"Recapitulating tumor micro-environment in biomaterial based-3D *in-vitro* tumor models"

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Statement of Purpose: Until recently, two dimensional (2-D) petridish culture was considered the gold standard for studying the effect of chemotherapeutic agents on tumor genesis and its progression. However, it may not accurately match the characteristics representative of tumors '*in vivo'* in terms of structural architecture, cell invasiveness and tumor micro-environmental conditions (low pH and low oxygen), thereby demonstrating a poor correlation between the 2-D culture and *in vivo* conditions (1). Recently, polymeric three-dimensional (3-D) degradable scaffolds have gained importance in the field of tumor biology due to their ability to bridge the gap between 2-D surfaces and animal models by providing a closer mimic of the tumor micro-environment in an *in-vitro* setting (2). Hence in this work, we attempt to develop an *in-vitro* lung tumor model using 3-D degradable scaffolds generated from chitosan and gelatin that act as chemical mimics of extracellular matrix components- glycosaminoglycans (GAG) and collagen respectively.

Methods: 3-D porous scaffolds were synthesized by freeze drying method at -80°C using chitosan (viscosity <200 mPa.s) and gelatin (Type A 300 Bloom) in the ratio of 1:2 (w/w) with genipin as the cross-linker (2.25% w/w of polymer) (3). These scaffolds were characterized for their morphology [Scanning Electron Microscopy (SEM)], porosity (Micro-CT), swelling kinetics, degradation behavior and compressive modulus (3). NCI-H460 cells (a non-small cell lung cancer line) were seeded on these scaffolds under static conditions for nine days and MTT assay was performed at the end of 3rd, 6th and 9th day to study cell proliferation. The responsiveness of 3-D tumor models and 2-D monolayer culture to topotecan (a cytotoxic drug) was performed at the end of 6th day of culture. NCI-H460 cells were also injected into nude mice to obtain *in vivo* tumors. The tumoroids generated by NCI-H460 cells in 3-D scaffolds were characterized and compared with 2-D cells and *in vivo* tumors by immunohistochemistry (IHC) for vimentin [epithelial to mesenchymal transition (EMT) marker]. Reverse Transcriptase- polymer chain reaction (RT-PCR) was also performed for vimentin and was normalized for beta-2 microgloblulin (β_2M), a house keeping gene.

Results: The chitosan-gelatin scaffolds generated by freeze drying method showed interconnected pores (as determined by SEM images – data not shown) with a porosity of 86.68±0.85% (as determined by Micro-CT) that is suitable for migration and nutrient availability to cells. The ability of scaffolds to support tumoroid formation was demonstrated by SEM (data not shown) and confocal microscopy (**Figure 1**). In figure 1, cell nuclei were stained using propidium iodide (red) and cell boundaries were stained by phalloidin (green). The pink color represents the scaffold that acts as a support for the cells to attach and proliferate. Further, cell viability decreased significantly with an increase in scaffold thickness from 1mm to 3mm ($P<0.05$), due to limited cellular infiltration. Hence, 1 mm scaffold thickness was chosen for all future experiments. Cell proliferation and viability increased significantly from day 3 to day 6 to day 9 ($P<0.05$) demonstrating the biocompatibility of the scaffolding system (data not shown). Further, NCI-H460 cells seeded on scaffolds and grown on 2-D surfaces were subjected to equal concentrations of topotecan (**Figure 2**). Cells seeded on 3-D scaffolds were found to be more resistant to topotecan when compared to cells on 2-D surfaces, indicating the possibility of a role being played by 3-D culture systems in modulating cell responsiveness to cytotoxic therapy (4).

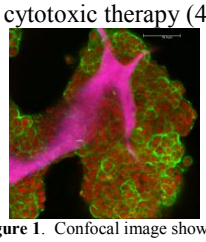

Figure 1. Confocal image showing NCI-H460 tumoroids on chitosan gelatin scaffolds at the end of day 9. (scale bar-50 microns)

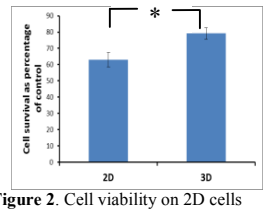

Figure 2. Cell viability on 2D cells and 3D scaffolds after topotecan treatment at the end of day 6 ($P<0.05$)

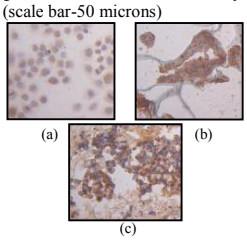

Figure 3: IHC for vimentin in NCI-H460 cell grown on (a) 2-D tissue culture polystyrene (b) 3-D scaffolds, and (c) *in vivo* tumors. (All images at 20X).

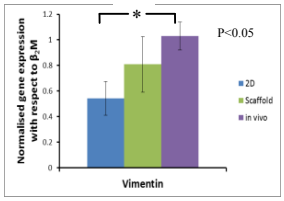

Figure 4. Relative expression of vimentin in H460 grown on 2-D, 3-D scaffold and *in vivo* with respect to β_2M

IHC for vimentin expression suggests its higher expression in cells grown on 3-D scaffolds and *in vivo* as compared to 2-D cells (**Figure 3**). This supports the fact that inherent metastatic nature of NCI-H460 cells is maintained in 3-D scaffold. The RT-PCR data for vimentin showed that there was no significant difference in gene expression pattern between 3-D scaffolds and *in vivo* tumors ($P>0.05$). However, there was a significant difference between 2-D and *in vivo* expression of vimentin ($P<0.05$) which corroborates the IHC data (**Figure 4**).

Conclusions: Chitosan-gelatin 3-D scaffolds demonstrated the ability to support NCI-H460 cell adhesion and proliferation thereby leading to the generation of tumoroids. The IHC and RT-PCR data indicated that 3-D scaffold based tumoroids closely mimic *in vivo* tumors as compared to 2-D cultures. Therefore, these chitosan-gelatin scaffolds show potential to be developed as a biomimetic model of lung tumors.

References: 1.Kim JB. *Sem Cancer Biol*;2005;15:365-377 : 2. Fischbach C. *et al. Nat Meth*. 2007;4:855-860: 3. Sardana *et al. Transactions of the Society For Biomaterials , Annual Meeting*, 2010:4.Faute *et al. Clin Exp Metastasis*;2002;19:161-168