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EFFECT OF L-929 DERIVED SECRETOME ON HUH-7 HEPATIC CANCER CELL- ON CELLULAR PROLIFERATION AND OXIDATIVE STRESS

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ABSTRACT

The tumor microenvironment (TME) plays a crucial role in cancer progression, where stromal components such as fibroblasts actively influence tumor cell behavior. This study investigates the effects of the secretome derived from L-929 fibroblasts on HuH-7 hepatic cancer cells on cellular proliferation and oxidative stress. The interaction between fibroblast-derived factors and hepatic carcinoma cells has not been extensively explored. Through in vitro assays, including MTT and ROS analysis, it was found that L-929 fibroblast secretome decreased the HuH-7 proliferation and also induced oxidative stress in HUH-7 cells. The findings contribute to a deeper understanding of tumor-stromal interactions and potential therapeutic implications for hepatocellular carcinoma (HCC).

Keywords: L-929 Fibroblasts, Huh-7 Cells, Secretome, Tumor Microenvironment, Hepatocellular Carcinoma, Cellular Proliferation, Migration, Oxidative Stress

1. INTRODUCTION

Cancer is characterized not only by the presence of malignant cells but also by a complex ecosystem comprising stromal components such as fibroblasts, inflammatory cells, and blood vessels, all embedded within an extracellular matrix (ECM). These non-malignant stromal cells, including cancer-associated fibroblasts (CAFs), actively participate in the communication network within the tumor microenvironment (TME) (Hanahan, D. & Weinberg, R. A., 2011).

Hepatic cancer or liver cancer is cancer that occurs in the liver. The liver is the largest internal organ. It performs several critical functions to help the body eliminate waste, absorb nutrients, and heal wounds. Among primary liver cancers, hepatocellular carcinoma (HCC) is the major histological subtype and accounts for 70–85% of total liver cancer cases. However, liver cancer is often difficult to treat surgically because many cases are diagnosed at an advanced stage, even at the time of initial diagnosis. Even after surgical treatment, liver cancer recurs frequently and metastasizes. The development of cancer recurrence, metastasis, and chemo- and radio resistance in a solid tumor is attributed to the presence of cancer stem cells (CSCs).

Recent research highlights the active role of stromal components in cancer progression. Stromal cells, particularly CAFs, contribute significantly to tumor growth and invasion by secreting cytokines, growth factors, and ECM proteins. These factors collectively create a supportive microenvironment that enhances the survival, proliferation, and metastatic potential of cancer cells (Kalluri, R., 2016).

L-929 cells, derived from murine connective tissue, have been pivotal in biomedical research since their establishment in the 1940s. These fibroblast-like cells are characterized by their spindle-shaped morphology and adherent growth pattern (Jones, E. et al., 2020). The average diameter of L929 cells is approximately 15-20 micrometres (Smith, M. L. and Forrester, L. M., 2018). L-929 cells are robust in culture, making them ideal for studying cytotoxicity and assessing the biocompatibility of various materials. They are extensively used in fields such as biomaterials and tissue engineering due to their reliability and versatility in experimental models (Coecke, S. et al., 2005). L929 cells are widely used in cytotoxicity assays due to their sensitivity to various compounds. They are a standard cell line for testing the biocompatibility of medical devices and materials (Smith, M. L. and Forrester, L. M., 2018). The L929/A variant, which is resistant to Adriamycin (doxorubicin), is used to study mechanisms of drug resistance and to develop novel anti-cancer treatments. Resistance can be modulated by agents such as verapamil and quinine (Wong, R. S. and Muschel, R. J., 2017).

HuH-7 cells, originating from hepatocellular carcinoma (HCC), have been a cornerstone in liver cancer research since their isolation in 1982. These cells are notable for their susceptibility to hepatitis C virus (HCV) and are widely used as models to study both hepatoma and viral infections. HuH-7 cells are typically grown as 2D monolayers and have specific growth requirements that support their use in studying drug effects and metabolic processes in liver cancer (Nakayama, K. H., et al., 2010). In culture, HuH-7 cells are typically maintained as adherent monolayers in growth media renewed periodically to support their metabolic needs and growth rates (Blight, K. J. et al., 2000).

The Secretome, defined as the complete set of proteins secreted by cells into the extracellular space, plays a critical role in cellular communication and signaling. Originally coined in 2004, the term "Secretome" was refined in 2010 to specifically encompass proteins that are actively secreted. In human biology, the Secretome comprises a significant portion of the proteome, influencing processes such as cell migration, signaling pathways, and interactions within the TME (Tjalsma, H. et al., 2000).

In liver cancer, especially hepatocellular carcinoma (HCC), CAFs are pivotal components of the tumor stroma. These cells can arise from various sources, including resident tissue fibroblasts, hepatic stellate cells (HSCs), and mesenchymal stem cells (MSCs). CAFs interact closely with tumor cells, immune cells, and endothelial cells within the TME, influencing tumor progression through direct cell-to-cell contact and paracrine signaling mechanisms. Their secretion of cytokines, growth factors, and extracellular vesicles support tumor growth, invasion, and resistance to therapies, highlighting their critical role in disease progression (Kalluri R., 2016). Studying the interaction between L-929 fibroblast cell Secretome and HuH-7 hepatocellular carcinoma cell offers insights into the complex dynamics within the tumor microenvironment. The secreted factors from L-929 cells, including cytokines, growth factors, and ECM proteins, may influence the behaviour of HuH-7 cells, impacting their growth, metastatic potential, and response to therapies. Understanding the interplay between stromal components, such as L-929 fibroblast cell's secretome and HuH-7 hepatocellular carcinoma cells, provides valuable insights into cancer biology and therapeutic strategies. These studies not only enhance our knowledge of tumor-stromal interactions but also pave the way for developing targeted therapies that disrupt the supportive microenvironment crucial for cancer progression.

Since the effect of L-929 fibroblast cells Secretome on HuH-7 cells has not been studied yet hence this study would provide an insight in understanding the interplay between L-929 cell Secretome and HuH-7 cells.

2. MATERIALS AND METHODS

All the chemicals used in the study were of tissue culture and molecular grade. Dulbecco's Phosphate Buffer Saline (DPBS) (TS1006), Penicillin-Streptomycin (Pen-strep) (A001-5X), Fetal bovine serum (FBS) (RM10832), Trypsin-EDTA (TCL007), MTT Reagent (RM1131), and Dulbecco's Modified Eagle's Medium (DMEM) (AL007A), Trypan blue dye (TCL005), were purchased from HI Media laboratories, Mumbai.

2.1. CELL CULTURE AND MAINTENANCE

L-929 fibroblast and HuH-7 hepatic cancer cells were procured from the National Centre for Cell Science, Pune, India, and maintained as per the supplier's protocol. Both cell lines were cultured in Full Growth Medium (FGM) consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, and 2 mM L-glutamine. Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. Culture media were replaced every 48 hours, and cells were passaged upon reaching 70-80% confluency using trypsin-EDTA.

2.2. SECRETOME COLLECTION

To prepare conditioned media, L-929 fibroblasts cells were seeded into T-75 flasks and grown until they reached 80% confluency. Cells were then washed twice with DPBS and incubated with serum-free DMEM for 48 hours. Subsequently the medium (known as conditioned media) in which cells were grown was collected, centrifuged at 1,500 rpm for 5 minutes to remove debris, and is called the Secretome. It was then filtered through a 0.22-µm filter. The filtered secretome was aliquoted and stored at -80°C for further use.

2.3. TRYPAN BLUE EXCLUSION ASSAY

Cell viability was assessed using the Trypan Blue exclusion assay. Huh-7 cells were plated in a T-25 flask in FGM and were allowed to grow in tissue culture incubator until 80 – 90 % confluency was achieved. Subsequently, these cells were trypsinized and 5×10^4 HuH-7 cells/ml per well in 1ml FGM were seeded in 24-well plates and allowed to adhere overnight. Cells were then washed with 1ml DPBS twice and then treated with FGM (1ml) containing L-929 derived conditioned media/filtered secretome with doses 5 µL, 10 µL, 20 µL, 40µL, 80µL and 160 µL for 24 hours while the control cells remained untreated. The plate was observed after 24 hours. Media was discarded followed by washing with 1ml DPBS and then cells were trypsinized and centrifuged at 4000rpm for 5mins. The pellet was resuspended in 1ml FGM. 10µl of this cell suspension was taken and added to 10µl 0.4% Trypan blue dye solution and mixed gently. After a 2-minute incubation at room temperature, 10 µL of this mixture was loaded onto a hemocytometer. Live (unstained) and dead (blue-stained) cells were counted under a light microscope. The percentage of viable cells was calculated using the formula:

Cell Viability (%) = (Number of unstained (viable) cells) / Total number of cells ×100

2.4. MTT ASSAY FOR CELL VIABILITY

The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the proliferation of cells in response to secretome treatment. Briefly, 5×10^4 HuH-7 cells/200µl per well in 200 µl FGM were seeded in 96-well plates and allowed to adhere overnight. Cells were then washed with DPBS (100 µL) twice followed by treatment with 200 µl FGM containing L-929 derived conditioned media/filtered secretome at doses 5 µL, 10 µL, 20 µL, 40µL, 80µL and 160 µL for 24 hours. The control cells remained untreated. After treatment, 10 µL of MTT reagent (5 mg/mL) was added to each well and incubated for 4 hours. Formazan crystals formed were dissolved in 100 µL dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader.

2.5. REACTIVE OXYGEN SPECIES (ROS) ASSAY

The intracellular ROS levels were measured using the dichlorofluorescein diacetate (DCFH-DA) assay. HuH-7 cells were seeded in black-bottom 96-well plates at a density of 5×10^4 cells/200µL per well and cells were then washed with DPBS (100 µL) twice and subsequently treated with FGM (200 µL) containing L-929 derived conditioned media at doses 5 µL, 10 µL, 20 µL, 40µL, 80µL and 160 µL for 24 hours. Control cells remained untreated. After treatment, cells were washed twice with DPBS (100 µL) and incubated with 10 µM DCFH-DA (100 µL) for 30 minutes at 37°C in the dark. Reactive oxygen species (ROS) levels were assessed using a spectrophotometer at 495 nm, measuring the absorbance of oxidized 2',7'-dichlorofluorescein (DCF) formed from DCFH-DA.

3. RESULTS

CELL VIABILITY (Trypan Blue Exclusive Test) of L-929 Fibroblast-Derived Secretome on HuH-7 Cells- The cytotoxic potential of L-929 fibroblast secretome on HuH-7 cells was evaluated using the trypan blue dye exclusion test. It was observed that adding L-929 fibroblast secretome for a period of 24 hours affected the HuH-7 cell viability in a dose-dependent manner, showing viability of cells in control was higher than the cells treated with L-929 fibroblast cell derived Secretome (dose) and also as the dose increased the cell viability decreased. The results obtained are shown in the Figure no. 1. Dosage of 160µl showed the lowest viability in comparison to all other dosages.

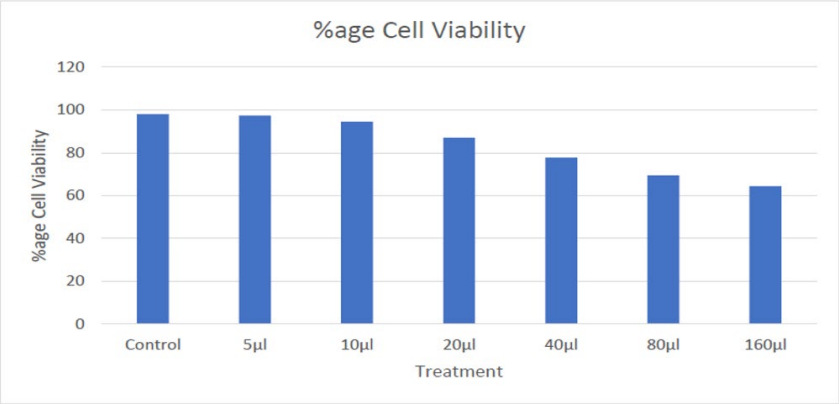

Figure 1 Graphical Representation of percentage cell viability using trypan blue exclusion test after 24 hrs. of treatment with L-929 Fibroblast-Derived Secretome on HuH-7 Cells

Cytotoxic Effect of L-929 Fibroblast-Derived Secretome on HuH-7 Cells: The cytotoxic potential of L-929 fibroblast secretome on HuH-7 cells was further evaluated using the MTT assay. This assay measures mitochondrial activity through the reduction of MTT dye into formazan crystals by oxidoreductase enzymes, thereby indicating cell viability. The colorimetric change was quantified using a plate reader at 570 nm (ELISA).

The results demonstrated that adding L-929 fibroblast secretome to HuH-7 cells for a period of 24 hours influenced HuH-7 cell viability in a dose-dependent manner. The results obtained are shown in the Figure no. 2. Lower doses (5 µL, 10 µL, 20 µL, and 40 µL) exhibited minimal cytotoxicity, with cell viability rates comparable to control conditions. However, at higher concentrations (80 µL and 160 µL), a significant reduction in viability was observed, indicating increased cytotoxicity. The highest dose (160 µL) demonstrated the most pronounced effect, suggesting that excessive fibroblast-derived factors may exert cytotoxic effects on hepatic cancer cells.

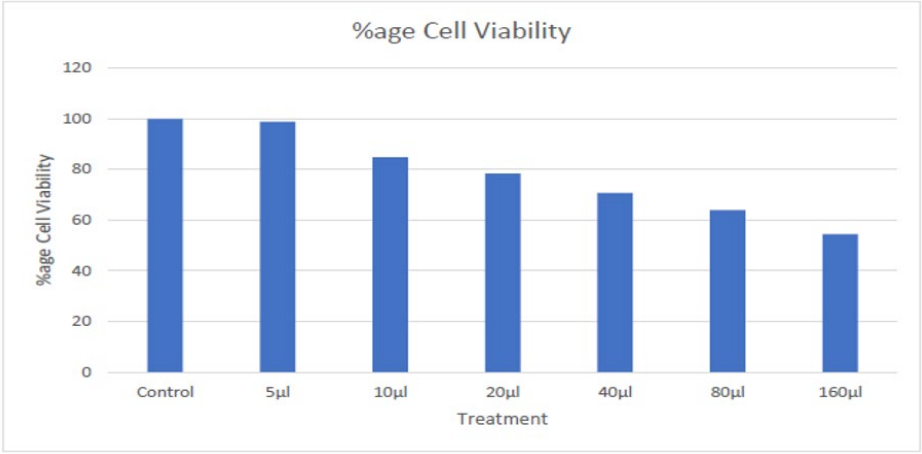

Figure 2 Graphical Representation of percentage cell viability using MTT Assay after 24hrs of treatment with L-929 Fibroblast-Derived Secretome on HuH-7 Cells

ROS of HuH-7 cells with L-929 Secretome - Superoxide anion, hydroxyl radical, and hydrogen peroxide are the three main physiologically significant reactive oxygen species (ROS) produced by cellular metabolism. They take part in normal cell functions when present in low quantities, but when present in excessive concentrations, they negatively impact cell signaling pathways (Birben et al., 2012). The results obtained are shown in the Figure no. 3. Reactive oxygen species (ROS) levels were assessed using a spectrophotometer at 495 nm, measuring the absorbance of oxidized DCF formed from DCFH-DA. It was observed that adding L-929 fibroblast secretome to HuH-7 cells for a period of 24 hours influenced generation of reactive oxygen species (ROS) by HuH-7 cells in a dose-dependent manner. It was found that cellular reactive oxygen species were high in higher concentration of L-929 cell Secretome i.e. 80µl and 160µl showing 0.317% and 0.428% of reactive oxygen species respectively, whereas lower concentrations of L-929 cell Secretome showed lesser amount of ROS. Control showed moderately lesser amount of ROS as compared to the treatment groups.

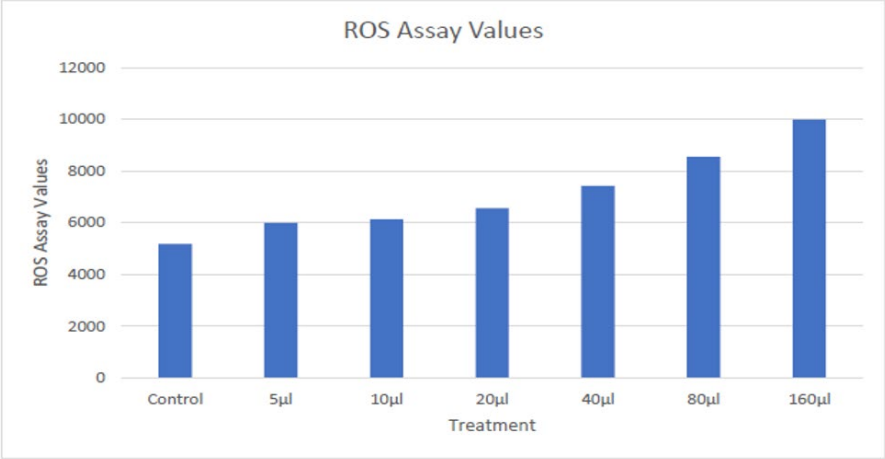

Figure 3 Graphical Representation of reactive oxygen species (ROS) generated by HuH-7 cells after 24 hrs of treatment with L-929 Fibroblast-Derived Secretome on HuH-7 Cells

4. DISCUSSION

In the present study, L-929 fibroblast cell Secretome was administered to HuH-7 cells at six different concentrations (5µl, 10µl, 40µl, 80µl and 160µl) in combination with full growth media. The results obtained in the present study are as follows:

The results were demonstrated employing assays such as Cell Proliferation using Trypan Blue Exclusion Test, MTT assay and Reactive Oxygen Species Assay. First of all, the cell viability was observed by trypan blue dye exclusion assay which represented the effect of L-929 fibroblast cell Secretome at different dose concentrations (5µl, 10µl, 40µl, 80µl and 160µl). The viability and proliferative effect on cells were found to be diminished following administration of L-929 fibroblast cell Secretome as compared to control. However, the viability was more in lower dose concentration till 40µl and was extremely diminished at 160µl of dose concentration as compared to lower dose concentration i.e. viability was low in high concentration and high in low dose concentration.

Cytotoxicity effect of L-929 fibroblast cell Secretome on Huh-7 cells was further validated by MTT assay. It was found that the higher dose (80µl and 160µl) concentration showed higher cytotoxic effect as compared to lower dose (5µl, 10µl, 20µl and 40µl) concentrations.

Similarly, number of Reactive Oxygen Species (ROS) generated by HuH-7 cells after administration of L-929 fibroblast cell Secretome at high dose (80µl and 160µl) concentrations produced more reactive oxygen species.

Based on these results, the study depicts that the fibroblast cell Secretome must contain some factors, proteins that decreased the proliferation of cancer cells and induced stress thereby generating reactive oxygen species and hence might decrease the rate of tumorigenesis in the cancer cells. Cell-cell communication and thus cellular microenvironment is the key factor of cells for their growth and differentiation. With altered microenvironment or altered microenvironment components, cells can undergo diverse form of changes which can conclude into either their proliferation or inhibition or can result in the onset of oncogenic transformation. Hence, the results obtained in the

present study strongly recommend that further elaborated studies are required to prove a possible relationship among the mentioned activities of effect of Fibroblast cell Secretome on this cancer cell line.

5. CONCLUSION

The interaction between fibroblast-secreted factors and hepatic carcinoma cells underscores the complexity of the TME. L-929 fibroblast secretome decreased the HuH-7 proliferation and also induced oxidative stress in HUH-7 cells. Targeting fibroblast-cancer interactions may provide novel therapeutic strategies for managing hepatocellular carcinoma (HCC). Future studies should investigate the potential of fibroblast-derived factors to reduce HCC progression. Additionally, in vivo validation of these findings might further elucidate the therapeutic relevance of fibroblast-cancer cell interactions.

CONFLICT OF INTERESTS

None .

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