American Journal of Bioscience and Bioengineering
2021; 9(4): 110-122
http://www.sciencepublishinggroup.com/j/bio
doi: 10.11648/j.bio.20210904.13
ISSN: 2328-5885 (Print); ISSN: 2328-5893 (Online)

A Novel Standardized Inflammatory Cell-Modulated 3D Tumor Tissue Model for Analysis of Tumor-Stroma Interaction and Drug Discovery

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To cite this article:

Sabine Hensler, Claudia Kuehlbach, Bianka Kotkamp, Dario Frey, Margareta Maria Mueller. A Novel Standardized Inflammatory Cell-Modulated 3D Tumor Tissue Model for Analysis of Tumor-Stroma Interaction and Drug Discovery. *American Journal of Bioscience and Bioengineering*. Vol. 9, No. 4, 2021, pp. 110-122. doi: 10.11648/j.bio.20210904.13

Received: June 29, 2021; **Accepted**: July 21, 2021; **Published**: August 11, 2021

Abstract: The last decades were marked by substantial progress in understanding the role of tumor-supporting inflammatory reactions in tumor growth and progression. While *in vivo* data substantiate the contribution of the inflammatory infiltrate and of tumor associated fibroblasts in promoting tumor growth and progression, little is known about the dynamic interaction of these two stromal cell types and their reciprocal influence on each other and on the tumor cells. Mechanistical analyses of these crucial interactions require a standardized and easy to manipulate environment. We therefore established a 3D organotypic *in vitro* model for epithelial tumors to analyze the interaction of macrophages, neutrophils and fibroblasts in the tumor microenvironment of malignant tumors. In the 3D model, epithelial tumor cells are grown on a collagen type I gel containing fibroblasts, macrophages and neutrophils. Comparable to the *in vivo* setting, the cytokine driven interaction between macrophages and fibroblasts markedly influences invasion and enhances M2 differentiation in the presence of tumor cells. Addition of neutrophils further leads to a strikingly enhanced tumor invasion associated with an increased expression of MMP-9 and a N2 differentiation of neutrophils. Thus, this novel 3D model provides an *in vivo* like tissue context to analyze tumor stroma interactions and presents an excellent tool for targeted interference. As such, the model is highly suitable for pharmaceutical screening of novel therapeutics. However, the use of collagen type 1 with its known batch to batch variability as ECM equivalent prohibits the model-standardization that is needed for pharmaceutical testing. Therefore, the 3D *in vitro* tumor-stroma model was adapted to the use of a bioinert dextran-hydrogel providing a highly standardized and easily modifiable scaffold material that allows the recovery of cells after pharmaceutical experiments. Comparable to the collagen-based model, cells maintained their physiological proliferation, migration and differentiation. Utilizing this standardized model, the efficacy and the tissue impact of novel pharmaceuticals can be investigated in detail with respect to cell morphology, behavior, viability as well as gene expression profiles thereby providing a 3D hydrogel tumor stroma a model that is of great interest for the pharmaceutical industry.

Keywords: Standardized 3D Model, Tumor Stroma Interaction, Inflammatory Cells, Dextran Hydrogel

1. Introduction

Enormous progress in the prevention, early detection and treatment of cancer has contributed to a steep decline in age-standardized cancer mortality for years. Nonetheless, the number of new cancer cases is steadily rising, making cancer still the second most common cause of death in industrialized countries [1]. While there is a clear influence of lifestyle i.e. consumption of alcohol, red meat, obesity etc. the main increase is due to the overall increase in life expectancy and the enhanced disease frequency in older people [1]. Thus, with regard to the demographic change the development of efficient tumor therapies and new and efficient anti-cancer drugs is essential. At present, extensive testing of novel drug

candidates in different experimental and clinical settings still makes the development of new therapeutics very time consuming and costly. Many of the experimental systems show very little resemblance to the human organism, leading to failure of numerous drug candidates late in this costly development, when they reach clinical trials. As a consequence the establishment of appropriate tests to predict therapeutic efficacy of lead substances early during development is urgently needed. Success of tumor therapeutics is strongly influenced by the three-dimensional (3D) tissue organization and the tumor environment, making it very difficult to predict therapeutic efficacy correctly with the currently used well established two-dimensional (2D) cell culture tests. While these models provide a well-controlled and homogeneous cell environment, they do not incorporate structural or mechanical tissue properties [2-5]. *In vivo* animal experiments, albeit less accessible for targeted manipulation, clearly provide more relevant data. However, their results suffer from the significant differences in human and animal physiology. Frequently this leads to the failure of drug candidates that were considered hopeful in animal tests. For this reason, human cell based 3D models have been established and optimized in recent years, to allow the analysis of interactions between different human cell types and the extracellular matrix (ECM).

In their tissue context *in vivo* tumor cells alter physiological tissue homeostasis, e.g. by secreting growth factors, inflammatory cytokines and angiogenic factors thereby activating fibroblasts, immune cells, inflammatory cells and blood vessels in the tumor microenvironment. This in turn contributes to tumor growth and progression by the secretion of cytokines, proteases and a tumor specific ECM [6]. Unlike 2D settings the use of humanized complex 3D *in vitro* cell culture models optimally reflects the human *in vivo* situation while still allowing an easy manipulation of the system [7]. The simplest and often used 3D model is the multicellular spheroid model that mimics micrometastases and represents a model of intermediate complexity [8]. More complex 3D models include different cell types, embedded in ECM materials. They provide an *in vivo* like mechanical support and can direct cell adhesion, proliferation, migration and gene expression [9, 10]. The ECM scaffolds used in these systems vary from natural ECM proteins to chemical inert hydrogels. Currently, tissue extracts such as rat collagen, fibrin or matrigel are most commonly used due to their biocompatibility and biological activities [6, 11]. However, the composition of these biological matrices varies from batch to batch and may contain unwanted components such as additional cytokines that influence the tissue reaction [12, 13]. This can limit the reproducibility of the data obtained, making them unsuitable for standardized tests in pharmaceutical industry.

The use of biomimetic hydrogels of synthetic origin characterized by their chemically defined, controllable and reproducible composition can circumvent this problem. Hydrogels generally consist of polymers and their crosslinkers that are connected via reactive groups such as thiol, maleimide and amino groups. They lack chemical and biological interaction to the embedded cells, and like e.g. thiol-maleimide or alginate hydrogels do not promote cell attachment. To achieve *in vivo* like cell adhesion properties, the hydrogels are modified to contain adhesion molecules like e.g. the fibronectin-derived RGD motif sequence [14]. The RGD peptide mimics the binding sites for cellular integrins that activate signaling cascades essential for cell survival, function and structure. To enable cellular degradation and remodeling of the ECM, the crosslinkers of hydrogels can be equipped with protease cleavage sites for specific matrix metallo-proteases (MMPs). Examples of such synthetic ECMs are PEO- (polyethylene oxide), PEG- (polyethylene glycol), PVA- (polyvinyl alcohol) and dextran-based hydrogels, which are combined with various biodegradable and non-degradable crosslinkers and have already been approved by the FDA as carrier materials for tissue engineering [15].

3D-Life hydrogels produced by Cellendes (Reutlingen, Germany) combine all these characteristics. Depending on the desired biological attributes of the artificial ECM, the building blocks can be combined and modified in various ways to produce bioinert, biocompatible hydrogels with different mechanical properties and pore sizes. The cell-matrix interactions, relevant for successful imitation of a natural ECM are achieved by covalent immobilization of peptides (e.g. RGD) and cleavage sites for MMPs. In addition the dextran based hydrogels can be degraded enzymatically by dextranase allowing the release of the embedded cells, thus enabling e.g. gene expression analyses.

In the present study 3D-Life hydrogels were used to establish a unique 3D *in vitro* tumor stroma model combining tumor cells with stromal fibroblasts and inflammatory cells. This model provides the basis for extensive studies of tumor stroma interactions and can be used for testing the response of complex tumor tissues to drug candidates under controlled *in vitro* conditions.

2. Material and Methods

2.1. Cell Lines

2.1.1. Fibroblasts

Primary human dermal fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (10.000 U/10.000 µg/ml Biochrom, Berlin, Germany) (D10) and upon confluency were subcultured at a split ratio of 1:3.

2.1.2. Immune Cells

U937 cells and *HL-60* cells were maintained in RPMI 1640 medium (Life Technology, Darmstadt, Germany) 10% FCS and 1% penicillin / streptomycin (10000 U/10000 µg/ml) (RPMI 10) and subcultured twice a week at a concentration of $1x10^6$ cells/10 mL medium.

Two days prior to establishing the 3D model, U937 cells were differentiated to macrophages by a 48 h incubation with 50 nM phorbol 12-myristate-13-acetate (Sigma-Aldrich,

Steinheim, Germany) in RPMI 10 [16]. 3×10^6 HL60 cells per 75 cm^2 were differentiated to neutrophils by incubation for four days in 30 ml RPMI 10 with 1.25% DMSO [17]. Medium was changed once after 2 days. Differentiation was determined based on cell morphology of the now adherently growing cells. Nonadherent cells were removed by washing with phosphate buffered saline (PBS).

2.1.3. Tumor Cells

(i) Malignant HaCaT-ras Tumor Keratinocytes

Malignant HaCaT-ras A-5RT3 cells [18] and malignant HaCaT-ras A-5IL-6 cells [19] were maintained in D10 containing 200 ng/ml G418 (Biochrom, Munich, Germany) and D10 containing 200 µg/ml Zeocin (Fisher Scientific, Schwerte, Germany) respectively at 37°C, 5% CO_2. Cells were subcultured once a week, using 0.01% EDTA followed by 0.1% EDTA/Trypsin.

(ii) MCF-7 Mamma Carcinoma

MCF-7 human mamma carcinoma cells were maintained in D10 containing 25 mM Hepes buffer, 1% sodium pyruvat and 1% non-essential amino acids (all Biochrom, Berlin Germany). Cells were subcultured twice a week.

(iii) H838GFP Lung Carcinoma

H838 non-small cell lung carcinoma (NSCLC) cells were transfected with pTracer-CMV2, containing the coding sequence for the green fluorescent protein GFP (Fisher Scientific, Schwerte, Germany). Cells were maintained in D10 containing 200 µg/ml Zeocin (Fisher Scientific, Schwerte, Germany) and subcultured twice a week.

2.2. Preparation of Organotypic Co-culture (OTCs)

OTCs were established as described before [6, 20]. 2.5 ml type I rat collagen (3.59 mg/ml), containing 0.8 x 10^5 human fibroblast, U937 macrophages and HL-60 neutrophils each were added to 6 well filter inserts (Falcon, Becton Dickinson, Heidelberg, Germany). After 24 h HaCaT-ras tumor keratinocytes, MCF-7 cells or H838 cells (1 x 10^6 cells) were seeded on top. 24 hours later, the medium on top of the OTCs containing HaCaT-ras tumor keratinocytes was removed to allow for air-exposed cultivation. Medium (D10 with 50 mg/ml L-ascorbic acid (Sigma- Aldrich, Steinheim, Germany) was changed three times a week. OTCs were cultivated for 21 days. Samples and conditioned media were harvested on day 7, 14 and 21.

2.3. Preparation of the 3D Bioinert Hydrogel Tumor Stroma Model

The 3-D-Life Dextran-CD Hydrogel SG Kit (Cellendes GmbH, Reutlingen, Germany) was used for the hydrogel based tumor stroma model. In a total volume of 150 µl. Components 1-3 (table 1) were mixed together, RGD Peptide was added and the mix was incubated for 20 minutes (min) at room temperature. Finally, CD-Link and the cells in PBS (table 2), were added and the gel was transferred into a 24 well transwell insert plate (Corning, Wiesbaden, Germany) and left to polymerize for 1 hour at 37°C and 5% CO_2. Subsequently D10 medium was added and changed again after 1 hour. Medium was aspirated the next day and 30.000, 40.000 or 50.000 tumor cells were seeded on top of the gel. 24 hours later, cultures containing HaCaT-ras tumor keratinocytes were lifted to the air medium interface (day 0) and grown air-exposed for up to 21 days. All other cultures were grown submersed.

Table 1. *Components and volumes for the preparation of the hydrogel cultures.*

Components	reagents	Volume (µl)
1	10x CB, pH 7.2	10
2	ddH_2O	53.5
3	SG-Dextran (28 mmol/l SH groups)	15
4	RGD-Peptide (20 mmol/l SH groups)	4
5	CD-Link (20 mmol/l SH groups)	17.25
6	cells in PBS	50

Table 2. *Cells used for the preparation of the 3D tumor stroma model.*

Cells	Cell number per 150 µl hydrogel
U937, differentiated to macrophages	6522
HL-60, differentiated to neutrophils	5000
fibroblasts	5000

2.4. Histology

Cryosections (10 µm) were mounted on glass slides and air dried. Histology was assessed by standard hematoxylin and eosin (H&E) staining.

2.5. Immunofluorescence Staining

Cryosections (10 µm) were fixed in 80% methanol for 5 min at 4°C and 100% acetone for 4 min at -20°C and re-hydrated in PBS. Slides were blocked with 12% bovine serum albumin for 30 min. Primary antibodies were added and incubated over night at 4°C. Slides were then washed in PBS, incubated with the fluorescence labelled secondary antibody for 1 h at RT, washed and mounted. Sections were photographed using a Leica microscope with epifluorescence. Primary antibodies: pankeratin (guinea pig-polyclonal, Progen, Heidelberg, Germany), CD15 (mouse-monoclonal, Dianova, Hamburg, Germany), anti-TNF-α (N1-neutrophil) (rabbit-monoclonal, VWR, Darmstadt, Germany), anti-MMP-9 (N2-neutrophil (rabbit-monoclonal, VWR, Darmstadt); secondary anti-bodies: donkey anti-guinea pig Cy2, donkey anti-mouse Cy3 (Dianova, Hamburg, Germany), donkey anti-mouse Alexa488 (Invitrogen, Darmstadt, Germany) and donkey anti-rabbit Cy3 (Dianova, Hamburg, Germany). Nuclei were stained with 10 µg/ml Hoechst 33258/bisbenzimide.

2.6. ELISA

Concentration of IL-1ß and IL-8 in the conditioned media was determined using Quantikine Immunoassay kits (R&D Systems, Darmstadt, Germany). Samples were tested in duplicate. Data shown are mean values ± SD.

3. Results

3.1. Establishment of a 3D Organotypic Tumor Stroma Culture Containing Neutrophils

Reproducible and simple *in vivo* like models are essential for a better understanding of the molecular interactions between the tumor and its stroma and for the development of new therapeutic strategies. For this purpose a basic 3D organotypic model containing epithelial tumor cells cultivated on top of a collagen type I gel with human dermal fibroblast and macrophages is available [20-22]. M2 differentiation of macrophages that occurred in the presence of fibroblasts was accompanied by enhanced tumor cell invasion into the collagen gel [23].

To even better mimic the *in vivo* situation, neutrophils - a second important cell type of the innate immune system – were included in the organotypic model. Neutrophils isolated from human blood have a very short survival time resulting in an extreme limitation of the 3D cultures´ life span. For this reason HL-60 leukemia cells differentiated towards a neutrophil phenotype following the protocol of Collins [17] were chosen instead. To ensure that the differentiated HL-60 neutrophils are an appropriate replacement for the very short lived primary neutrophils, their adequate differentiation was confirmed by their CD15 expression 12, 24, 48 and 72 hours after differentiation (data not shown) and by the expression of a cytokine profile characteristic for neutrophils. The known neutrophil-derived cytokines interleukin-1ß (IL-1ß, [26]) and interleukin-8 (IL-8, [27]) were measured in the culture supernatant. While undifferentiated HL-60 cells show a lack of IL-1ß or IL-8 expression, HL-60 cells differentiated to a neutrophil phenotype express both IL-1ß and IL-8 comparable to primary neutrophils (table 3 and literature used therein).

Table 3. *IL-1ß [28, 29] and IL-8 [30] expression of neutrophils, of HL-60 cells and of differentiated HL-60 neutrophils (ELISA-data) after 48, 72 and 96 hours.*

	neutrophils	**HL-60 cells**	**Differentiated HL-60 neutrophils**		
			48h	**72h**	**96h**
IL-1ß [pg/ml]	50 – 200	0	144.1	17.3	4.5
IL-8 [pg/ml]	4 – 7	0	13.4	431.4	375.7

Based on these data we considered HL-60 cells differentiated to a neutrophil phenotype as suitable replacement for primary neutrophils in the 3D organotypic model.

Neutrophils are known to contribute significantly to angiogenesis and tumor progression *in vivo* [31, 32]. To investigate their contribution to tumor malignancy and their interaction with other stromal and tumor cells in the *in vivo* like 3D tumor-stroma cultures, fibroblasts, U937 macrophages and HL-60 neutrophils were combined in a collagen gel with A-5IL-6 tumor keratinocytes cultivated as a tumor epithelium on top. Histological sections of these cultures were compared to cultures with either fibroblasts and U937 macrophages or fibroblasts and HL-60 neutrophils in the gel (Figure 1).

After two weeks, 3D cultures with fibroblasts and HL-60 neutrophils or fibroblasts, U937 macrophages and HL-60 neutrophils in the gel showed an increase in epithelial thickness compared to cultures containing only fibroblasts and U937 macrophages (Figure 1 A, B versus C). In addition, in 3D cultures containing all three stromal cell types, tumor cell infiltration into the gel was enhanced showing islets of tumor cells in the collagen gel (Figure 1 B).

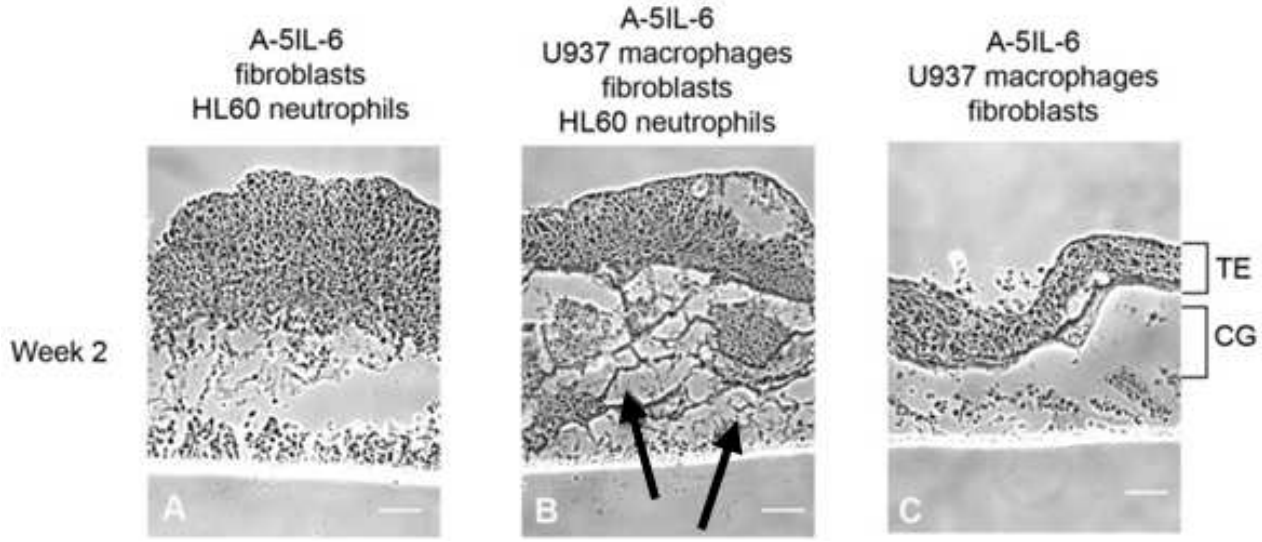

Figure 1. *Photomicrographs of histological section of 3D cocultures with A-5IL-6 keratinocytes after one and two weeks with U937 macrophages and fibroblasts (C), with HL-60 neutrophils and fibroblasts (A) and with U937 macrophages, HL-60 neutrophils and fibroblasts (B) in the collagen gel (H&E staining). Representative images of two independent experiments are shown. A-5IL-6 tumor cells form a tumor epithelium (TE) on top of the collagen gel (CG). Spindle-shaped fibroblasts, as well as macrophages and neutrophil granulocytes are embedded in the gel. Arrows mark infiltrated areas or islands of tumor cells in the gel. Measuring bar: 100 µm.*

Localization, survival and differentiation of stromal cell types as well as organization of the tumor epithelium in organotypic cultures were monitored by immunofluorescence staining against the neutrophil-specific antigen CD15 (red) and keratin (green) (nuclei were stained in blue with Hoechst 33258). Epithelial thickness and infiltration of keratin positive tumor cells into the collagen gel as determined by staining against epithelial keratin confirmed the enhanced infiltration of tumor cells into the gel in cultures with all 3 stromal cell types (figure 2 B, arrow). While the number of HL-60 neutrophils was similar in all cultures, confirming their terminal differentiation (Figure 2 A-D), they exhibited a characteristic localization in cultures containing all three stromal cell types where they were often found in large numbers close to or even within the epithelium or the invasive tumor cell islands (Figure 2 B and H, arrows).

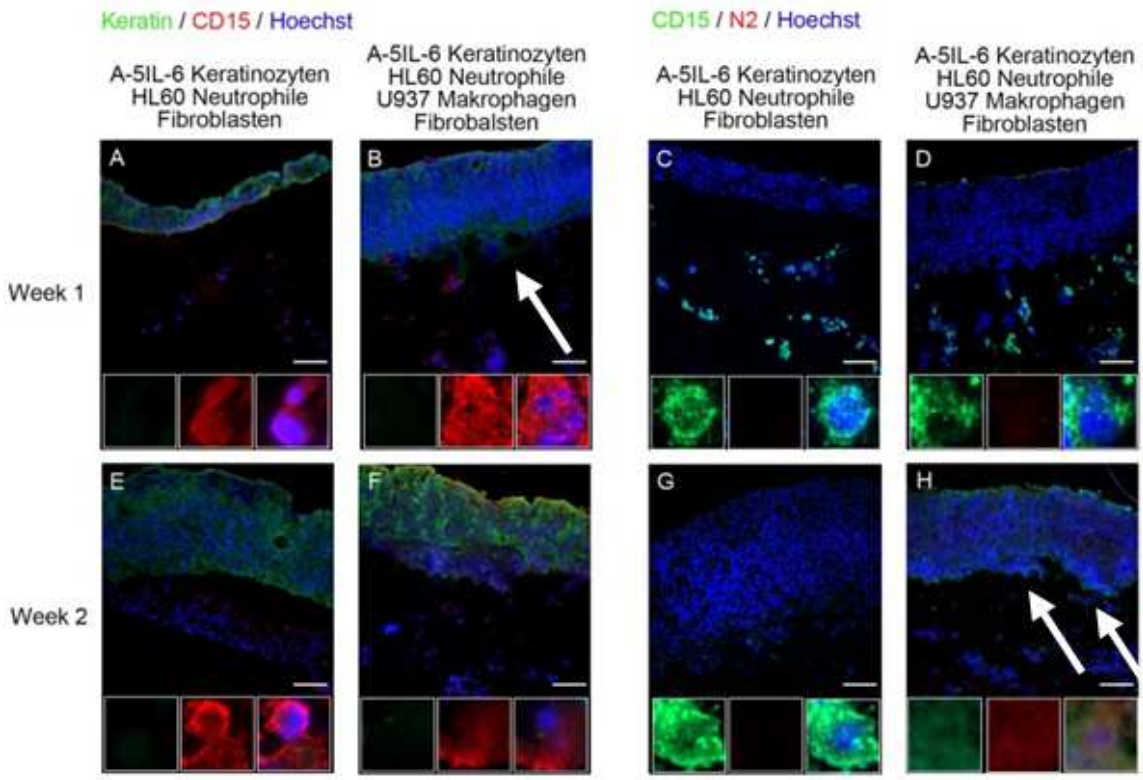

Figure 2. *A, B, E, F Immunofluorescent staining of A5 IL-6 epithelial tumor cells against keratin (green) and HL-60 neutrophil against CD15 (red) in OTCs. Nuclei are stained in blue with Hoechst 33258. Cultures containing fibroblasts, U937 macrophages and HL-60 neutrophils in the gel (B, F) exhibit enhanced epithelial thickness (week 1) and infiltration of tumor cells into the collagen gel when compared to cultures containing only HL-60 neutrophils and fibroblasts (A, E). C, D, G, H: Staining of HL-60 neutrophils against CD15 (green) and the N2 differentiation marker (anti-MMP-9) (red) in OTCs of A-5IL-6 tumor keratinocytes containing either HL-60 neutrophils and fibroblasts (C, G) or HL-60 neutrophils, U937 macrophages and fibroblasts (D, H) in the collagen gel. Nuclei are stained in blue with Hoechst 33258. Spontaneous N2 differentiation of neutrophils is exclusively found in cultures containing all three stromal cell types after 2 weeks of cultivation (H). Measuring bar: 100 µm.*

Neutrophils are known to exhibit different activation and differentiation states depending on their tissue context [31]. TGF-ß [33] and IFN-ß [34] influence neutrophil polarization with IFN-ß inducing N1 polarization while inhibiting the tumor-promoting N2 phenotype and TGF-ß inducing polarization to the N2 phenotype, while blocking the tumor suppressing N1 phenotype [31]. To determine the neutrophil phenotype in the OTCs, cultures were stained against the neutrophil antigen CD15 in green and an N1- (anti-TNF-α, data not shown) or N2- (anti-MMP-9) differentiation marker in red (Figure 2). Remarkably, while all cultures initially showed a N1 differentiation of neutrophils (data not shown), cultures containing fibroblasts, HL-60 neutrophils and U937 macrophages in the stromal equivalent, exhibited a spontaneous shift towards a N2 phenotype in week two (Figure 2 H).

Thus, the inclusion of neutrophils in the organotypic system nicely reproduced the tumor stroma interaction *in vivo*. This was reflected by an increased tumor invasion that depended on the activated stromal compartment with its previously shown pro-tumor M2 macrophages [23] and the spontaneous pro-tumor N2 differentiation of neutrophils that was found in the presence of fibroblasts and macrophages.

3.2. Adaptation of the OTC Model to a Synthetic Hydrogel Matrix

To better adapt the in vitro 3D tumor-stroma model for the high reproducibility and standardization required in pharmaceutical testing, the biological matrix collagen type I was replaced by a chemically inert synthetic matrix with a defined and highly reproducible composition. A biomimetic

maleimide-dextran hydrogel (Cellendes 3-D Life Technologie) was used. The hydrogel is functionalized with RGD peptides as integrin binding sites and MMP2 cleavage sites to allow cell adhesion and matrix reorganization that are typical in tumor tissue.

The nature of the matrix is of crucial importance for cell physiology and contributes significantly to the formation of the tumor promoting microenvironment. To determine the optimal matrix, different hydrogel compositions were tested with stromal cells i.e. either fibroblasts alone, fibroblasts and U937 macrophages or fibroblasts, U937 macrophages and HL-60 neutrophils. Dextran hydrogels with a 2.5 or 3.5 mmol/l SH groups for cross-linking and 0.5 mmol/l immobilized RGD peptide were employed. All cells survived in the hydrogels for 12 days. However, after 14 days, no viable cells could be found in either gel type (data not shown). In addition, handling of the hydrogels with 3.5 mmol/l SH groups caused problems, due to the very short polymerization time. Consequently dextran hydrogels with 2.5 mmol/l SH groups and 0.5 mmol/l RGD peptide were used for further optimization. The significantly shorter survival of stromal cells in the hydrogels compared to the collagen based tumor stroma cultures was attributed to the lack of tumor cell derived cytokines that might contribute to the survival of stromal cells in the tumor microenvironment.

Thus, the next optimization step dealt with the optimal adhesion peptide concentration to allow cell spreading and survival of tumor cells and the formation of a functional tumor epithelium on top of the hydrogel.

The maleimide dextran polymer with 2.5 mmol/l SH groups and 0.5 mmol/l RGD peptide was additionally coated with different concentrations of RGD peptide (0, 2, 4, 6, 8 and 10 mmol/l). Epithelial tumor cells (HaCaT-ras tumor keratinocytes) cultivated on top of the hydrogels without stromal cells for 8 days showed a relatively low adhesion combined with an irregular growth pattern. Therefore instead of the RGD peptide solution the hydrogel functionalized with 0.5 mmol/l RGD-peptide was coated with a thin layer of a second hydrogel containing 2, 4 and 6 mmol/l RGD peptide. Best growth conditions were achieved using a maleimide dextran polymer with 2.5 mmol/l SH groups and 0.5 mmol/l RGD peptide in the first gel and a second thin gel layer with 4 mmol/l RGD peptide (Figure 3). The cells now adhered considerably better but still retained their irregular growth pattern in patches.

The system was now extended to include additional epithelial tumor types. Gels were seeded with 30.000, 40.000 and 50.000 MCF-7 cells breast carcinoma cells [35] or 50.000 and 75.000 H838 NSCLC cells [36] respectively. With both RGD peptide concentration, MCF-7 cells initially exhibited local cell accumulations that showed some resemblance to acini and only very slowly formed a monolayer on the gel (data not shown) whereas H838 cells reproducibly established a confluent monolayer on day 5 (Figure 3).

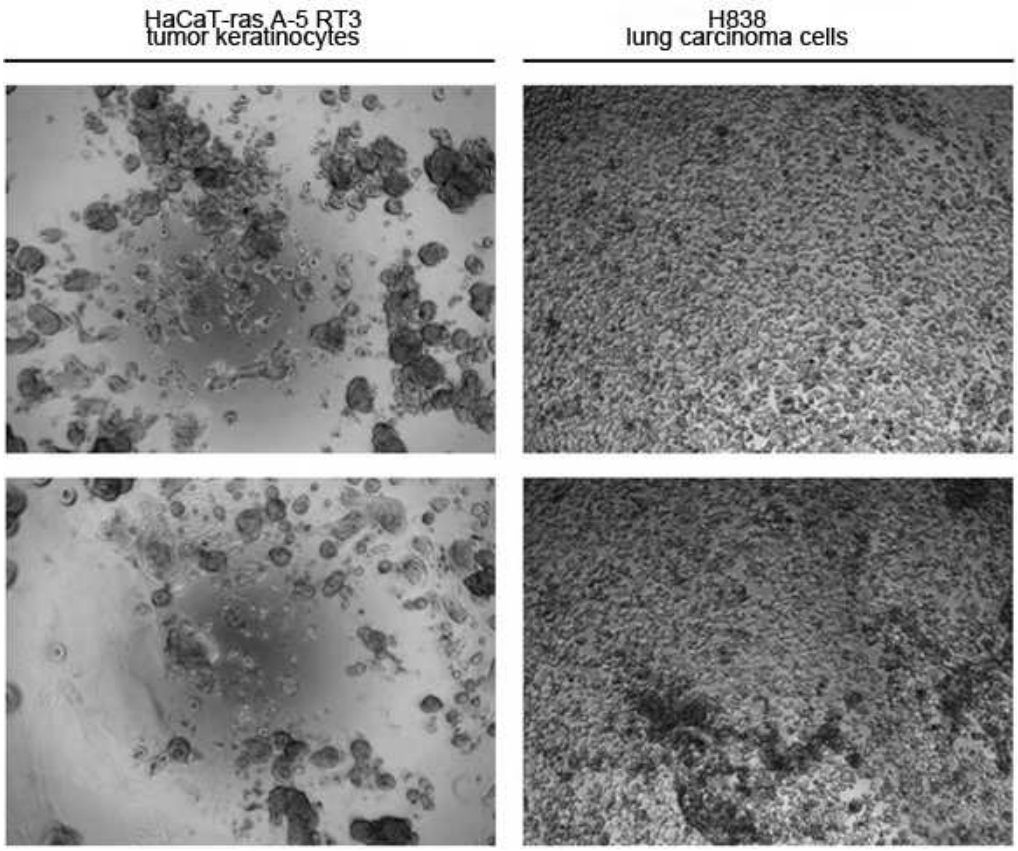

Figure 3. *Representative photomicrographs of vital HaCaT-ras A-5RT3 tumor keratinocytes and H838 lung carcinoma cells on dextran hydrogels after 5 days of culture. 50.000 cells were seeded on top of the hydrogels (without stromal cells). While A-5RT3 tumor keratinocytes showed a patchy growth pattern, H838 lung carcinoma cells formed a sub-confluent but homogeneous monolayer on top of the gel.*

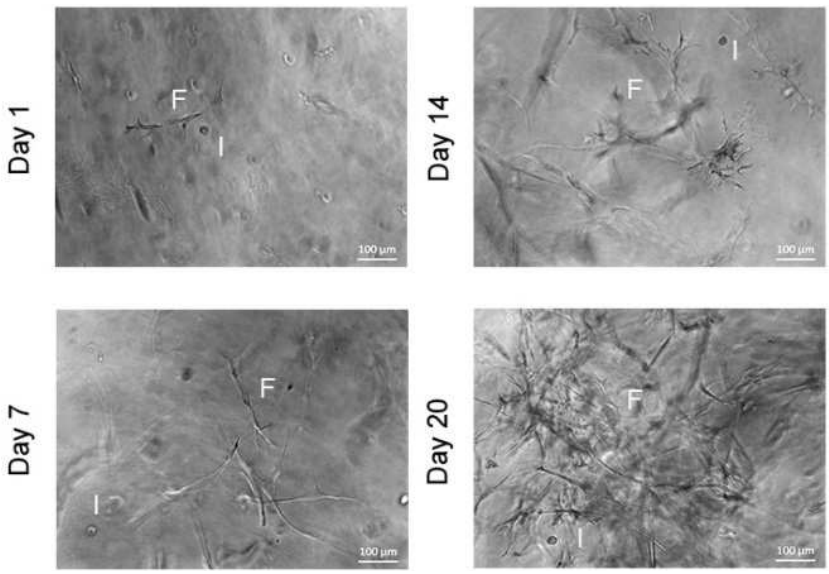

Figure 4. *Representative photomicrographs of vital stromal cells in dextran hydrogels at different time points. Fibroblasts (F), U937 macrophages and HL-60 neutrophils (I - inflammatory cells) were cultivated in CD-Link (2.5 mmol/l SH groups) cross-linked dextran hydrogels with immobilized adhesion peptide RGD (0.5 mmol/l) and a tumor cell compartment on top for 21 days. Cell viability was demonstrated microscopically. All cell types were vital until day 21. At later time points during culture fibroblasts formed a branched network (arrow day 20). Bar: 100 µm.*

In a final step the established stromal cell compartment i.e. the dextran hydrogel containing fibroblasts, U937 macrophages and HL-60 neutrophils and the epithelial tumor compartment i.e. H838 lung cancer cells were combined using dextran hydrogels with 2.5 mmol/l (SH groups) and 0.5 mmol/l immobilized RGD peptide and a second thin hydrogel layer containing 4 mmol/l RGD peptide on top. In this combined approach, the stromal cells survived 21 days (Figure 4) i.e. considerably longer than in the cultures without tumor cells (see above). The fibroblasts generated branched networks after a longer cultivation period (Figure 4 day 20). In addition, the combination of stroma and tumor compartment also improved the growth behavior of the H838 cells, which formed a confluent monolayer at 5 days (data now shown) i.e. significantly faster than, when the epithelial tumor cells were cultivated alone.

To improve the microscopic observation of the tumor cells on top of the gel the overall hydrogel volume was reduced to half. RGD peptide was again added on top in a second thin gel at a concentration of 4 mmol/l. The cell count of the stromal cells was proportionally adapted to the reduced volume. The modifications were tested with 30.000 HaCaT-ras tumor keratinocytes and MCF-7 breast carcinoma cells seeded on the hydrogels, containing stromal cells. In these combined cultures even MCF-7 cells (Figure 5) and HaCaT-ras tumor keratinocytes formed a confluent tumor cell monolayer on top of the hydrogel containing fibroblasts and inflammatory cells (Figures 5, 6, 7).

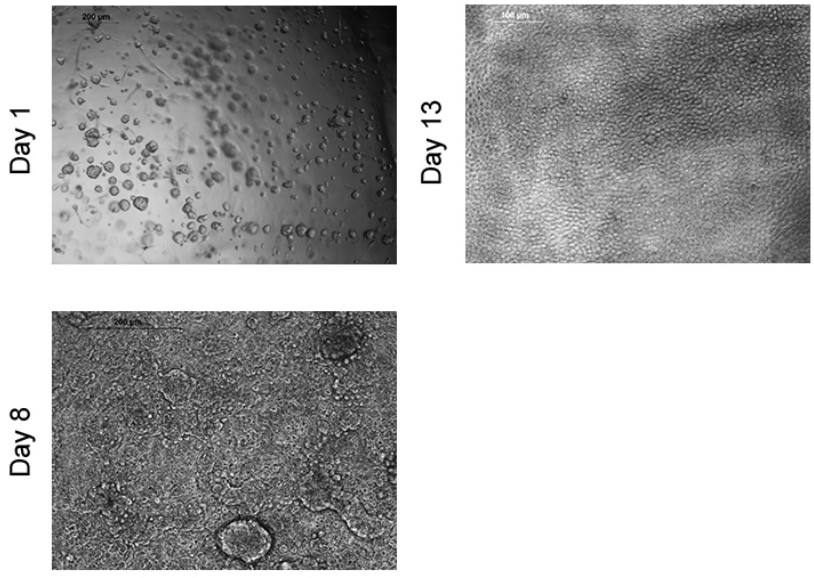

Figure 5. *Representative photomicrographs of vital tumor cells on dextran hydrogels containing fibroblasts, U937 macrophages and HL-60 neutrophils at different time points. MCF-7 breast carcinoma cells initially grow in clusters but subsequently expand into a confluent monolayer after 8 days. Bar: 200 µm.*

The hydrogel used so far exhibited a very short polymerization time and was not suitable for use in a semiautomatic setting or a potential 3D printing process as would be desirable for pharmaceutical tests. Therefore a slower polymerizing SG-dextran was tested in the 3D tumor-stroma-cultures. The chemical reaction albeit slower is still a Michaelis Menten addition during which the thiol groups establish stable thioether bonds with thiol-reactive groups. The properties of the new slower gelling hydrogel were otherwise adapted to those of the fast polymerizing gel.

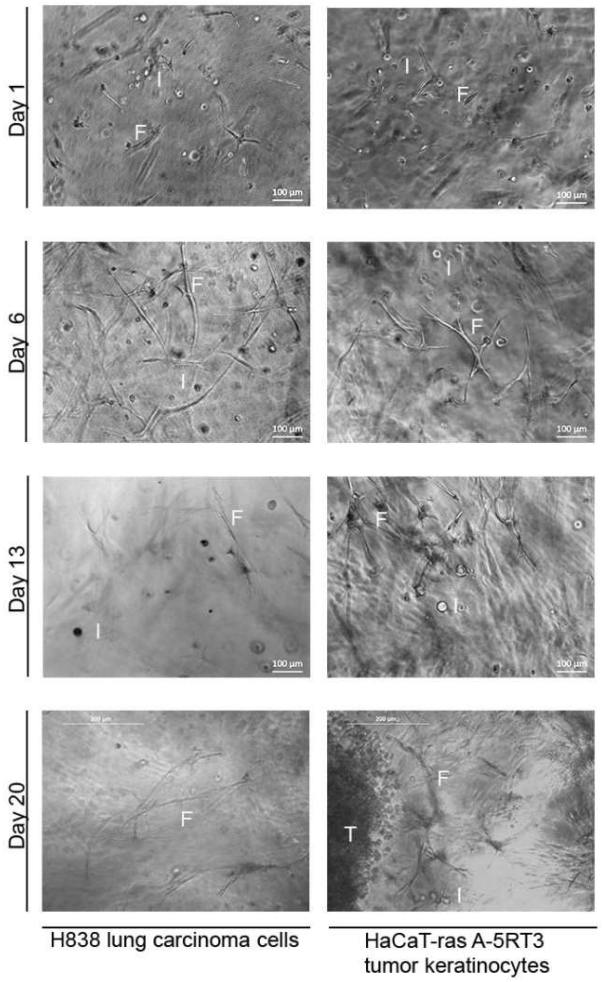

Figure 6. *Representative photomicrographs of vital stromal cells in slow polymerizing dextran hydrogels at different time points. Fibroblasts, U937 macrophages and HL-60 neutrophils were cultivated together in dextran hydrogels with immobilized adhesion peptide RGD (0.5 mmol/l) and either H838 lung carcinoma cells or A-5RT3 tumor keratinocytes on top for 20 days. Cell vitality was demonstrated microscopically. All cell types were vital until day 20 in the two independent experiments with H838 or A-5RT3 cells respectively. T- tumor epithelium. Bar: 100 µm.*

Using this more slowly polymerizing dextran polymer the stromal equivalent was prepared with fibroblasts, HL-60 neutrophils (5000 cells/gel each and U937 macrophages (6500 cells/gel) in a SG-Dextran hydrogel with a 2.5 mmol/l (SH-groups) and 0.5 mmol/l immobilized RGD-peptide (polymerization time was now 1 h). For the epithelial equivalent 30.000 H838 cells per gel were seeded on an additional thin gel layer containing RGD peptide (4 mmol/l). Cell growth was observed for 21 days. Stromal cells survived again for 21 days with fibroblasts generating extended networks after a longer cultivation period (Figure 6 day 6, 13, 20). H838 lung carcinoma cells formed a confluent cell layer after 6 days while HaCaT-ras tumor keratinocytes initially showed irregular growth in these cultures but subsequently also formed a confluent monolayer (Figure 7).

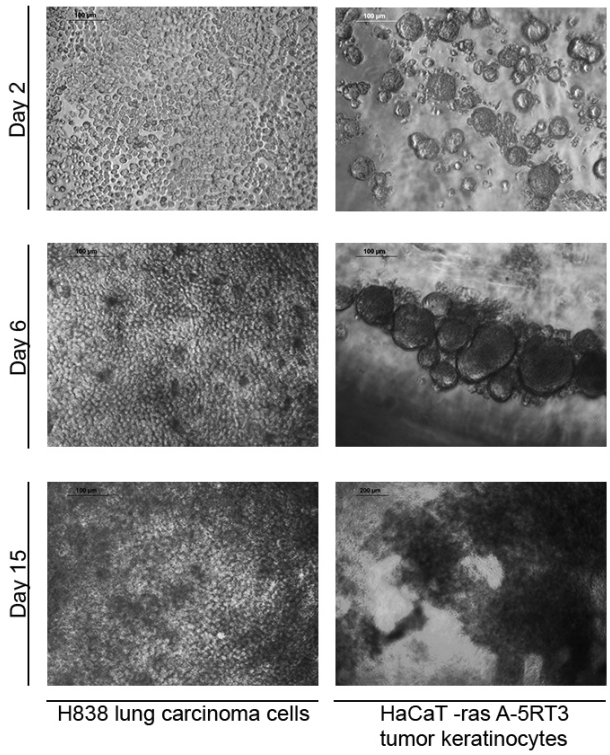

Figure 7. *Representative photomicrographs of vital H838 lung carcinoma cells as well as A-5RT3 HaCaT-ras tumor keratinocytes on dextran hydrogels containing stromal fibroblasts, U937 macrophages and HL-60 neutrophils at different time points. H838 cells show a confluent monolayer at day 6, A-5RT3 cells initially exhibit a patchy growth patter but expand into a monolayer after about 6 days and subsequently grow into a multilayer epithelium.*

Taken together the data shown here substantiate the establishment of an *in vivo* like 3D tumor-stroma model that allows the analysis of complex interactions between different stromal cell types and the tumor compartment and demonstrate the successful adaptation of the model to a standardized dextran-hydrogel. This provides a valuable tool to analyze the efficacy of novel therapeutics in an easy to manipulate yet still highly standardized 3D- tumor-stroma interaction model.

4. Discussion

4.1. Establishment of an Organotypic Tumor Stroma Culture Including Neutrophils

In light of the now generally accepted fact that 3D tissue organization crucially influences tissue function 3D cell culture models have gained increasing importance in tumor

research in academia and pharmaceutical companies, making 3D systems of human cells potential new high-throughput systems for chemical genomics and pharmaceutical screenings [24, 25, 37].

Based on extensive data from human tumors and animal experiments [21, 22, 38, 73] an organotypic cultivation model based on the natural matrix collagen type I incorporating stromal fibroblasts as well as human macrophages as inflammatory cells was developed that allowed a spontaneous *in vivo* like differentiation of M2 invasion supporting macrophages. This M2 differentiation could be inhibited by treatment with anti-IL4 antibodies [23] thereby providing a first *in vitro* 3D model to mimic at least in part a functional inflammatory compartment. To achieve another important step towards the adaptation of this 3D organotypic culture model to the *in vivo* situation, the model harboring fibroblasts and macrophages in the stromal compartment was extended to additionally include (HL-60) neutrophils, the second important cell type of the innate immune system in tumor development and progression [39, 6, 40, 32].

Inflammatory cells and their myeloid precursors in spleen, bone marrow and blood play an important and regulatory role during angiogenesis and tumor progression [39, 6]. Immunohistochemical-based studies in various human tumors revealed an increased number of neutrophils compared to healthy tissue e.g. an increased level of neutrophils was observed in biopsies of adenocarcinomas of the colon compared to surrounding healthy tissue. In these biopsies, neutrophils were found throughout the tumor, but their number was dramatically increased in the invasive and ulcerated areas [41]. Increased neutrophil numbers that correlated with a significantly worse prognosis were also observed in bronchioloalveolar lavages and in biopsies of patients with bronchioloalveolar carcinomas compared to the control groups [42]. Similar observations were reported in patients with myofibrosarcoma, gastric carcinoma and melanoma [43-45]. A functional connection between neutrophils, induction of angiogenesis, tumor growth and invasion was demonstrated in the HaCaT heterotransplantation model for human squamous cell carcinomas of the skin. In this model persistent recruitment of neutrophils into the tumor stroma was required for sustained angiogenesis, which in turn was a prerequisite for malignant tumor growth [21, 6, 22]. Further confirmation of a functional contribution of neutrophils to tumor progression came from studies on the activation of neutrophils by lung cancer cells and their subsequent promotion of tumor progression [46].

While neutrophils can clearly promote tumor progression, their role in tumors may be ambivalent as discussed by Piccard et al. 2012. Similar to macrophages, neutrophils can assume different activation or differentiation states depending on the tumor microenvironment [31]. Neutrophils act tumor-inhibiting by inducing cytotoxicity, mediating tumor rejection and inducing immunological memory against tumor cells (N1 phenotype). In contrast, neutrophils can also support tumor progression by promoting angiogenesis, invasion and metastasis and by promoting immunosuppression (N2 phenotype) [31, 32]. The cytokines TGF-ß [33] and IFN-ß [34] influence polarization to the N2 tumor-promoting or the N1 tumor-suppressing phenotype, respectively [31]. Due to their ambivalent role in tumor progression a better understanding of their functional contribution to either tumor growth or the antitumor response is important for the development of novel therapeutic strategies [39]. Consequently, the inclusion of neutrophils in a 3D *in vitro* system provides a crucial means for the testing of therapeutic candidates and the relevance of these tests for the situation in the patient.

In agreement with the above described *in vivo* observations concerning a tumor-promoting role of neutrophils, inclusion of fibroblasts, macrophages and neutrophils in the stromal compartment of the 3D organotypic tumor-stroma culture resulted in increased growth of the tumor epithelium and enhanced infiltration of tumor cells into the stromal neighborhood mimicked by the collagen gel when compared to cultures lacking neutrophils.

In the context of these findings, the organotypic co-cultures were also examined regarding neutrophil differentiation towards a N1 or N2 phenotype. While the total number of HL-60 neutrophils remained constant throughout the 3 week observation period, cultures containing all three stromal cell types frequently revealed an accumulation of neutrophil granulocytes close to the tumor epithelium or in the invasive tumor areas. After 2 weeks of cultivation neutrophils in these cultures were even found in the epithelial tissue itself. Importantly, a shift towards a N2 differentiation of the neutrophils was observed after 2 weeks of cultivation exclusively in the 3D tumor stroma cultures containing all three stromal cell types**.** These findings coincide with previous observation in animal transplantation experiments of different epithelial tumor types where neutrophils were recruited to the tumor epithelium within the first two weeks after transplantation and subsequently promoted angiogenesis and tumor invasion [21, 47]. This suggests that the established 3D collagen based model described here is a valid *in vitro* model mimicking the tumor promoting mechanisms of tumor stroma interaction *in vivo*.

4.2. Adaption of the 3D Tumor Stroma Model to a Chemically Inert Synthetic Matrix

To successfully imitate the *in vivo* microenvironment in an *in vitro* culture setting, strength and molecular composition of the scaffold materials need to be considered carefully. Collagen, hyaluronic acid and matrigel were the first native matrices used in the establishment of 3D *in vitro* cultures in tumor research. However, these matrices have various disadvantages, such as fixed pore size, non-modifiable structure or even e.g. in the case of Matrigel a non-physiological growth factor content. In addition these native scaffolds exhibit a considerable batch to batch variability which results in difficulties in standardization and impairs the reproducibility of the results [48]. Thus, these systems are not suitable for efficacy tests of therapeutics in pharmaceutical industry. Consequently in many cases two-dimensional cell culture systems are preferred, although

the results have a lower transferability to actual *in vivo* conditions in the patient [10, 49]. This is where the importance of biomimetic hydrogels of synthetic origin comes into play. These synthetically produced scaffold materials allow a standardized and highly reproducible composition and experimental set-up. Their mechanical and chemical properties can be designed individually. Factors such as pore size, degree of cross-linking and association strength can be changed flexibly according to individual requirements. The major disadvantage of these synthetic carrier materials is however, that they do not contain any signal motifs that can be recognized by cells as target sequences for proteases, adhesion receptors, etc. To circumvent this shortcoming, the carrier materials can be functionalized with integrin binding sites and interfaces for proteolytic enzymes [15, 48].

Accordingly, synthetic hydrogels have been developed with cleavage sequences that allow degradation by active matrix metalloproteinases [50] and with modifications by proteins or peptides that allow cells to spread and migrate and to form cell-cell contacts to fibroblasts, smooth muscle cells and mesenchymal stem cells. Many hydrogels are based on polyethylen glycol (PEG). These gels have a low strength and are thus difficult to handle. Dextran, a degradable polysaccharide offers a viable alternative to PEG [51]. The dextran polysaccharide consists mainly of a linear chain of α-1, 6 linked D-glucopyranose. Dextran hydrogels, in contrast to PEG, do not offer any protein adsorption and cell adhesion sites. However, dextran offers three hydroxyl groups per glucopyranose unit each of which can be used for chemical modifications. e.g. with crosslinkers or biologically active molecules [50] such as MMP cleavage sites [50] and RGD binding sites, to generate a physiological growth environment for different types of cells and spheroids [51]. In addition and differently from PEG hydrogels, dextran-based gels can be degraded enzymatically by dextranase [52]. This degradation ultimately allows the recovery of the embedded cells from the *in vitro* cultures for further analysis.

The use of a dextran-based hydrogel functionalized with protease cleavage sites and RGD binding motifs in this study offers additional advantages. While collagen based matrices strongly contract during cultivation due to the interaction of the embedded fibroblasts with the collagen fibers, the dextran-hydrogel maintains its initial size during the entire cultivation period. Due to the contraction, microscopy of the stromal cells in the collagen gel and even of the epithelial cell layer on top of the gel proves highly difficult at later stages of culture. Even, the use of ascorbic acid to reduce gel contraction, does not significantly improve the results. Here the use of the dextran hydrogel provides a clear advantage. The hydrogels retain their initial size and since they remain transparent during the whole cultivation time, they allow live-cell imaging of stromal cells in the gel. Taking advantage of these characteristics a successful adaptation of the collagen-based 3D tumor stroma model to the use of dextran based hydrogels in a 24-well format was achieved. Survival of stromal cells in the cultures was enhanced by the presence of tumor cells, and vice versa. Tumor cell growth and survival was normalized by the presence of stromal cells suggesting an *in vivo* like cross talk between both cell compartments that was accompanied by *in vivo* like differentiation pattern of the stromal cells (data shown for fibroblasts) in these hydrogels.

Taken together, the approach of a 3D tumor stroma culture using a dextran-based hydrogel in which stromal cells of the tumor microenvironment are combined with epithelial tumor cells on top of the gel is unique and has enormous potential with respect to possible modifications and its standardization as a test system in pharmaceutical industry.

4.3. Organotypical Cultivation Models in Tumor Research

3D *in vitro* models offer a unique perspective to explore the behavior of different cells and the development of a (tumor-) tissue in an organ context [24, 53]. Organotypic cultivation models imitate the complexity of biological tissues *in vitro* [54] and are of great interest e.g. as models which reproduce the typical multi-layered structure of the skin and show characteristic pathological properties of the epidermis [55, 56]. While 3D skin models are nowadays considered state of the art, the most commonly used *in vitro* models in the search of tumor drugs are still cytotoxicity and clonogenicity assays. These 2D assays are often performed with established cell lines and certainly have advantages with respect to their suitability for high-throughput screening [57]. However, their results never take the context of physiological differences between monolayer cells and a 3D-tissue into account. Indeed, in order to be able to investigate active substances within a biologically relevant 3D environment, the first organ cultures were developed in 1960 by cultivating tissue explants in culture medium [58].

Comparison of results from 2D monolayer models with any 3D model system, reveal a significant influence of the microenvironment on the experimental outcome. E.g. when tumor cells are embedded in a three-dimensional collagen matrix as small or larger cell aggregates, different cell morphology and also different cell behavior with regard to migration and invasion can be observed [24, 59, 60]. This is in part due to gradients in the supply of oxygen and nutrients as well as of degradation products along the volume of the 3D cell aggregates [61]. Another important and decisive property of cells *in vivo* that is nicely mimicked in 3D models is cell polarity, which depends on both the cell type and the microenvironment. In epithelial cells the apical versus basal polarization is crucial for tissue organization and targeted secretion of molecules. While the basal cell pole is located on a thin basement membrane consisting of different matrix proteins, the apical side of many epithelial tissues, especially in secreting organs, is organized in spherical 3D structures surrounded by a lumen to function as acini of e.g. the mammary gland, pulmonary alveoli or kidney glomeruli. This tissue organization that coincides with functional properties like the secretion of mucus or milk proteins into the lumen is lost when cells are cultivated in 2D [62, 63]. Interestingly, even fibroblasts, although they do not show this highly polarized apical-basal organization *in vivo* in 3D, exhibit different morphologies and physiologies when cultured in 2D versus 3D [60]. When fibroblasts are cultivated 2D, they acquire a forced polarity with

a dorsal and ventral side, the latter adhering to the matrix. This polarization is immediately lost upon cultivation in a mesenchymal 3D matrix [64-66]. While cell polarization and phenotype may change in embryonic development and during tumor development and progression (e.g. epithelial cells can undergo epithelial-mesenchymal transition losing their polarity and acquiring an enhanced capacity to migrate [67, 68]), the physiological characteristics of cells are nonetheless induced by the tissue context and play a crucial role in cell function and tissue physiology [60]. As a consequence 3D models are urgently needed to allow a deeper analysis of biological mechanisms, a better testing of hypotheses and the analysis of gene function before gene targeting experiments are performed in animals [53]. Clinical research, in particular, benefits from the third dimension and associated physiologically relevant models [69]. Experiments with 3D model systems have repeatedly shown the complex interactions of the composition and strength of the matrix, of integrins, growth factor receptors and signaling cascades during normal and malignant growth and development [70, 71].

5. Conclusion

The 3D tumor stroma model introduced here provides a physiological interaction of relevant cell types in the tumor microenvironment with each other and with the tumor cells. Consequently it can be considered suitable for more detailed analysis of the signaling molecules and pathways released by stromal cells and epithelial tumor cells. The adaptation of the model to degradable hydrogels allows the use of an easily modifiable and standardized scaffold material where cells are still capable of physiological proliferation, migration and differentiation in 3D [15, 72]. With this model, the mechanism of action and the tissue impact of different pharmaceuticals can be investigated in detail with respect to cell morphology, behavior, viability, as well as concerning gene expression profiles and quantitative analyses of the released proteins. As a consequence of its reproducibility and its defined properties mimicking the *in vivo* environment the 3D hydrogel tumor stroma model could therefore be of great interest for the pharmaceutical industry.

Acknowledgements

This work was supported by BMBF-funding to MM Mueller (grants MICROMET and 3D TuMiMo). The authors thank Brigitte Angres and the Cellendes GmbH for providing test reagents and for continuous technical support during the establishment of the hydrogel cultures.

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