mimics the clinical observations of TAM localizations and subsequent functional performance, a cancer cell/macrophage spheroid model is described. The central component of the model is a triple negative breast cancer spheroid embedded in a three-dimensional collagen gel. Macrophages are incorporated in two different ways. The first is a heterospheroid, a spheroid containing both tumor cells and macrophages. The heterospheroid mimics the population of TAMs infiltrated into the tumor mass, thus being exposed to hypoxia and metabolic gradients. In the second model, macrophages are diffusely seeded in the collagen surrounding the spheroid, thus modeling TAMs in the cancer stroma. The inclusion of macrophages as a heterospheroid changes the metabolic profile, indicative of synergistic growth. In contrast, macrophages diffusely seeded in the collagen bear the same profile regardless of the presence of a tumor cell spheroid. The macrophages in the heterospheroid secrete EGF, a cytokine critical to tumor/macrophage co-migration, and an EGF inhibitor decreases the metabolic activity of the heterospheroid, which is not observed in the other systems. The increased secretion of IL-10 indicates that the heterospheroid macrophages follow an M2/TAM differentiation pathway. Lastly, the heterospheroid exhibits resistance to paclitaxel. In summary, the collagen embedded heterospheroid model promotes TAM-like characteristics, and will be of utility in cancer biology and drug discovery.

Graphical Abstract

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Embedded Multicellular Spheroid

Keywords

spheroid; tumor associated macrophages; heterospheroid; breast cancer; paclitaxel

1. Introduction

A malignant tumor is more than a single, mutated cell population replicating without regard to the otherwise healthy tissue, within which it resides. Rather the surrounding stroma maintains a dynamic relationship with the tumor, through which it is intimately involved in cancer initiation, growth, and progression. Furthermore the stroma can exert such influence that a normal architecture suppresses a malignant phenotype [1] and an activated stroma promotes neoplastic progression [2]. Both acellular and cellular components of the stroma influence tumor progression [3].

Cellular components of the tumor stroma include fibroblasts, myofibroblasts, endothelial cells, pericytes, macrophages, and a variety of inflammatory cells [4]. Tumor associated macrophages (TAMs) are a macrophage subset that drew early interest due to histological observations of tumor infiltration [5,6]. Macrophage content in human tumors varies from 50–80%, with one study in breast cancer quantifying infiltration as 490 and 343 macrophages/mm² for medullary carcinomas and ductal carcinomas, respectively [7,8]. Clinically, high TAM infiltration indicates worse overall and relapse-free survival, emphasizing their importance in cancer progression and prognosis [9,10]. TAMs classified as M2, or alternatively activated macrophages, are generally associated with tissue repair and remodeling, tumor promotion, metastasis, and immunoregulation [11,12]. This is in contrast to M1 or classically activated macrophages that destroy intracellular pathogens by triggering a proinflammatory response [13].

Given the critical role TAMs play in tumors, the development of *in vitro* models, which recapitulate the interplay between TAMs and cancer cells, is of significant basic and clinical interest. TAMs and tumor cells have been modeled *in vitro* using monolayer co-culture [14] and supernatant transfer [15], both of which are sufficient for tumor cells to promote M2 pathway activation associated with TAMs. However, a monolayer culture is insufficient in providing the environmental cues that define a tumor microenvironment, and is unable to replicate the 3D localization of macrophages with respect to the tumor. Pollard et al, identified three distinct TAM populations present in both human and murine tumors: 1) in the surrounding stroma; 2) in necrotic, hypoxic areas of the tumor and; 3) aligned with the abluminal side of vessels [16]. These locational differences within the tumor demonstrate the need for TAM-cancer cell models that reflect clinical observations.

Spheroids offer a unique opportunity to mimic elements of an *in vivo* tumor, such as the multicellular nature, metabolic gradients, and inclusion of stromal factors such as extracellular matrix (ECM) and secondary cell types. Spheroids were first prepared in the 1970s based upon the premise of forming a multicellular structure by denying cells an attachment site [17,18]. Multiple cell types have been incorporated in a spheroid during formation to form a heterospheroid [19,20]. However, the most common method to prepare a macrophage spheroid model consists of a spheroid in a non-adherent well exposed to macrophages in the surrounding media. Using this configuration, spheroids of breast cancer cells or cancer associated fibroblasts were used to characterize infiltration of macrophages into a tumor versus its fibroblast rich stroma [21]. TAMs laden with gold nanoshells were also used as a novel drug delivery system to treat adjacent tumor cells after irradiation [22]. In another study, the role of TAMs in tumor angiogenesis was characterized by implanting an infiltrated spheroid into a murine model and documenting subsequent TAM-driven angiogenesis [23]. TAM inclusion led to the release of vascular endothelial growth factor (VEGF) that increased angiogenesis as demonstrated by the increased vessel number and length. The use of these models has provided key insights into TAM biology.

Unlike the previous models, Hauptman et al, incorporated an ECM mimic by first preparing a spheroid on an agarose coated well and then transferring it onto a layer of collagen. They studied the complexity of tumor/macrophage interaction by demonstrating that the inclusion of different macrophage phenotypes had significant effects on colon cancer cell migration and proliferation [24]. For example, one subtype, similar to macrophages found in central tumor regions, increased proliferation, but prevented migration. Although an improvement, the model does not provide a fully 3D ECM, where macrophages can populate and migrate along fibrillar collagen surrounding a tumor, as is found *in vivo* [25,26]. Furthermore, these models do not enable the concurrent study of two different TAM subpopulations such as those found in the surrounding stroma or necrotic regions. Herein, we describe two different cancer cell/macrophage spheroid models that include: 1) a triple negative breast cancer cell line derived from metastatic cells; 2) a spheroid recapitulating tumor macrostructure; 3) collagen as an ECM mimic; 4) incorporation of macrophages within the spheroid or in the surrounding microenvironment; 5) measurement methods that include quantitative whole spheroid analyses; 6) a paracrine interaction as an example of an important cytokine-based interaction between the macrophages and breast cancer cells; and 7) treatment with a

chemotherapeutic that demonstrates the protective effect of macrophage presence on cancer cells.

2. Materials and Methods

2.1 Cell Culture

MDA-MB 231, a human adenocarcinoma cell line derived from a metastatic site, and RAW 264.7 (ATCC, USA), a murine Abelson leukemia transformed macrophage/monocyte line were cultured in Dulbecco's Modified Eagle Media supplemented with fetal bovine serum (10%) and penicillin/streptomycin (1%, 10,000 IU/mL penicillin; 10,000 mg/mL streptomycin) (Invitrogen, USA). Cell lines were kept at 37°C in a humidified chamber with 5% CO₂. Propagation was performed as recommended by ATCC, with macrophages requiring mechanical removal.

2.2 Spheroid Formation

The method for spheroid formation follows previously published method, with the addition of 2.5% Matrigel (Corning, USA) and centrifugation at 1,000 g for 10 minutes as described by Ivascu et al [27,28]. RAW 264.7 cells were added to agarose-coated plates in quantities ranging from 500–5,000 with 10,000 MDA-MB 231 cells to create a heterospheroid as shown in Figure 1b. The diffuse model was formed by inclusion of the RAW 264.7 cells in collagen during the spheroid embedding, which was otherwise performed as previously described [27]. Media was changed daily on all collagen-embedded models.

2.3 Imaging

Brightfield images of spheroids on agarose were captured using an Olympus IX70 microscope with an Insight camera. Resulting images were analyzed on ImageJ (available at <http://imagej.nih.gov/ij/> National Institute of Health, USA) [29]. DIC images were acquired on a DMI600B microscope (Leica, Germany) with an Imagem EM-CCD Camera (Hamamatsu Photonics, Japan) in a spinning disc confocal setup (Yokogawa, Japan). Confocal imaging was performed with the addition of lasers (excitation 488 and 561 nm). Imaging was done using Micro-Manager 1.4 Software. Assembling of frames into a single image was done with a custom Matlab program.

2.4 Fluorescently Stained Heterospheroids

MDA-MB 231 cells and RAW 264.7 cells were stained with green and red cell trackers, (Molecular Probes, USA) respectively at 20 µM prior to spheroid formation. Heterospheroids were imaged after 72 hours on agarose.

2.5 Oxygen Saturation

In order to assess metabolic rate, oxoplates, which measure the partial pressure of oxygen in the media, were used. Measurements were obtained in 96 well oxoplates (PreSens Precision Sensing, Germany) using a SpectraMax M5 plate reader (Molecular Devices, USA). Data was analyzed according to manufacturer's instructions. The sensor of the oxoplate does not have the same surface chemistry as a standard tissue culture plate, therefore some collagen

gels detach, thus altering the sensing of the oxygen consumption of the embedded cells and potentially the ability of the sensor to get an accurate readings. Data points that exceeded two standard deviations from the averages were removed as outliers.

2.6 Protein Secretion

Media was exchanged daily with samples taken for the last 24-hour interval. Secreted proteins were detected on the last day of culture via a mouse EGF ELISA (Sigma-Aldrich, USA) or mouse Interleukin-10 (IL-10) ELISA (Abcam, USA). The mouse EGF ELISA was selected since the capture antibody shows no cross reactivity with the human analogue, and the detection antibody has less than 0.01% cross reactivity. The mouse Interleukin-10 ELISA has no cross reactivity with human IL-10.

2.7 Inhibitor Treatment

Systems were treated with EGFR inhibitor Tyrphostin AG 1478 (Sigma-Aldrich, USA) at concentrations utilized by other investigators, ranging from 0.2–200 nM for 24 hours prior to oxoplate measurement [30].

2.8 Drug Treatment

The heterospheroid and associated control systems were treated with paclitaxel (10 ng/mL) and cultured for an additional 72 hours. Efficacy was assessed via a colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell proliferation assay. When the MTS reagent was applied directly to the 3D systems, diffusion through the collagen was limited resulting in very low absorbance readings. Therefore, cells were harvested from the 3D systems and cultured as a monolayer overnight before the addition of the MTS reagent. Collagen-based matrices were disaggregated with a treatment of collagenase (1.5 mg/mL) in phosphate-buffered saline. The breast cancer spheroids required subsequent disaggregation with trypsin. Absorbance was read at 490 nm. Cell viability was calculated as a percentage of the untreated control of each model.

3. Results

3.1 Macrophage Incorporation into the Spheroid Model

The impact of macrophage (RAW 264.7) inclusion on tumor spheroids was studied in the collagen embedded breast cancer (MDA-MB 231) spheroid model in two different ways as shown in Figure 1A. Collagen embedding was performed as previously described, where a spheroid was prepared on an agarose-coated well before transferring into a 4 mg/mL collagen gel [27]. The first method of macrophage incorporation was a two cell type “heterospheroid,” and the second was by diffusely incorporating macrophages into the collagen gel surrounding a spheroid of breast cancer cells. In order to form a spheroid containing cancer cells and macrophages, a combination of macrophages, breast cancer cells, and Matrigel was added to the agarose-coated wells as shown in Figure 1B. The resulting heterospheroid contained two cell types in different specified ratios with non-embedded spheroids shown in Figure 2A–C. When 10,000 breast cancer cells were included with a varied number of macrophages (500–5,0000), the corresponding spheroid diameter increased as shown in Figure 2D. For the entire range of macrophage concentrations used

(500–5,000 cells), spheroid formation occurred. The diameter appeared to plateau at ~800 μm when 2,500 or 5,000 macrophages were added. Higher macrophage concentrations were not explored as increasing macrophage content decreased the robustness of the spheroid. To investigate localization by cell type prior to collagen embedding, cells were labeled with a fluorescent cell tracker before spheroid formation. The resulting image of a spheroid composed of 1,000 macrophages and 10,000 breast cancer cells showed a homogeneous mixture of cell types throughout the structure (Figure 2E). In this text, heterospheroids will be described by the number in thousands of the cancer cells followed by macrophages, such that 10C5M refers to a heterospheroid with 10,000 cancer cells and 5,000 macrophages.

The second design of a cancer cell/macrophage spheroid model has the macrophages embedded in the collagen gel surrounding the cancer cell spheroid. A 10,000 cancer cell spheroid surrounded by 5,000 macrophages diffusely embedded in the collagen will be referred to as 10Cd5M. A tumor cell only model, consisting of a 10,000 cell breast cancer spheroid embedded in collagen (10C), and a macrophage only model with 5,000 diffusely embedded macrophages in a collagen gel (d5M) served as controls. Images of all four systems with 5,000 macrophages are shown in Figure 3 on the first and last day of culture in collagen. The corresponding images with 1,000 macrophages are shown in Supplemental Figure 1. The cell systems were grown for eight days in collagen. The method of macrophage incorporation significantly affected overall morphology. Diffusely seeded macrophages formed multiple, large clusters which achieved diameters greater than 100 μm by day eight, in the presence or absence of the tumor spheroid. In contrast, heterospheroids grew as a singular, large, dense structure containing both cell types.

3.2 Synergistic Growth in Spheroid Systems

Oxoplates, which monitor the percent oxygen saturation via sensors at the bottom of the well, were used to observe the overall metabolism of the four cell model systems. The relative oxygen level for the four different models using 5,000 macrophages and/or 10,000 tumor cells is shown in Figure 4: cancer cell spheroid alone, a heterospheroid, diffusely seeded macrophages with a cancer cell spheroid, and a macrophage only model. The analogous graph for 1,000 macrophages is found in Supplemental Figure 2. The percent oxygen levels declined steadily from 100% to approximately 16% at the end of eight days in the spheroid (10C) model. The oxygen consumption of a spheroid surrounded by macrophages (10Cd5M) followed the trajectory of a single spheroid until day three when consumption increased and oxygen levels rapidly decreased. This pattern was mirrored by the diffusely embedded macrophages (d5M), where the abrupt decrease in percent oxygen saturation occurred between day four and five. However, the heterospheroid (10C5M) rapidly consumed oxygen from day one, starting at an initial oxygen level of only 79% and continuing with an exponential decay. The slope decreased over time, with all macrophage containing samples reaching the same relative oxygen level by day seven and resulting in significantly lower levels than with cancer cell spheroids alone. The highest rate of oxygen consumption was observed with the heterospheroids, suggesting a synergistic growth pattern of the two composite cell types.

3.3 Cytokine Based Interactions

Media samples from each of these four model systems were assessed for the presence of macrophage-derived mouse epidermal growth factor (EGF), as an indicator of paracrine interactions between the two cell types. The measured EGF is secreted by the macrophages and not the human cancer cells because the antibodies used have minimal (<0.01%) cross reactivity with human EGF. The EGF levels measured by ELISA, on samples taken on day eight (n=4), from the four different models are shown in Figure 5A. The 10C5M heterospheroids secreted the highest concentration of EGF (50.0 ± 10.7 pg/mL). This was statistically higher than EGF produced by all other models ($p < 0.005$). The 10Cd5M model secreted 12.8 ± 3.9 pg/mL EGF versus 4.7 ± 2.9 pg/mL by diffusely seeded macrophages (d5M), which was statistically lower than the 10Cd5M model ($p < 0.05$). Secretion levels for 10C were the lowest at 2.8 ± 6.0 pg/mL, which was not statistically different from levels secreted by d5M.

We hypothesized that increased cellular growth due to paracrine interactions, may be responsible for the increased oxygen consumption demonstrated by 10C5M heterospheroids. Therefore we determined EGF expression for each of the four systems, and then treated with an inhibitor in order to block interactions between the macrophage-derived EGF and the EGF receptor (EGFR) known to be upregulated on carcinoma cells, including MDA-MB 231 [31,32]. Specifically, the 10C5M heterospheroids were treated with increasing doses (0.2–200 nM) of the EGFR inhibitor Tyrphostin AG1478. Within 24 hours of EGFR inhibition, the 10C5M spheroid responded to concentrations from 0.2–200nM in a dose dependent manner (Figure 5B). The lower doses of 0.2–2 nM did not afford a statistically different response from untreated heterospheroids. However, the relative oxygen saturation significantly increased from $50.4 \pm 4.5\%$ for 10C5M without inhibition to $72.3 \pm 9.8\%$ at the maximum EGFR inhibitor dose of 200 nM ($p < 0.005$). Treatment of 10C5M with 200 nM Tyrphostin AG1478 decreased the oxygen presence to levels previously noted for heterospheroids with only 1,000 macrophages ($75.1 \pm 6.9\%$). No concentration dependent response with Tyrphostin AG1478 was observed when analogous models 10Cd5M and 10C were treated (Supplemental Figure 3).

3.4 Macrophages Skewed to M2 Differentiation Subset

IL-10 is a known marker for M2 differentiation in TAMs using RAW 264.7 macrophages [33]. Macrophages diffusely cultured in collagen secreted less IL-10 in the presence of a spheroid (Figure 6; d5M versus 10Cd5M: 5.4 ± 1.7 pg/mL versus 1.8 ± 0.8 pg/mL, respectively $p < 0.05$). Heterospheroid culture afforded the largest production of IL-10 at 30.2 ± 18.3 pg/mL, which was statistically higher than all other values ($p < 0.05$). As expected, the spheroid alone (10C) had negligible IL-10 secretion at 0.3 ± 4.0 pg/mL. The production of IL-10 can be directly attributed to the macrophage cell line, as only murine, not human IL-10 was measured.

3.5 Drug Treatment of Heterospheroid

The previous data demonstrate that of the models presented, the heterospheroid most closely mimicked a TAM-infiltrated tumor. Therefore the heterospheroid, along with the single cell models were treated with a clinically used chemotherapeutic, paclitaxel, before assessing

their NAD(P)H dehydrogenase activity as a surrogate for metabolic activity. The heterospheroid 10C5M was more resilient to paclitaxel treatment than 10C, ($82.4 \pm 26.0\%$ versus $28.2 \pm 14.5\%$, $p < 0.05$) but not statistically different from the d5M model ($98.0 \pm 6.7\%$, $p > 0.05$) as shown in Figure 7.

4. Statistical Methods

All experiments were performed with an n of three or greater, and the data is presented as an average with standard deviations. A $p < 0.05$ is considered significant.

5. Discussion

As our knowledge of cancer biology grows, it has become clear that monolayer systems are too simplistic to adequately model the complexity of the interactions between a growing tumor and the surrounding stroma. The cellular and extracellular stromal components known to support the progression and growth of a tumor are potential targets for novel treatments. Given the role of tumor-associated macrophages in the migration, invasion, and metastatic potential of carcinomas, TAMs are of particular interest.

In response to this interest, we explored two cancer cell/TAM spheroid models mimicking solid tumor within a surrounding collagen gel as a model ECM. Due to the effect of tumor microenvironment on TAM function, macrophages were incorporated and studied in these models in two different ways – either diffusely embedded within the surrounding collagen (10Cd5M) or within the spheroid forming a heterospheroid (10C5M). Incorporation of macrophages within a heterospheroid mimics the population clinically found in necrotic and hypoxic areas by Pollard and others [34,35]. Interestingly, we have previously demonstrated that cell death and necrosis occur at the center of cancer cell/tumor spheroids resembling those used in the current study [27]. The second model consists of a cancer cell spheroid surrounded by a macrophage population diffusely embedded in the collagen (10Cd5M). This models the stromal population found in normoxic regions of breast cancer shown by Lewis et al [35]. Despite the same ratio of cells, these models respond differently, demonstrating the importance of localization, microenvironmental cues, cell clustering, and nearest neighbor effect.

The differences in the macrophage microenvironment between the 10C5M and 10Cd5M models result in different metabolic profiles despite the same number and ratio of cell types (Figure 5). The growth profile is characterized by changes in percent oxygen saturation as this has been correlated to viability [36]. The 10C5M heterospheroid exhibits an early increase in oxygen consumption unlike any of the other cell systems, suggesting that the 10C5M heterospheroid uniquely demonstrates synergistic growth between the two cell types – cancer cell and macrophage, similar to that demonstrated *in vivo*. In contrast, the oxygen levels with the 10Cd5M model resembles that of the d5M model, and, thus, is consistent with macrophage-driven growth rather than synergistic growth. The slightly decreased percent oxygen saturation noted in 10Cd5M compared to d5M alone is likely due to the addition of the cancer cell spheroid itself, which is not present in the macrophage only model. In contrast, despite the same number of cells, the 10C5M heterospheroid model has a

higher oxygen consumption (i.e. lower percent oxygen saturation) than the 10Cd5M model, indicating significantly greater cell growth and/or metabolism driven by synergistic interactions between the macrophages and breast cancer cells in the heterospheroid model.

On a cellular level, TAMs can follow an M2 or alternative activation pathway, which is generally associated with tissue repair and remodeling, tumor promotion, and immunoregulation [11]. Specifically, the M2 phenotype is associated with pro-tumor effects such as encouraging angiogenesis, as well as immunosuppressive functions such as inducing T regulatory cells to suppress T-cell mediated anti-tumor activity [7]. This is in contrast to the M1 phenotype, where the macrophages produce pro-inflammatory cytokines, kill microorganisms and tumor cells, and have a high capacity to present antigens [7]. Interestingly, tumor cells can promote a M2 activation pathway in macrophages through the secretion of cytokines (including CSF-1, CCL17 and CCL22) and ECM components [11]. In response, TAMs promote angiogenesis by secreting VEGF and COX-2 [37], and induce co-migration of both cancer cells and TAMs with the secretion of epidermal growth factor (EGF) [38,39]. The binding of EGF to the corresponding EGF receptor (EGFR), found to be over expressed in a number of cancers including breast cancer, stimulates the secretion of CSF-1, which further drives macrophages along the pro-tumor pathway in a positive feedback loop with nearby tumor cells [38,39]. In our model, secretion of EGF was found to be minimal for single cell models, including macrophages alone, but was significantly increased in the mixed macrophage/cancer cell models. This was particularly evident in the 10C5M model where the heterospheroids produced more than four times the amount of EGF seen in the 10Cd5M model. This increased EGF production observed in the heterospheroid is consistent with the paracrine loop between tumor-associated macrophages and cancer cells previously reported in the literature [38]. Furthermore, blocking this interaction markedly decreased the overall metabolism in the heterospheroid systems but not the other cancer cell containing systems, confirming that the EGF/EGFR interaction is at least partly responsible for the early and sustained increased metabolic activity as noted by a decrease in oxygen level with the heterospheroids (Figure 5). Previous *in vitro* TAM/cancer cell systems demonstrated that monolayer co-culture of TAM and cancer cells could induce EGF expression [38,39]. However, the current manuscript demonstrates that co-culture alone is insufficient in 3D environments, and that EGF secretion is significantly increased in the interactive environment of a TAM/cancer cell heterospheroid.

IL-10 is a cytokine involved in various anti-inflammatory activities that is frequently upregulated in a number of cancers, including breast cancer, correlates with stage [40], and its production is associated with TAM conversion to an M2 activation pathway [41]. In the literature, the conversion of RAW 264.7 cells to an M2/TAM phenotype was confirmed by IL-10 secretion [33] and can be achieved by treatment with exogenous cytokines such as IL-4 [42], monolayer co-culture with cancer cells,[14] or in response to nanoparticle surface chemistry [43]. The role of IL-10 is complicated by its implication in both induction of the M2/TAM phenotype, and as evidence of the M2/TAM phenotype [7]. The combination of the mouse and human cell line allows identification of murine IL-10 as opposed to human IL-10 secreted by the tumor cells, which can promote M2/TAM phenotype. Inclusion of the murine RAW 264.7 line also enables the use of its well-characterized phenotypes. The high concentration of IL-10 produced by the macrophages of the heterospheroid system, shows

that the combination of the microenvironment, ECM mimic, and relatively close proximity of macrophages to cancer cells within the heterospheroid is sufficient for TAM conversion. Although invasion of the cancer cells into the surrounding collagen results in cell/cell interactions between macrophages and tumor cells in the 10Cd5M model, this remains distinctly different from the dense, intermingled architecture of the heterospheroid, and was not sufficient to induce macrophage IL-10 production.

To ascertain if the MDA-MB 231 cells in the heterospheroid model responded differently to a chemotherapeutic compared to cancer cells alone (10C), the samples were treated with paclitaxel. When treated with paclitaxel, the 10C5M heterospheroid is less sensitive than the associated breast cancer only model, 10C indicating TAM-mediated resistance. A similar treatment response was observed with the cancer cells in the d5M model. The resistance to paclitaxel in breast cancer, due to TAM interaction, is supported by results published by Yang et al, who reported that the source of the resistance is due to IL-10 secretion by TAMs, which activates the IL-10/Stat3/bcl-2 signaling pathway. The 3D heterospheroid, described herein, replicates results that would otherwise require artificial polarization of macrophages by exposure to a cocktail of cytokines. Additionally, it sets the stage for future work that examines the contributions of a subset of specific macrophages to chemotherapeutic response and/or chemoprotective effect (via fluorescent protein expressing cell lines, genetic and proteomic expression, apoptotic markers). The heterospheroid model offers an easy to implement *in vitro* system for drug discovery aimed at TAMs as well as a co-culture system to characterize the roles of TAMs in drug response.

The motivation for early studies on TAMs was the clinical observations of macrophages in the tumor environment. Continuing clinical interests are fueled by recent evidence that TAMs can enhance or suppress the efficacy of existing therapeutics on cancer cells and drive reparative mechanisms post-treatment [44,45]. For example, skewing TAM polarization away from the pro-angiogenic/immune-suppressive M2 phenotype toward a tumor-inhibiting phenotype [46] or the use of a novel chemotherapeutic, trabectedin, that selectively kills monocytes, including TAMs, [47] are both being investigated as novel treatments. However, the lack of *in vitro* models of cancer cell/TAM interactions limits progress given the significant challenges of using animal models for these studies [46–48]. *In vivo* growth of human tumor xenografts requires the use of immunocompromised animal models, which by default will have altered immune responses, and thus tumor microenvironments [49]. Therefore there is a critical need for the development of *in vitro* tumor models that study TAMs in the context of the tumor macrostructure and physiologically relevant TAM/cancer cell co-localization.

6. Conclusion

We present four distinct iterations of a collagen embedded model: a spheroid composed of only cancer cells (10C), diffusely seeded macrophages (d5M), a cancer cell spheroid diffusely surrounded by macrophages (10Cd5M), and a macrophage/cancer cell heterospheroid (10C5M). The heterospheroids recapitulated EGF-based paracrine interactions, promoted tumor growth in a model dependent manner, and secreted cytokines consistent with an M2/TAM polarization. The heterospheroid exposes macrophages to a

microenvironment more consistent with that found within an *in vivo* tumor, thus reducing the need for exogenous agents that are often utilized to attain M2 polarization. The source of differentiating cues is limited to the accompanying breast cancer cells, and the multicellular macrostructure. However in the spheroid model, TAM differentiation is not solely achieved through the co-culturing of breast cancer and macrophage lines, as is found with monolayer co-culture, since the 10Cd5M model demonstrates significantly lower TAM conversion than 10C5M heterospheroids. Rather, it is the synergistic combination of co-culture and macrophage-tumor proximity and interactions present within the heterospheroid that results in M2 differentiation and the accompanying increases in oxygen consumption and metabolism. Upon treatment with paclitaxel, the heterospheroid demonstrates a chemoresistant effect. In summary, we describe TAM/cancer cell interactions within the context of an activated tumor stroma and promote the use of *in vitro* heterospheroid and diffusely seeded systems as models of breast tumor infiltrated with TAMs for future discoveries in cancer biology, agent mechanism studies, drug screening, and drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

Two in vitro collagen-embedded multicellular spheroid models are described that mimic the clinical observations of macrophage localization within a tumor. Incorporation of macrophages within a breast cancer spheroid emphasizes cell-cell interactions with subsequent differentiation toward a tumor-promoting TAM phenotype. In contrast, macrophages seeded around the tumor spheroid display decreased interaction with cancer cells and no indication of a TAM phenotype. Finally, the presence of macrophages in the heterospheroid increases resistance to paclitaxel. This study demonstrates that cell-cell interactions and 3D collagen matrix direct macrophage activity, and, thus, highlights the important role the local environment itself plays in macrophage behavior.

Figure 1.

Macrophage Incorporation into Spheroid Model: Starting with an embedded spheroid model, the influence of macrophages (shown in green) was studied using four different models (A). The first model was a breast cancer spheroid with 10,000 cancer cells (shown in red) embedded in a collagen gel (10C). The second model consists of 5000 diffusely embedded macrophages within a collagen gel (d5M). The third model combines these with a spheroid surrounded by diffusely seeded macrophages (10Cd5M) within a collagen gel. The fourth model is a heterospheroid (10C5M) composed of both cell types. B) Heterospheroids are formed by including breast cancer cells and macrophages during spheroid formation on agarose in various ratios.

Figure 2.

Heterospheroid Formation: Macrophages were incorporated during spheroid formation to form a heterospheroid with a defined number of macrophages. Images show spheroids with tumor cells and 0 (A), 1,000 (B), or 5,000 (C) macrophages. D) Inclusion of a greater number of macrophages resulted in increased spheroid diameter. E) A spheroid with stained cancer cells (green) and macrophages (red) shows the position of cell types throughout the spheroid in an overlaid image. The scale bar is 100 μm for all images.

Figure 3.

Collagen Embedded Models: DIC images show the growth of the models in collagen from day one to day eight. The models are a spheroid composed of 10,000 cancer cells (10C); a heterospheroid with 10,000 cancer cells and 5,000 macrophages (10C5M); a 10,000 cancer cell spheroid surrounded by 5,000 macrophages (10Cd5M); and 5,000 macrophages singly embedded in collagen (d5M). All scale bars are 500 μm.

Figure 4. Synergistic Growth in Spheroid Systems: The relative oxygen saturation provides an indication of how macrophage incorporation method affects overall metabolic activity and cell growth rate (n=6).

Figure 5. Cytokine Based Interaction of Macrophages and Tumor Cells: A) EGF concentration detected by ELISA from the four models (n=4). B) Addition of an EGFR inhibitor at concentrations from 0.2–200 nM in the 10C5M systems disrupts the EGF/EGFR interaction (n=4). The relative oxygen saturation is an indicator of overall metabolic activity (** p<0.005, *p<0.05).

Figure 6. Heterospheroid Enhances TAM Conversion: Secretion of IL-10 indicates the alternative M2 activation pathway associated with TAMs. IL-10 secretion is detected via ELISA (* p<0.05).

Figure 7. Heterospheroid Imparts Chemoresistance

When treated with paclitaxel, the heterospheroid is more resistant than the analogous cancer cell only model ($n=6$; $p<0.05$). The DIC images show the morphological effect of paclitaxel treatment upon the systems. All scale bars are 500 μm .