ENHANCING GENE EXPRESSION IN NON SMALL CELL LUNG CANCER CELL LINE NCI H23 BY 3D AGGREGATE FORMATION AS EVIDENCED BY PROTEIN PROFILING

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ABSTRACT:

Background and Objectives: *Cancers are important contributors for high mortality rate globally. Cancer biomarkers are important for research and these uniquely expressed or over-expressed are useful for cancer detection, monitoring and prognosis. Cancer cell lines are invaluable for biomarker discovery. From two-dimensional cultures, cells are now being cultured in three-dimensions to mimic in vivo systems closer. Objective of this study is to optimize simple 3D culture conditions of cancer cell line NCI H23, isolate proteins in a 'three-fraction' model and compare the protein profiles of 2D and 3D cell cultures to ascertain expression changes.*

Methods: *Agarose hydrogels were optimized to obtain healthy 3D aggregates. NCI H23 cells formed floating aggregates with extracellular matrix. Cytoplasmic, membrane and nuclear protein fractions from 2D and 3D cultured NCI H23 cells were extracted and were analyzed both qualitatively (SDS PAGE) and quantitatively.*

Results: *The 3D culture conditions were optimized as 1 ml of 0.5% agarose gel. The 3D aggregates showed pronounced acellular and necrotic cores. Protein profiling by SDS PAGE showed differential expression for 2D and 3D cultures. Quantitative analysis also revealed that the ratio of the protein in the three fractions of 2D and 3D cultures were different.*

Conclusion: *Gene expressions vary in 2D and 3D cultures for the same cell type resulting in a variation of expressed proteins. This reiterates the usefulness of 3D cultures for cancer research including biomarker and drug discovery. Also, these 3D systems will throw more light on the mechanisms of carcinogenesis, metastasis and cancer cell behaviour.*

Key words: *Cancer cell line, 3D cultures, gene expression, protein biomarkers, NCI H23*

SRJM 2011;4:15-19

INTRODUCTION:

Cancer arises when a cell, for a variety of reasons, escapes from the normal constraints placed on its growth and begins to divide in an unregulated fashion.[1] Progressing tumors give rise to distant metastases, which are the cause of 90% of human cancer deaths. They are characterized by changes in Extra Cellular Matrices (ECMs) and their interactions with tumor cells.[2] Recent studies have shown that the molecules mediating adhesion are also capable of signal transduction. These adhesion proteins and motility factors involved could serve as better targets for new treatments for cancers and to prevent metastasis.

Biomarkers which are cancer-specific and capable of early detection are essential for cancer management and therapy of which the genomic and proteomic types being the primarily important ones.[3] Thus, development of new genomic and proteomic approaches towards cancer biomarker discovery can lead to the identification of novel DNA and Protein biomarkers.[4] Biomarkers have a great impact on cancer therapeutics as their role is increasingly promising, suggesting an integrated approach for treatment selection and patient management.[5] *in vitro* cell culture models are good choices for biomarker discovery owing to the limitations of other material available such as patient biopsies.[3]

While traditional monolayer cultures are powerful tools to understand how cells proliferate, grow and respond to stress, they do not recreate the property *in vivo*. Therefore, development of novel *in vitro* model which reflects the actual *in vivo* characteristics is essential.[6] Three-dimensional (3D) scaffolds reflect normal cell morphology & behavior for more realistic cell biology and function, *in vivo*-like morphology, and better intercellular interactions.[7] 3D cultures of tumor cells have been obtained by promoting the aggregation of cells in spheroids via several different methods or by using scaffolds. Extensive studies have shown that growing cells within 3D scaffolds diminishes the gap between cell cultures and physiological tissues. Therefore, a 3D cell culture system may prove to be of tremendous advantage over conventional 2D cell culture system.[8]

The objective of this study is to ascertain if the 3D culture systems can induce a differential expression of proteins when compared to the 2D systems for a same given cell line. A 'Three-Fraction' protein model obtained from both 2D and 3D cultured cells was used for the comparative analysis. Also, the 3D culture characteristics were studied and optimal culture phases as ideally suited for obtaining a particularly localized protein fraction (cytoplasmic, membrane bound and nuclear) were identified.

Materials and Methods

The cell line NCI H23 obtained from National Center for Cell Sciences, Pune, India was cultured as traditional monolayer attachment cultures in RPMI 1640 medium

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supplemented with 10% Fetal Bovine Serum (FBS). The cell line required 1 to 2 medium changes before attaining confluency in a T-25 culture flask. Passaging, harvesting by trypsinization, cell count along with viability checks were performed in accordance to standard procedures. A freshly thawed stock was cultured for 3 passages and the harvested cells of the third passage were used for 3D cultures. Agarose hydrogels prepared in serum free medium were used as the matrix to obtain 3D aggregates of the cell line. 0.5% of agarose hydrogel was prepared in serum free RPMI 1640 medium by melting at 80ºC. 6 well plates with 1ml of the agarose hydrogels, sterilized by UV exposure inside a laminar air flow cabinet were used. The UV exposure for 45 minutes, apart from ensuring sterility of the matrix also is useful for the polymerization and 'setting' of the low melting agarose used for the gels. As each cell line has a unique optimal 3D culture condition requirements, we have optimized the parameters for the cell line chosen. The various parameters included the concentration of Agarose (0.25%, 0.5% and 0.75%) and the volumes (1ml, 750μl, 500μl and 250μl) as suitable for a 6 well plate. The cells harvested by trypsinization of a monolayer were cultured as 3D aggregates in the optimized conditions. NCI H23 cell line forms floating aggregates in the culture medium above the hydrogels and were harvested by aspirating the culture medium. The floating aggregates harvested were tested for cell counts and viability. Healthy 3D aggregates thus harvested were washed thrice in plain medium and were used for protein fractionation as a 'Three-Fraction' Model. The cytoplasmic, membrane bound and nuclear proteins were extracted as distinct fractions using the Bio-Rad Readyprep Sequential Extraction Kit. Essentially, the kit contains three protein extraction reagents for step wise sequential extraction of cytoplasmic, membrane bound and nuclear proteins from cells and tissues. The Reagent 1 contains 40 mM Tris base, the Reagent 2 contains 10 ml of 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, and 0.2% (w/v) Bio-Lyte 3/10 ampholyte and the Reagent 3 contains 10 ml of 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB 3-10, 40 mM Tris, and 0.2% (w/v) Bio-Lyte 3/10 ampholyte. 200 mM tributyl phosphine (TBP) in 1-methyl-2-pyrrolidinone (NMP) sealed under nitrogen gas was used as the reducing agent along with reagents 2 and 3.

The cell pellet obtained from the harvested 2D monolayers and the 3D aggregates were re-suspended in 400μl of Bio-Rad ready Prep Protein Extraction Reagent 1 and centrifuged at 4000 rpm for 10 minutes. The supernatant containing the protein fraction I (cytoplasmic proteins) was transferred to a 1.8 ml Eppendorf tube, labeled as supernatant 1 and stored at -20^{0}C. The pellet obtained after the first fractionation step was re-suspended in 200μl of Ready Prep Protein Extraction Reagent2, vortexed and was centrifuged at 1000rpm for 10 minutes at 25^{0}C. The supernatant containing the protein fraction II (membrane-bound proteins) of intermediate solubility was collected and stored at -20ºC. The pellet obtained from the extraction 2 was re-suspended in 100μl of Ready Prep Protein Extraction Reagent 3 and vortexed. The solution containing protein fraction III (nuclear proteins) were also stored at -20^{0}C. The three protein fractions were quantified by using a modified Bradford method. Serially diluted bovine gamaglobulin of concentration 1.5mg/ml was used as the standards. The protein estimation was done in 96 well flat bottom plates in triplicates for each sample; the spectrophotometric absorbance measured at 595 nm (A_{595}). The three fractions were also qualitatively analyzed by SDS PAGE. The reagents for the SDS PAGE included 30% Acrylamide mixture, Lower Tris (pH 8.8), Upper Tris (pH 6.8), 10% APS and 10% SDS. The running buffer was composed of 1.875 g of Tris, 9 g of glycine, 0.625 g of SDS dissolved in 300ml of dd. H_20. The pH was set to 8.3 and the final volume made upto 500ml. The sample buffer was composed of 2.1 ml of 1.5M Tris HCl (pH.6.8), 1ml of 20% SDS, 0.5 ml 0f 100% glycerol, 0.5 ml of mercaptoethanol. 2.5 mg of bromophenol blue was added to 0.4ml of dd. H_20. The Staining solution was prepared from 2 g of CBB added to 500 ml of alcohol to which 70 ml of acetic acid was added. The final volume of the solution was adjusted to 1000 ml with dd. H_20. The Destaining solution was 35 ml acetic acid in 200 ml alcohol with the final volume made upto 500 ml with dd. H_20. 30 μl of the samples were loaded to each of the wells along with the standard marker proteins for electrophoresis at 100V till the tracking dye reached the bottom of the gel. The gel was carefully removed from the mold and immersed in staining solution overnight with uniform shaking at 37ºC.

RESULTS:

Healthy confluent NCI H23 cells were obtained on Day 3 (72 hours) of seeding a T-25 flask as monolayers. In 3D conditions, small aggregates of cells were observed in the NCI H23 3D culture plates at 24 hours. On day 5 (168 hours) of incubation, a single large aggregate surrounded by small aggregates was observed. The aggregates were not embedded on the agarose and were found to be floating. The presence of extracellular matrix was found more apparent in the floating aggregates at this stage along with acellular regions within the aggregates. Upon harvest, cells in 2D culture formed a solid pellet on centrifugation whereas 3D aggregates formed large, loosely packed cell pellets. Also, the 3D aggregates were visible as floating structures within the centrifuge tubes upon harvest, even before centrifugation. For studies on the shifts in cell culture phase durations the cells in 2D and 3D were harvested every 24 hours. Cell count was performed and the viability was tested using trypan blue dye exclusion for both 2D and 3D cells. The total cell count for 2D and 3D cultures were found to be 132 x 10^4 and 30 x 10^4 cells/ml at the end of 144 hours and the cell viability at 72 hours was 97.4% and 88% respectively. A marked shift in the time frames for the cell culture phases was observed for 2D and 3D. (Fig. 1) Of the 0.25%, 0.5% and 0.75% of agarose in volumes of 1ml, 750μl, 500μl and 250 μl, lower concentrations and lower volumes showed a

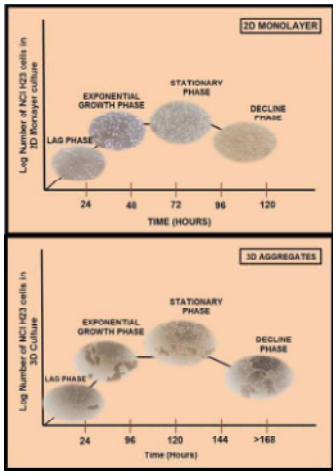

Figure 1: A marked shift was observed in the duration of culture phases for NCI H23 cell line in 2D and 3D cultures. Usually, the various cell culture phases, the lag, log (exponential), stationary and the decline phases are determined by the doubling time of a particular cell line/ type and also the nutritional requirements and carrying capacity of a particular culture system. Given the same culture area, medium and supplements, NCI H23 cells showed much longer durations for the progress through each of the culture phases when compared to the monolayers. This is attributed to the fact that the surface area available for monolayer cultures depletes rather rapidly and space available for cell proliferation diminishes faster depending on the doubling time of the cell line. However, in a 3D condition, cells do not depend on a surface as they loose the dependence on attachment to culture surfaces. This induces the aggregate formation where more cells are accommodated in the aggregates. This finally results in a 3D system supporting cell cultures for much longer duration, thus extending each of the cell culture phases in comparison to the 2D systems. When cells from each of the culture phases are harvested and cell counts taken, the 3D system provides more numbers of cell for a given culture phase and also protein expressions differ markedly as the culture phases progress among the 2D and 3D cultures.

varied growth on the hydrogel. The cells were not aggregated and found as single cells among which few were attached like a monolayer. Cells seeded on 0.25% agarose and volume 250 μl formed typical monolayer. 0.5% and 0.75% of agarose were found to support good aggregate formation. A 1 ml volume of 0.5% gel was found to be optimal for cell aggregate formations. Comparison of culture phases in 2D and 3D as measured by cell counts at 24 hour intervals for 6 days showed lengthy/extended durations in 3D system. (Table 1)

Sequential protein extraction yielded final volumes of 800 μl, 400 μl and 200 μl of CF, MF and NF respectively. Protein estimation of the fractions yielded a concentration of 2.2, 0.9, 2.9 mg/ml of CF, MF, NF in 2D and 0.8, 0.8 and 0.72 mg/ml of CF, MF,NF in 3D. The results of

Table 1. The culture feature differences of NCI H23 cell line in 2D and 3D systems showed marked variations apart from the morphology. 3D systems showed a slower proliferation rate with the presence of extracellular matrix surrounding the aggregates and acellular zones as the cultures progressed. Also, the presence of conditioned medium had a positive effect on 3D system but a similar effect was not seen in 2D systems.

Features	**2D**	**3D**
Cell proliferation	Faster	Slower
Necrotic cores	Absent	Present
Acellular regions	Absent	Present
Extra cellular matrix	Absent	Present
FBS requirement	High	Low
Conditioned medium	Not Significant	Significant
Cell count upon culture progress		
24 hours	1 x 10^4/ml	2 x 10^4 /ml
48 hours	2 x 10^4 /ml	3 x 10^4 /ml
72 hours	6 x 10^4 /ml	3.5 x 10^4 /ml
96 hours	8 x 10^4 /ml	4 x 10^4 /ml
120 hours	17 x 10^4 /ml	4.5 x 10^4 /ml
144 hours	22 x 10^4 /ml	5 x 10^4 /ml

qualitative analysis by SDS PAGE showed that higher molecular weight proteins in the range of 70-85kDa were expressed in the cytoplasmic fractions of both 2D and 3D whereas lower molecular weight proteins in the range of 34-26kDa were expressed in the 2D and 3D nuclear fractions. In the membrane fractions differential expression was observed in 2D and 3D. (Fig. 2) The lag, log and plateau phases were found best suitable for the identification and

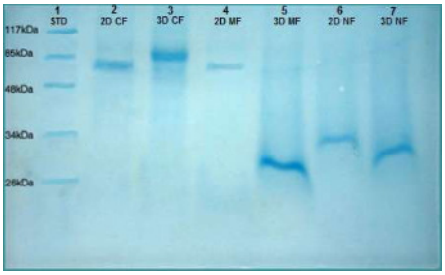

Figure 2 : Qualitative analysis of the three protein fractions extracted from 2D and 3D cultures by SDS PAGE showed similar profiles for the Cytoplasmic and Nuclear fractions. However, slight molecular weight differences were noticed with 3D cytoplasmic fractions showing slightly higher molecular weights compared to the 2D fraction. On the other hand, 3D nuclear fractions show lower molecular weight components when compared to the 2D counterparts. A strikingly different profile was observed for the membrane fractions where the 3D fraction showed a much lower molecular weight protein group at around 30 kDa when compared to the 2D fraction which was in the molecular weight rage of 60-65kDa.

characterization of the cytoplasmic, nuclear and membrane proteins respectively.

DISCUSSION:

Cancer cells behave differently as monolayers and in a 3D environment. These differences include morphological, metabolic, cell communication, signaling and gene expressions. Thus, cells in a 3D environment are good models as '*near-to-in vivo*' systems and give us useful insights from a variety of ways.[8] They can serve as a cost effective screening platform for drug development & testing and improve the predictive value of cell based assays for safety and risk assessment studies. Cell lysates from lung cancer cell lines and lung cancer animal models can be used for lung cancer biomarker discovery. But 2D culture model do not fully represent the complex circumstances of lung cancers. Therefore, use of cell lysates from well established 3D aggregates can overcome this limitation and facilitate the identification of potential new protein biomarkers. This could be fulfilled by use of natural and synthetic hydrogels which provides essential insights into nearly all aspects of cell behavior, including cell adhesion, migration, and differentiated function. Agarose hydrogels are convenient for obtaining 3D aggregates and optimizing conditions is essential for a given cell type.[9] 3D scaffold used for aggregate formation can be optimized with respect to a variety of parameters. We have shown that decreased gel concentration of 0.25% and decreased volume do not support aggregate formation and results in a monolayer. Agarose concentration of 0.5% and volume of 1 ml was found to be ideal for good aggregate formation. Thus, optimal parameters on standardization, would present us with a simple tool and technique to obtain the required 3D aggregates.

As lung cancer cell lines are very useful for cancer research,[10] it makes them all the more important as 3D aggregate. The 3D structure of cells and interactions with their neighbors significantly influences their ability to grow and function. A marked shift in the time frames for the cell culture phases was observed. An extended lag to log phase duration was observed for the 3D aggregates. Cells in 3D culture generally undergo a slower proliferation. Also 3D cultures stay healthy for a longer period of time (more than 6 days) whereas cells in 2D culture reach decline phase in 72 hours. For the same seeding density, the number of confluent aggregates in 3D culture is lower than the number of cells in 2D culture.

Adding three-dimension to a cell's environment creates significant differences in cellular characteristics and behavior. Therefore, development of bioimetic scaffolds which emulate the natural environment of their native extracellular matrix, ultimately provide a better understanding of lung biology. 3D aggregates of NCI H23 expressed more extracellular matrix around them and also pronounced acellular zones within the aggregates, probably filled with the same extra cellular matrix. The buoyancy of the NCI H23 cell aggregates making them to float might be due to the extracellular matrix around the aggregates and also the core acellular regions. It also showed dark necrotic regions on the surface which reflects the tumor morphology.

Protein estimation was performed using Modified Bio-Rad protein assay using the bovine gamma globulin as standard. The protein concentration of the cytoplasmic, membrane and nuclear protein fraction was much higher for 2D than in 3D. This might be due to the higher number of cells in 2D compared to 3D culture. Also in 2D culture, the ratios of the three fractions followed the order NF > CF > MF whereas 3D cultures showed an order of CF > MF > NF (Fig. 3). These differences in the expression of the three protein fraction ratios indicate that 3D cultures are more suitable towards protein biomarker discovery.

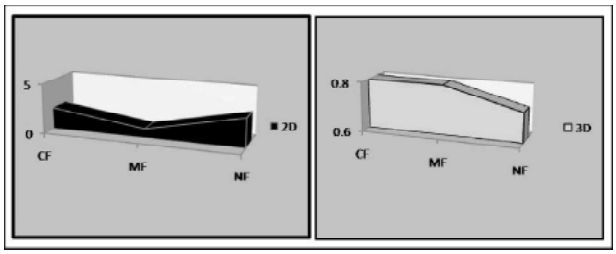

Figure 3: The ratios of the three protein fractions (the cytoplasmic, membrane and nuclear) were different in 2D and 3D systems. While the 2D system showed NF > CF > MF order, the 3D fractions showed CF > MF > NF. This indicated differential protein expressions in the 2D and 3D culture systems.

SDS PAGE results have again proven the differential expression of proteins in 2D and 3D culture. In both 2D and 3D cultures, the cytoplasmic protein fractions expressed high molecular weight proteins. In 3D cytoplasmic fractions, certain proteins which were not expressed in 2D were found to be expressed. The representative bands were faint of molecular weight in the range of 50-60 kDa. These proteins might constitute the matrix proteins of the ECM which was a unique characteristic observed in 3D cultures. Membrane protein fractions of 2D and 3D showed a striking difference in the expression pattern. In 2D high molecular weight proteins in the range of 60-65 kDa were expressed whereas in 3D low molecular weight proteins of molecular weight 30 kDa were expressed. The expression pattern of nuclear fractions was almost similar in 2D and 3D. In both the fractions, low molecular weight proteins in the range of 30kDa were abundant indicating active cell proliferation. Because previous studies describe that nuclear proteins responsible for cell proliferation are of low molecular weight. Thus it is very clear that proteins are differentially expressed when cells are provided a three dimensional environment. Therefore more protein profiling studies with 3D cultures can lead to the identification of new potential biomarkers.

Monoclonal antibodies raised against these novel proteins can have potential application in imaging techniques used for cancer diagnosis and also in targeted cancer therapy. Since high molecular weight proteins are highly accumulated in cytoplasmic fractions, raising antibodies through *in vivo* immunizations will be optimal.[11] Nuclear fractions can be a good choice for *in vitro* immunization as they have abundant low molecular weight proteins.[12][13] Membrane fractions which express high molecular weight proteins in 2D culture and low molecular weight proteins in 3D culture will be ideal for *in vivo* immunizations with *in vitro* stimulation which is a new strategy.[14] Further development through better proteomic tools, including more sensitive mass spectrometry analysis, is required for the identification of specific proteins that are differentially expressed in 2D and 3D cultures which has great potentials for the discovery of new biomarkers.

CONCLUSION:

Biomarkers have contributed tremendously in cancer research, diagnostics and therapy. Cancer cell lines are contributing important insights into the cancer mechanisms, drug discovery and biomarker identification. 3D cell cultures serve as efficient *in vitro* models for cancer research which include novel biomarker discovery owing to differential gene expressions when compared to 2D culture systems.

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