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QUANTITATIVE EVALUATION OF GROWTH PROPERTIES OF MYOBLASTS THROUGH MICROSCOPIC IMAGE PROCESSING TO OPTIMIZE SEEDING DENSITY FOR EXPANSION

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The quantitative in vitro approaches are considered as valuable predictors of cellular properties in medicine and biological research. Microscopes incorporated with specialized software for acquisition and analysis of digital images makes possible the quantitative evaluation of cellular properties both invasively and non-invasively. In our study, quantitative evaluation through analysis of microscopic images was employed for optimizing the seeding density (X_0) for in vitro culture of myoblasts. For this purpose, myoblasts were seeded at density ranges from $X_0 = 1\times10^2$ to 3.5×10^3 cells/cm^2, and the parameters such as lag time, growth index and ratio of proliferative population were quantitatively evaluated. It was found that lag time for myoblasts were decrease with increasing seeding density, and at $X_0 = 1\times10^3$ cells/cm^2 it was 1.4 folds lower than that of at $X_0 = 1\times10^2$ cells/cm^2. In contrast, the growth index at $X_0 = 1\times10^3$ cells/cm^2 was 2.3 times higher than that of at $X_0 = 1\times10^2$ cells/cm^2. These results indicated that myoblasts seeded at 1×10^3 cells/cm^2 provides the best condition for expansion. In conclusion, processing of microscopic images provides valuable tool to express the biological behaviors of cells in quantitative manner that helps to optimize the culture process for clinical uses.

Keywords: Myoblasts, Lag time, Growth index, Proliferative population

INTRODUCTION

Tissue engineering and regenerative medicine is an exciting multidisciplinary research field involving biology, medicine, engineering, and physical, chemical and material science. Over the past decade, tissue engineering research makes revolutionary development in repairing, restoring, maintaining or enhancing tissue or organ function to address medical needs for partial or complete damage of tissue or organ [1]. For clinical applications, cells harvested from the human tissue samples is cultured *in vitro* to expand (expansion phase), seeded onto biomaterials to form 3D tissue construct (tissue formation phase) and grown either *in vitro* or *in vivo* prior to transplant to the same patient (autograft) or other patient (allograft). The safety and efficacy of the tissue engineered construct is depends on the quality of cultured cells that demands the continuous assessment of cellular properties during culture process. The quantitative *in vitro* approaches are considered as valuable predictors of cellular properties. Microscopes incorporated with specialized software for acquisition and analysis of digital images makes possible the quantitative evaluation of cellular properties both invasively and non-invasively

For current set up of tissue engineering process, expansion phase is the rate limiting step that determines the duration for transplantation as well as the quality of cells. Thus it's required to optimize the expansion process to achieve the required amount of cells in quickest possible time without compromising the quality of cells. Non-invasive monitoring system for the cellular behavior during the expansion process can facilitate determine cell number and cellular morphology that can help to evaluate kinetic parameters of cells such as attachment, lag time, cell area, and growth rate through image processing. Few of the culture variables like seeding density, confluency, culture environment and medium composition were shown to affect the cellular kinetic parameters that determine the expansion efficiency [2-5]. This study was aimed to optimize the seeding density for expansion phase of human skeletal muscle myoblast cells (HSMMs) and used to treat the myocardial infraction.

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MATERIALS AND METHODS

Culture of HSMMs

HSMMs purchased from Cambrex Bio Science, Walkersville, Inc., USA was used for the experiment. Cryopreserved cells were thawed to initiate the subculture as described elsewhere [3]. In brief, thawed cells were mix with DMEM (Sigma, USA) containing 10% FBS (Sigma) and seeded on the culture vessels (Nalgen Nunc., USA), and incubate at the atmosphere of 37°C and 5% CO_2 as a primary culture (N_p = 1). The waste medium was replaced in every 24 hours. After reaching 70% confluency cells were detached from the culture surface by enzymatic treatment with trypsin for 3 minutes and trypsin was neutralized by trypsin inhibitor. To remove the trypsin and trypsin inhibitor centrifugation was performed at 1000 rpm for 5 minutes, and pellet was re-suspend with the fresh medium. Cellular viability was evaluated using trypan blue staining and seeded for the next passage (N_p = 2). The subculture was continued until N_p3, and cells from N_p4 were used for further experiments.

To determine the optimal seeding density (X_0), the HSMMs were seeded at various density ranging from $X_0 = 1\times10^2$ to 3.5×10^3 cells/cm^2, and incubate in the atmospheric condition of 5% CO_2 at 37°C. The waste medium was replaced every 24 hours. The culture was continued until reaching 60% confluency, which was determined using custom-made software, as described elsewhere [3].

Analysis of Kinetic parameters

Processing of captured images was performed to evaluate the kinetic parameters non-invasively for analyzing of cellular status in monolayer culture. The kinetics parameters which include lag time and growth index were estimated by determining the adherent cell concentration (X_a) on captured images. For this purpose, the phase contrast images of culture surface was captured from five different positions in each culture well at every 24 h using a digital camera attached to an optical microscope. The number of cells was counted manually on the images obtained at a given culture time (t). The time profile of HSMMs were used in the culture simulation software (custom made) to evaluate the lag time as describe elsewhere [2]. The growth index was obtained as the ratio of adherent cell concentration at 74 h of incubation to seeding density.

Evaluation of proliferative population

To estimate the ratio of proliferative population, the HSMMs were stained with 5-bromo-2-deoxyuridine (BrdU; Roche Applied Science, Germany) and 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, USA), as describe elsewhere [3]. In brief, prior to staining, the cells were incubated with 10 mM BrdU in DMEM for 12 h followed by the fixation of cells and denaturation of the DNA double strand. After masking the nonspecific binding sites with 10% FBS, the cells were kept for 2 h at 37°C with a mouse monoclonal anti-BrdU antibody (1: 250 dilution; Sigma-Aldrich). The cells were then immunolabeled with goat antimouse IgG (1:400 dilution, Alexa Fluor 568; Molecular Probes) accompanied by nuclear staining using DAPI (1:15,000 dilution). Images were captured from each sample using a fluorescence microscope, and the populations of BrdU-positive and DAPI-positive nuclei as proliferative and total nucleus numbers, respectively, were evaluated. The ratio of proliferative nucleus was evaluated from the proliferative (BrdU-positive) nucleus number to total (DAPI-positive) one. To calculate the number of proliferative and total cells, an image processing tool was developed using LabVIEW software (National instrument, USA). The software processed the original image, captured by fluorescent microscope, by extracting color plane based on intensity, followed by filtering particle background noise. Finally, the BrdU-positive and DAPI-positive cells were counted automatically. The automatic counting is as accurate as manual counting (r = 0.9985, Data not shown).

F-actin staining

For F-actin staining, the cells were fixed for 10 min at room temperature with 4% paraformaldehyde in PBS, followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min. The cells were then treated with Alexa Fluor Phalloidin 488 (Molecular Probes) for F-actin staining at room temperature accompanied by nuclear staining using DAPI (1:15,000 dilutions).

Statistical analysis

All the data were recorded as an average of triplicate determinations. The statistical analyses were performed using unpaired t-test. P value less than 0.05 was considered significantly different.

RESULTS AND DISCUSSION

In vitro culture of HSMMs

The clinical application using HSMMs demanded large number of cells that requires the expansion of patient cells through multiple passages. However, during *in vitro* culture, mononuclear myoblasts (proliferative cells) fused to form multinucleated myotubes (the differentiated cells), as shown in Fig. 1, causes the reduction of growth ability of HSMMs in culture [3].

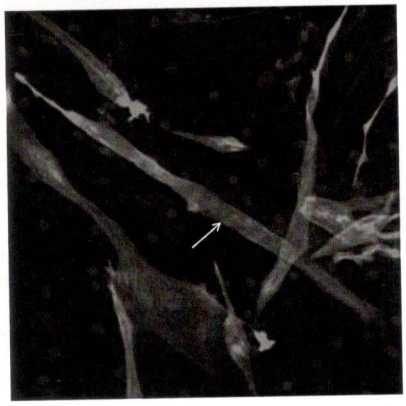

Fig. 1. Formation of multi-nucleated myotubes (white arrow) during primary culture of Human Skeletal Muscle Myoblast Cells (HSMMs).

In previous study [6-7], it was shown that myoblasts fusion was induced by the cell-cell contact. Cell seeded at high density are more susceptible to came in contact with each other at early stage of culture, and myoblasts seeded comparatively at high density shown to facilitate the myotube formation, both at 2-D and 3-D culture [8]. Moreover, considering tissue engineering application, lower seeding density is preferable provided minimal number of cells harvested from patient's tissue, although it could affect the other kinetic parameters. Thus, for *in vitro* expansion, it requires to optimize the seeding density of HSMMs for culture.

Effect of seeding density on HSMMs kinetic parameters

To evaluate the optimal seeding density, HSMMs were inoculated at different concentrations of X_0 = 1.0×10^2, 5.0×10^2, 1.0×10^3 and 3.5×10^3 cells/cm^2, and cellular kinetic parameters of lag time and growth index were evaluated. As shown in the Fig. 2, the lag time of HSMMs was kept constant at seeding density of X_0 = 1.0×10^2 to 5.0×10^2 cells/cm^2, decreased steeply at 1.0×10^3cells/cm^2, which was approximately 1.4 folds lower than that at X_0 = 1.0×10^2 cells/cm^2. However, the lag time at X_0 = 3.5×10^3 cells/cm^2 was almost similar with that at X_0 = 1.0×10^3 cells/cm^2. In contrast, the growth index of HSMMs at 74 hours was found to be increased with increasing X_0 in the range up to 1.0×10^3 cells/cm^2, whereas the values declined afterwards (Fig. 3B). The growth index at X_0 = 1.0×10^3 cells/cm^2 was evaluated to be 3.46, which is approximately 2.3 times higher than that at X_0 = 1.0×10^2 cells/cm^2. Similar phenomena of kinetic parameters were previously reported for *in vitro* culture of epithelial cells [2]. It was assumed that in case of low seeding density such as X_0 = 1.0×10^2 cells/cm^2, higher dispersion of cells in the culture surface causes lack of cellular paracrine effect and cell-cell communication that ultimately leads to elongation of cellular lag time and reducing growth index. In case of high seeding density (for example, X_0 = 3.5×10^3 cells/cm^2), the culture surface became near confluent within 74 h culture resulting the frequent cell-cell contact that may contributed in reduction of growth index.

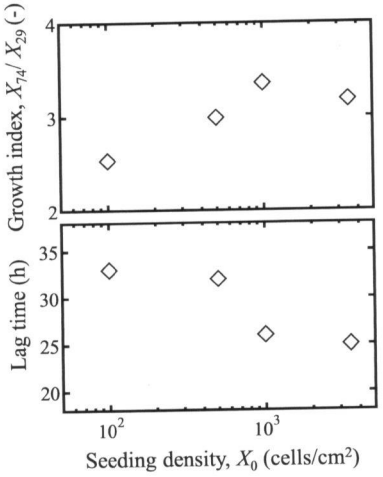

Fig. 2. Effect of seeding density on growth kinetics of lag time and growth index of HSMMs evaluated quantitatively through processing of digital images.

Effect of seeding density on proliferative population

To understand the effect of seeding density on proliferative population, HSMMs were cultured until 60% confluency, and cells were stained with anti-BrdU and DAPI to evaluate the ratio of proliferative (BrdU positive) cells. As shown in Fig. 3A, cell seeded at low density ($X_0 < 1.0\times10^3$ cells/cm^2), tends to grow as a colony and distributed heterogeneously resulting higher cell-cell contact. In contrast, cell seeded $X_0 \geq 1.0\times10^3$ cells/cm^2, cellular distribution was homogenous throughout the culture. The cellular distribution affected the number of proliferative population. As shown in Fig. 3B, the ratio of proliferative population is increase with increasing seeding density, and at $X_0 = 1.0\times10^3$ cells/cm^2, it was 1.5 times higher than that of $X_0 = 1.0\times10^2$ cells/cm^2, and significantly different ($p<0.05$). Considering all these results indicated that seeding density affect the cellular properties of HSMMs in culture. Taking consideration of tissue engineering application $X_0 = 1.0\times10^3$ cells/cm^2 provides the most favorable seeding density for *in vitro* expansion.

CONCLUSION

In last decade, HSMMs became a potential cell source for the treatment of cardiac failure [9]. Clinical application demanded large amount of cells, thus required to expand *in vitro* through serial culture. HSMMs cultured on conventional culture technique shows the low expansion potential (unpublished data), and requires the optimization of culture processes. Major approaches to improve the cellular growth properties is the modulation of the culture environment, which includes seeding density, surface, availability of the medium component and boundary conditions for culture operations. In this study, we optimize the seeding density for culturing HSMMs in culture. It was found that cell seeded at 1.0×10^3 cells/cm^2 provides suitable condition for expansion of HSMMs based on the result achieved for growth kinetics and proliferative population. In conclusion, information acquired by processing of digital images and culture simulation, the kinetic parameters was evaluated quantitatively that helps to optimize the seeding density for the culture of HSMMs.

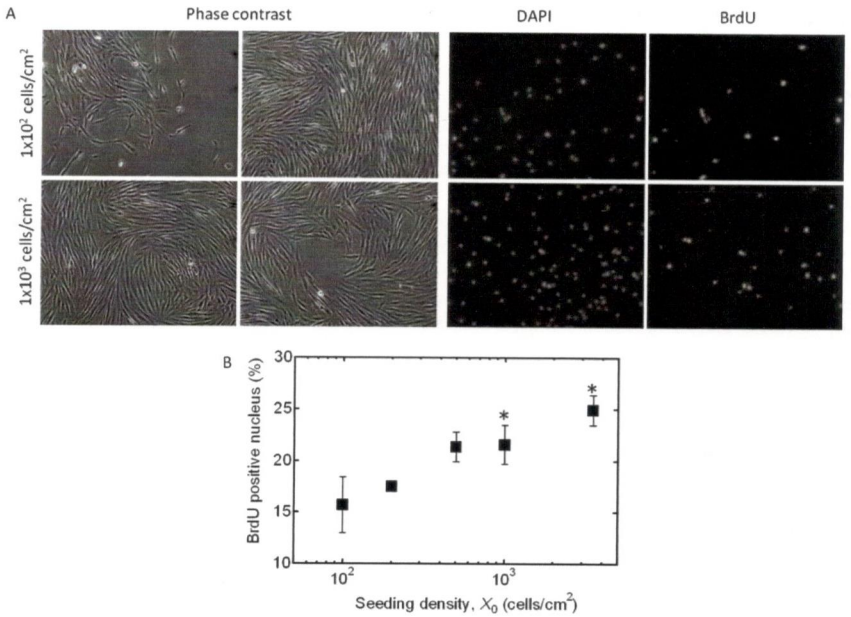

Fig. 3 Effect of seeding density on cell distribution (A) and proliferative population (B) of HSMMs. A) Representative phase contrast (two different positions of same culture vessel) and fluorescence images (BrdU and DAPI positive nuclei) of HSMMs at 60% confluency. B) Co-relation between seeding density and ratio of BrdU positive nucleus at 60% confluency.

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