Biotechnology and Bioprocess Engineering 28: 467-472 (2023)
DOI 10.1007/s12257-022-0260-0

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

Mechanical Stretch Promotes Invasion of Lung Cancer Cells via Activation of Tumor Necrosis Factor-alpha

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Received: 31 August 2022 / Revised: 13 November 2022 / Accepted: 27 November 2022
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Abstract Most of the gas exchange in the human body is carried out by the lungs, and the physiological activities of the lungs are uninterrupted. Due to the deterioration of the external environment, pulmonary cell lesions are common clinical lung diseases. Mechanical cyclic stretching is one kind of bionic technology to observe lung cancer cells. The A549 cell line is the human lung adenocarcinoma cell line derived from a primary lung tumor. This study investigated the effects of mechanical cyclic stretching on A549 cell activity and gene expression profile. Whereas mechanical cyclic stretching had no significant difference in colony formation and cell migration of A549 cells, the cell invasion increased significantly in A549 cells after stretching. In addition, the microarray data showed that mechanical cyclic stretching altered gene expression, induced inflammation of cells, and activation of Wnt/β-catenin and tumor necrosis factor pathways. More importantly, mechanical cyclic stretching activated the expression of tumor necrosis factor-alpha (TNF-α) protein. Therefore, the increase of cell invasion induced by mechanical cyclic stretching might be associated with the activation of TNF-α in human lung adenocarcinoma cells.

Keywords: mechanical stretch, invasion, lung cancer, microarray

1. Introduction

The physiological functions of the human body operate infinitely, especially in terms of the heart and lungs, which are important organs of physiological operation. The lungs are an important organ of the respiratory system. To maintain the energy required for life, the physiology must absorb oxygen from the outside world and discharge the carbon dioxide produced by physiological metabolism. The lungs are the organs that accomplish this gas exchange [1]. Physiological movements of the lung can cause the effects of intracellular organelles, which in turn lead to affected gene regulation, epithelial-mesenchymal transition is due to the induction of transcription factors that alter gene expression to promote loss of cell-cell adhesion, resulting in cytoskeletal dynamics and a shift from epithelial morphology and physiology to changes in mesenchymal phenotype [2]. Mechanical forces on cells induce conformational changes in transmembrane proteins that alter the cellular phenotype, such as cell adhesion molecules such as integrin, surface receptors, and ion channels, and then reorganize the cytoskeleton. Cycling epidermal cells

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in the lung by mechanical forces results in the reorganization of filamentous actin (F-actin) into ligated surrounding actin rings [3].

Chronic respiratory diseases, including tuberculosis, pulmonary fibrosis, bronchiectasis, and chronic obstructive pulmonary disease, have a higher chance of developing lung cancer. Lung cancer is the most common cancer in Canada and the leading cause of cancer death [4]. Non-small cell lung cancers account for almost 85% of lung cancer cases [5]. During the neoplastic progression of tumors, cancer cells can invade surrounding tissues, subsequently metastasize to lymph nodes, and ultimately spread to distant sites, resulting in a poor prognosis for the patients [6]. This directed migration is proposed to occur through a complex interaction of tumor cells with surrounding microenvironments, including non-cancerous cells, growth factors, chemokines, and extracellular matrix components [7]. In addition to specific biochemical signals, mechanical forces and electrical fields have been studied to play crucial roles in tumor migration and thus metastasis [8].

The physical environment critically affects cell shape, proliferation, differentiation, migration, and invasion by exerting mechanical forces on cells [9]. The cytoplasm is sensed and transduced into intracellular signals and responses by cells. The cell membrane and cytoplasmic proteins have been implicated in sensing mechanical forces, but the cell structure is not complete, and the cell transduction pathways remain largely elusive. Furthermore, the mechanical operation is associated with the chemical operation. This study is major to mechanical forces experienced by the cells that critically shape physiological processes and can be sensed by a range of genes. Mechanical force is not only imposed on the cell but also generated within cells contraction, migration, invasion, and morphogenesis [10]. The mechanical operation needs to be integrated and interpreted by cells in the context of a chemical operation stimulus that equally influences and affects cells, to build a comprehensive picture of the cellular environment and allow the initiation of appropriate responses. This study generated the profile of gene expression in response to cyclic mechanical stretch in human lung cancer cells and indicated the candidate pathway associated with cancer invasion.

2. Material and Methods

2.1. Cell culture

Human adenocarcinomic alveolar basal epithelial cells A549 were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% of fetal bovine serum (FBS) (Biological Industries, Cromwell, CT, USA) and L-glutamine (HyClone, General Electric Co., Lafayette, CO, USA) in a humidified atmosphere with 95% air-5% CO_2 at 37°C.

2.2. Mechanical cyclic stretching

The cells were stretched by a stretching device, Boxer™ Cyclic Stretch Culture System (ATMS Boxer™; TAIHOYA Corp., Kaohsiung, Taiwan). A total of 5×10^5 cells were seeded on a 20 mm × 20 mm polydimethylsiloxane surface that had been coated with collagen type I and left to incubate overnight. On the second day, the cells were subjected to a 15% strain and a frequency of 0.5 Hz (6V) through stretching.

2.3. Colony formation assay

A total of 2×10^2 cells in 2 mL cell culture medium were incubated in each well of 6-well plate for 7 days. The plates were stained with 0.005% crystal violet to assay the colony formation.

2.4. Cell migration assay

The migratory ability was measured by seeding 6×10^4 cells in 70 μL cell culture medium into each well of the Ibidi Culture-Insert system (Ibidi GmbH, Gräfelfing, Germany) and incubated overnight. After the cells had attached to the culture insert, it was removed. The migration of the cells was documented over a period of 24 hours, with three pictures being taken at each time point.

2.5. Cell invasion assay

1X Matrigel (Costar; Corning Inc., Corning, NY, USA) was loaded into the upper chamber at 37°C for at least 1 h until polymerization. A total of 1×10^5 cells in 200 μL FBS-free cell culture medium were seeded on the polymerized Matrigel. A total of 700 μL cell culture medium supplemented with 10% of FBS and subsequently the upper chambers were transferred into replicate wells of 24-well plates. After incubating at 37°C for 18 h, Giemsa stain was used to color the cells and the number of cells that had migrated into the Matrigel was counted.

2.6. Microarray

Total RNA was extracted using RNeasy Mini Kit (Qiagen, CA, USA) following the manufacturer's instruction. Total RNA from each sample was quantified by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was amplified and transcribed into cRNA and cDNA was labeled and hybridized to the GeneChip Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA) according to respective manufacturer's protocols. The raw data were submitted to the NCBI Gene Expression Omnibus database GSE214041.

2.7. Functional analysis

Ingenuity Pathway Analysis (IPA) (Qiagen) was used to perform the enrichment pathways. The analysis parameter to selecting significant pathways was based on a 2-fold change minimum in the gene profile and p value < 0.05.

2.8. Immunofluorescence confocal microscopy staining

After washing with 1× PBS three times, the stretched cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, and blocked with normal serum for 1 h at room temperature. The samples were stained with primary antibodies overnight at 4°C, washed, and incubated with fluorescein-conjugated F-actin, tumor necrosis factor-alpha (TNF-α) and nuclei antibodies at 37°C for 1 h. All staining was observed under a confocal microscope or an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan) after counterstaining with DAPI.

3. Results and Discussion

3.1. Effect of mechanical cyclic stretching on colony formation

Two groups were used to estimate the effect of mechanical cyclic stretching on the ability to form colonies. The results of the colony formation were no significant difference in two groups (Fig. 1). Mechanical stretch did not significantly affect the colony formation ability.

3.2. Effect of mechanical cyclic stretching on cell migration ability

Migration and invasion are the key driving factor that for cell motility and mechanical stretch regulated the cytoskeleton associated with migration and invasion [11]. Mechanical cyclic stretching promoted migration but inhibits invasion of rat bone marrow stromal cells [12]. In addition,

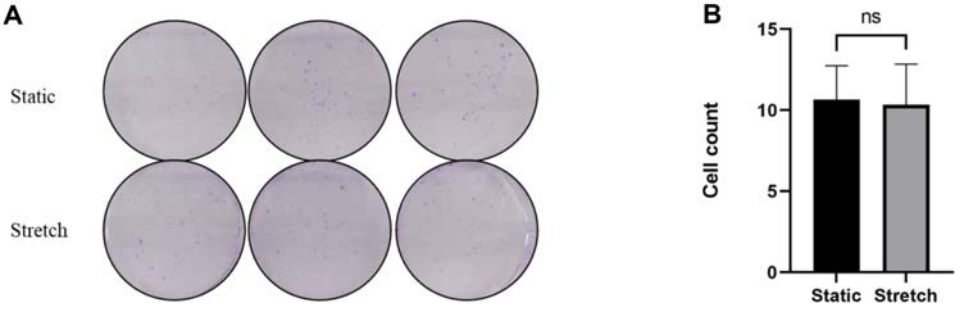

Fig. 1. Colony formation ability of A549 cells with or without mechanical cyclic stretching. (A) A549 cells were seeded at a low density in triplicates and culture for 7 days. Pictures were representative for the static group and the stretch group. (B) Cell colonies greater than 0.5 mm was counted. Histogram showed the numbers of cell colonies in two cell groups. Statistical significance was determined using the Mann–Whitney *U*-test (mean ± SD, n = 3). ns: not significant.

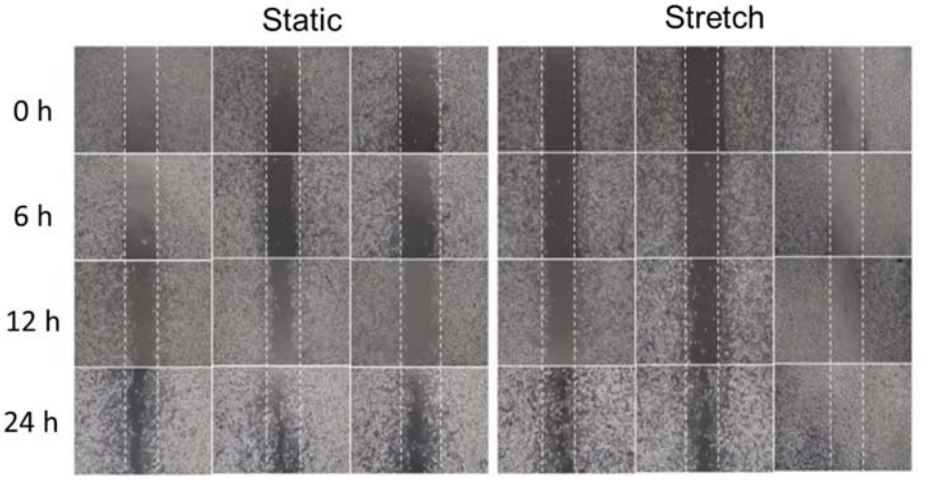

Fig. 2. Cell migration after mechanical cyclic stretching. A549 cells were seeded into an ibidi Culture-Insert system (Ibidi GmbH, Gräfelfing, Germany) to create a defined cell-free gap. Gap closing was observed and documented for 24 h. Dashed lines represented day 0. Pictures for 0, 6, 12, and 24 h were depicted after removing the culture-insert.

mechanical cyclic stretching inhibited migration of rat alveolar epithelial cells [13]. In this study, we aim to investigate the effects of mechanical cyclic stretching on migration and invasion of lung cancer cells. Gap closing was observed for 24 h and documented at 0, 6, 12, and 24 h. The data at 24 h showed the remaining gap width in the stretch group was similar to that in the static group (Fig. 2). Mechanical stretching also did not significantly affect the migration rate of the A549 cells.

3.3. Effect of mechanical cyclic stretching on cell invasion

The result of cell invasion assay showed that the numbers of invasive cells at 18 h were significantly increased in the stretch group (Fig. 3A). The quantitative analysis demonstrated that the invasive abilities were significantly increased in the stretch group (Fig. 3B). In this study, the experimental results demonstrated that mechanical cyclic stretching promoted the invasive ability in lung cancer cells.

3.4. Mechanical cyclic stretching alters gene expression profiles

Microarrays are used to examine changes in gene expression of a large number of genes simultaneously by fluorescent labeling of complementary DNAs [14]. Several studies generated the gene expression data on different cell models with mechanical cyclic stretching condition [15-20].

Microarray was used to investigate which genes were regulated by mechanical cyclic stretching and whether the changes in these genes would affect the cell invasion rate. The heatmap of differentially expressed genes across the two groups were constructed and are shown in Fig. 4A and Table S1. Gene enrichment using IPA and the top 10 enrichment pathways were shown in Fig. 4B. The result demonstrated that mechanical cyclic stretching indeed induced inflammation of cells and activation of Wnt/β-catenin and TNF pathways mediates increased invasion in lung cancer cells. The most enriched pathway was TNF

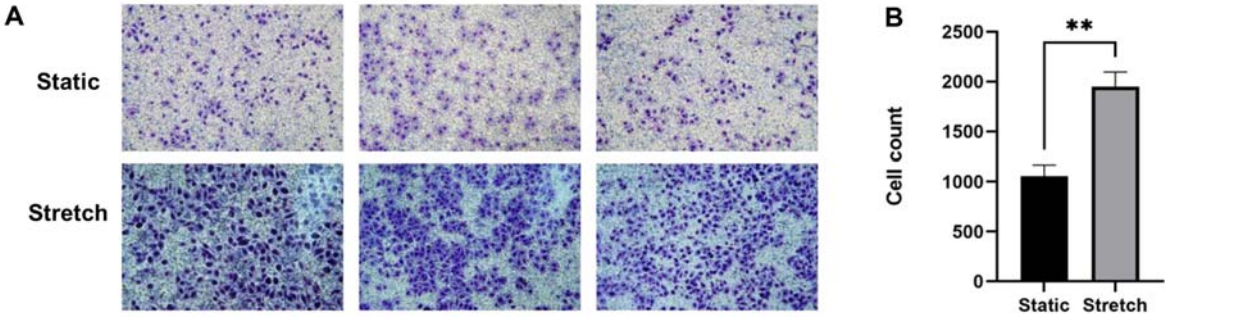

Fig. 3. Effect of mechanical cyclic stretching on cell invasion. After 18 h, invaded cells were stained with Giemsa, photographed (A), counted in three high power field and represented in the form of bar graph (B). Statistical significance was determined using the Mann–Whitney *U*-test (mean ± SD, n = 3). **p < 0.01.

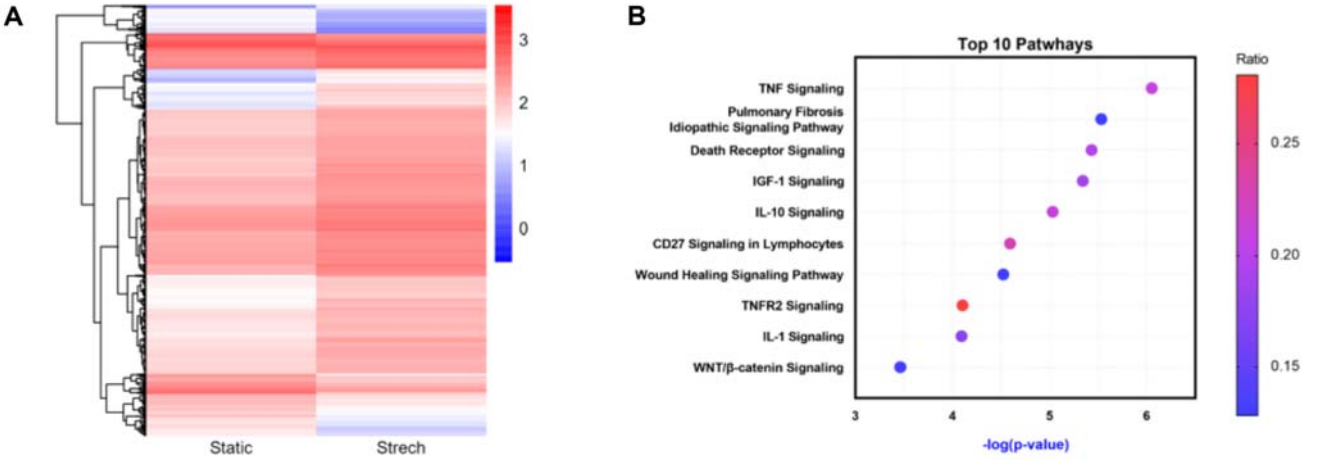

Fig. 4. Gene expression and functional pathways. (A) Heatmap of differentially expressed genes of A549 cells after stretching. (B) Ingenuity Pathway Analysis top 10 canonical pathways (p < 0.001). The most statistically significant canonical pathways identified are listed according to their p value (−log). TNF: tumor necrosis factor, IGF-1: insulin like growth factor-1, IL: interleukin.

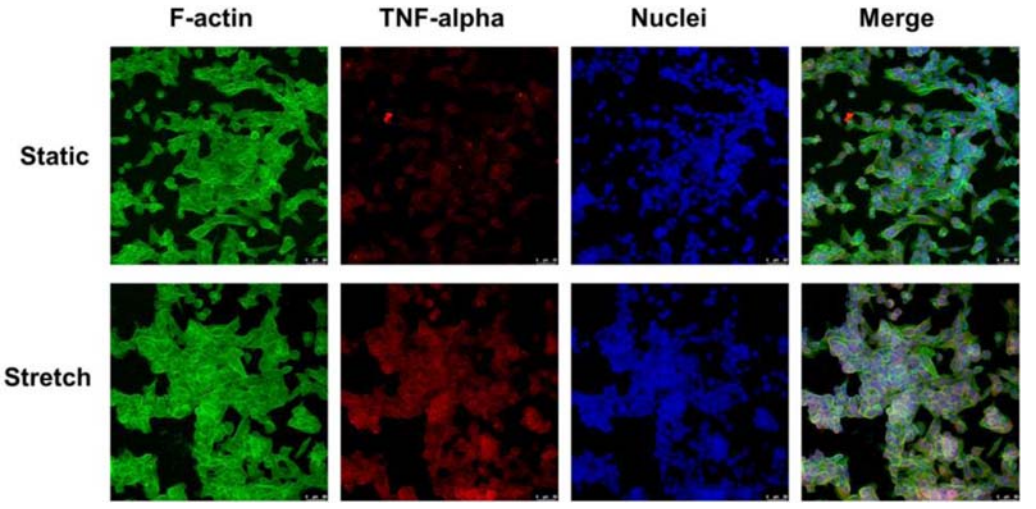

Fig. 5. Morphology of nuclei and filamentous actin (F-actin), and tumor necrosis factor-alpha (TNF-α) of human lung cancer cells. Representative pictures of immunofluorescence staining of the F-actin, TNF-α and nuclei on the static and the stretch groups. Scale bar = 50 μm.

signaling pathway. TNF-α gene is the gene in TNF signaling pathway and a critical cytokine. A lot of evidences demonstrated TNF-α enhance cell invasion by regulation with multiple lines [21]. Therefore, immunofluorescence assay was used to investigate whether mechanical cyclic stretching enhanced cell invasion by increasing expression of TNF-α in lung cancer cells. Immunofluorescence staining was used to observe the activation of TNF-α in the static and stretched cells. Whereas the level of TNF-α protein was low expression in the static cells, TNF-α expressed as well as nuclei expressed in the stretched cells (Fig. 5). These results suggested that TNF-α affected the regulation of gene and protein expression induced by mechanical stimulation in lung cancer cells.

4. Conclusion

The physiological function of the lung causes cells to stretch mechanically. We have confirmed that the mechanical stretching of lung cancer cells directly affects the invasion of cancer cells. We demonstrated that stretching lung cancer cells in a biomimetic mechanical cycle could enhance the invasive ability of lung cancer cells, whereas not affect the colony formation and cell migration. DNA microarray analysis indicated the mechanical stretching might regulate the inflammation pathway. TNF signaling pathway was the most significant pathway and the activation of TNF-α was significantly increased in the stretch group. Therefore, mechanical stretching promoted the cell invasion might through activation of TNF-α to in lung cancer cells.

Acknowledgements

We would like to thank National Core Facility for Biopharmaceuticals (NCFB, MOST 106-2319-B-492-002) and National Center for High-performance Computing (NCHC) of National Applied Research Laboratories (NARLabs) of Taiwan for providing computational resources and storage resources. This study was supported by grant from Show Chwan and Chan Bing Show Chwan Memorial Hospital (BRD109024), NCU&LANDSEED Chronic disease research center and the Ministry of Science and Technology (MOST 111-2221-E-008-066 – and 105-2314-B-008-002-).

Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

Electronic Supplementary Material (ESM)

The online version of this article (doi: 10.1007/s12257-022-0260-0) contains supplementary material, which is available to authorized users.

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