再生医療用足場材料上の間葉系幹細胞の分化のシグナル伝達に関する研究

The Study on the Signal Transmission System for the Differentiation of Bone Marrow-derived Mesenchymal Stem Cells seeded on Type-1 Collagen-glycosaminoglycan Scaffolds Matrix

小林正典*

Masanori Kobayashi

Summary

In the regenerative tissue engineering using the mesenchymal stem cells (MSCs) and biomaterials as a scaffold, methodology for differentiation and tissue regeneration of MSCs in vitro is keenly required.

Although the differentiation mechanism of MSCs in scaffold matrix have been still unclear, recent studies on the regenerative tissue engineering have reported that the differentiation and tissue regeneration of stem cells might be controlled by some outside-in signals via the cell adhesion site from matrix other than the biochemical stimulations such as exogenous growth factors.

To investigate the presence of signal transmission system for differentiation of MSCs on collagen-GAG matrix, cell culture experiment with the cyclic mechanical stress stimulation was performed using MSCs and collagen-glycosaminoglycan (GAG) matrix.

As a result, the 7 days cultured MSCs showed the presence of differentiated type-I collagen positive cells without type-II collagen. On the other hand, immunohistochemical staining for α-smooth muscle actin (α-SMA) revealed some cells containing α-SMA expression which meant the contracture and adherence to matrices.

Regarding the relation between the α-SMA and the type-I collagen expression rate of MSCs, the type-I collagen expression correlated with the α-SMA expression in non-stress and lower mechanical stress group, while type-I collagen in high mechanical stress group expressed in irrelevance to α-SMA expression. This result suggested that the type-I collagen expression of MCSs depended on the mechanical stress on the matrix, the MSCs might receive the outside-in signals for differentiation from matrix via the attachment site.

キーワード：間葉系幹細胞、タイプ- I コラーゲン・グルコサミノグルカン、アルファ平滑筋アクチン抗体、分化

Keywords：Mesenchymal stem cells (MSCs)　Type-I Collagen-glycosaminoglycan (GAG), α-smooth muscle actin (α-SMA), Differentiation

1. Introduction

Resent strong interest in the regenerative tissue engineering using Embryonic stem cell (ES cell), mesenchymal stem cells (MSCs) can be attributed to their high potential of multi-differentiation. However, up to now few knowledge and technique have been found concerning the regulation of the differentiation, proliferation of their

＊ 工学部総合機械工学科

stem cells. For this background, the regenerative medical treatment implanting MSCs seeded resolvable material scaffolds have been tried in order to accelerate the healing process for injured or defective tissue [1)-7)].

In this method, on the premised that the biomaterials as a scaffolds and the implantation technique could be improved, it is very important for successful regenerative tissue engineering to understand and control the differentiation of MSCs in the scaffold matrix in vivo. Although some experiments for tissue engineering using MSCs-seeded scaffold matrix have already performed, the differentiation mechanism of MSCs in scaffold matrix have been still unclear.

However, recent many studies on the regenerative tissue engineering have reported the new basic knowledge concerning the proliferation, differentiation of some kinds of stem cells. According to these studies, stem cells might have other channels for signals by outside stimulations except the biochemical stimulations such as exogenous growth factors [8)]. Especially, as for the relation between stem cells and scaffolds, it has demonstrated that the differentiation and tissue formation of stem cells might be controlled by some outside-in signals transmitted via the cell adhesion factors from matrix (Fig-1). The studies for tissue engineering using the collagen-glycosaminoglycan (GAG) developed in our laboratory has also supported this report [9-13)]. Therefore, understanding the mechanism of the differentiation of MSCs in this collagen-GAG matrix should be accomplished for successful regenerative medical application using this scaffold materials.

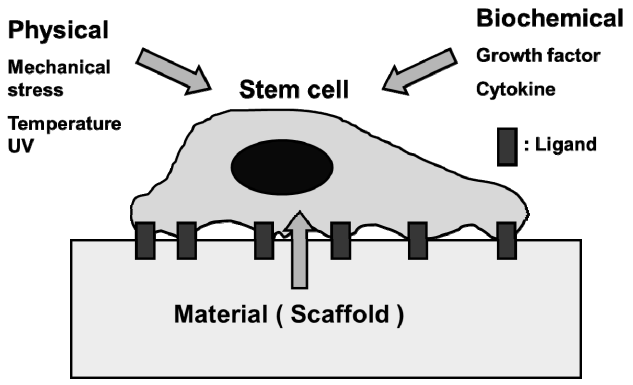


Fig-1. The schema of the control system for differentiation and tissue regeneration of MSCs.

The objective of this study was to investigate the presence of signal transmission system for differentiation of MSCs via the cell's adhere site such as channel or receptor etc. on collagen-GAG matrix using two factors regarding the interaction between the cell and scaffold matrix, which were the immunohistochemical staining for α-smooth muscle actin (α-SMA) and the cyclic mechanical stress stimulation.

2. Materials and Methods

2.1 Materials

2.1.1Type-I Collagen-glycosaminoglycan matrix

The porous collagen-GAG scaffold matrix was prepared according to a previous published procedure. Briefly, a suspension of type - I bovine tendon collagen (Integra Life Sciences, Plainsboro, NJ) and chondroitin 6-sulfate (Sigma, St.Louis, MO) in dilute acetic acid (pH 3.2) was freeze-dried to form a porous sheet (>95% porosity) 4 mm in thickness. By varying the temperature at which the collagen-GAG suspension was frozen, collagen-GAG matrices of an average pore size 84 μm were produced. The matrix were cross-linked by dehydrothermal treatment (DHT cross-linking), which consisted of exposure to vacuum of 30 mTorr at a temperature of 105 ºC for 24 hrs. Gained matrix sheets were all sterilized, and cut out as a 10 mm × 50 mm sheet as a scaffold for cell culture.

2.1.2 Mesenchymal stem cell isolation and culture

Canine bone marrow was aspirated from the tibia by a aseptic surgical procedure. An incision was made over the proximal medial anterior aspect of the tibia of dog, periosteum was exposed, and a small hole was drilled through the cortex. Several milliliters of bone marrow was drawn up using a injection syringe.

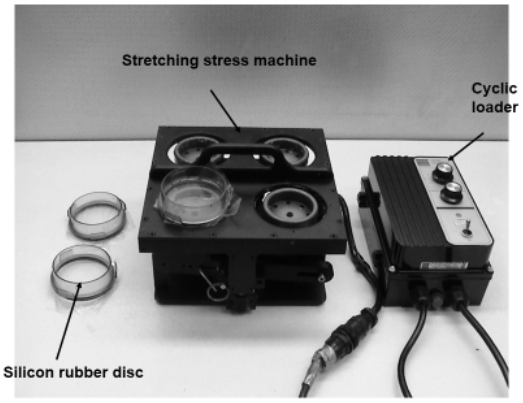
MSCs were separated by gradient under the aseptic condition, and cultured in flasks with medium (DMEM/F-12, Gibco Life Technologies) supplemented with 10 % fetal bovine serum (FBS, Hyclone Tecnoloies), 25 μg/ml ascorbic acid (Wako Chemical, Japan) and Penicilin/Streptomycin/Fungizone cocktail (Gibco). The culture flasks were incubated at 37 ºC and 5 % CO2 and 95 % humidity under the change of medium in 3 times in a week.

2.1.3 Mechanical stress loading instrument

The laboratory mechanical strain apparatus were designed for mechanical stress examination on cultured stem

cell-seeded matrix (Fig-2(a)). The mechanical strain is produced by pushing the center of silicon rubber disc in culture chamber up with an actuator driven by cyclic load cell, as shown in Fig-2(b). The magnitude of pressure and cyclic load of actuator are controlled by an electronic sensor to adjust to induce mechanical strain and load frequency of silicon rubber.

(a)



(b)

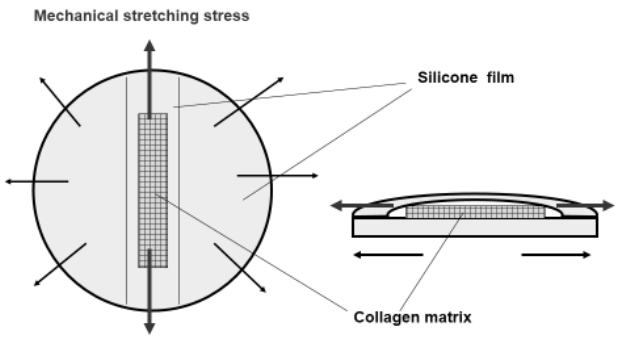


Fig-2. (a) The appearance of mechanical stretching apparatus.
(b) The schema of producing of mechanical stretching strain on the type-I collagen-GAG matrix specimens.

2.2 Methods

2.2.1 Cell - seeded matrix specimen

The collagen-GAG sheets were rinsed in Dulbecco's phosphate-buffered saline (DPBS) prior to the cell-seeding. Three – five passage mesenchymal stem cells were collected by trypsinization and resuspended in complete medium at a concentration of 2.0×10^6 cell/ml. Each one milliliter of this cell suspend medium was added to each matrix sheet, and specimens with the cell density = 2.0×10^6 (cell / sheet) were prepared for mechanical stress examination.

2.2.2 Culture and mechanical stress loading

To regulate the adhering of MSCs to scaffold matrix that might have a channel for signal transmission, the stem cell-seeded matrices were provided an initial load-bearing condition by mechanical stretching stimulator, grown in culture. Prior to the setting of cell-seeded matrix, the mechanical stimulation machine was sterilized by the washing by 70 % ethanol and the exposure to ultraviolet radiation (UV) for 24 hrs. The matrix sheets were set between two silicon rubbers of cell chamber of the loading system as shown in Fig-2, the medium was added and then the mechanical stimulation was applied. Cell culture were maintained under the cyclic mechanical stress condition at 37 ºC, 5 % CO2 , 95 % humidity and the medium change in 3 times in a week. Applied stress conditions were following : loading cycle; 15 / min, mechanical strains; 0 % (non-stress), 5 % and 15 %, culture and experiment period ; 7 days.

2.2.3 Histological and immunohistochemical analysis

For the evaluation for contracture, proliferation and differentiation of MSCs on matrices, histological and immunohistochemical analysis were performed. At 7 days post operation of mechanical stimulation, cell-seeded matrices were fixed in 10 % neutral buffered formalin, dehydrated in grated ethanol, and embedded in paraffin with non-stress matrix specimens (control). The embedded matrices were cut in cross-section at a thickness of 5 μm and stained with hematoxylin and eosin (H-E stain), and immunohistochemical stains for α-smooth muscle actin (α-SMA), type -I and II collagen.

The immunohistochemical stains for α-SMA was used in order to evaluate the adhere state of MSCs to matrix. For immunohistochemical staining for α-SMA, the mouse monoclonal antibody for α-SMA was used in the

immunohistochemical procedure. Briefly, slide-mounted sections were deparafinized, rehydrated and rinsed in PBS before blocking with 10% normal goat serum (Gibco). Sections treated using the α-SMA antibody (Sigma), which was used at a dilution of 1 : 400, were incubated with biotinylated goat anti-mouse IgG secondary antibody (Sigma) diluted 1 : 400 for 30 min, and reacted with 3-amino-9-ethylcarbazole chromogen kit (Sigma) to detect the positive stain for antibody.

The immunohistochemical staining for type-I and II collagen were used in order to evaluate the differentiation of MSCs. For immunohistochemical staining for type-I and II collagen, a standard adivin-biotin complex peroxidase-based antibody staining technique was used. Deparaffinized slides were prepared for immunostaining by digestion in 0.1% protease XIV (diluted in Tris-buffered saline, pH 7.4, TBS ; Sigma) for 60 min and non-specific staining was blocked with application of 30% goat serum (Sigma) for 20 min. Sections were incubated with the primary antibody for 2 hrs, followed by incubation with biotinylated goat anti-mouse IgG antibody for type-I and II collagen (Sigma ; 1: 200 in TBS) for 45 min. Endogenous peroxidase was quenched with 3% hydrogen peroxidase (10 min) and sections were then incubated with Extravidin-Conjugated Peroxidase (Sigma ; 1 : 50 in TBS) for 20 min and developed with AEC (Zymed Laboratotries Inc.) and countestained with Mayer's hematoxylin for 20 min.

3. Results

3.1 Histological observation (H-E stain)

Fig-3 shows the H-E histological appearances of each specimens with different mechanical strains. After 7 days in culture, most stem cells had migrated along the porous outer surface of the matrix, a few cells were observed in the interior of matrix. Quantitatively, the number of survival cells tended to decrease with the high stimulated mechanical strain. The spindle shaped stem cells attached to the pore walls, some cells often exhibited a flattened morphology in high strain group.

The degradation or breakage of structure of collagen-GAG matrix tended to increase microscopically according to the increasing of mechanical strain.

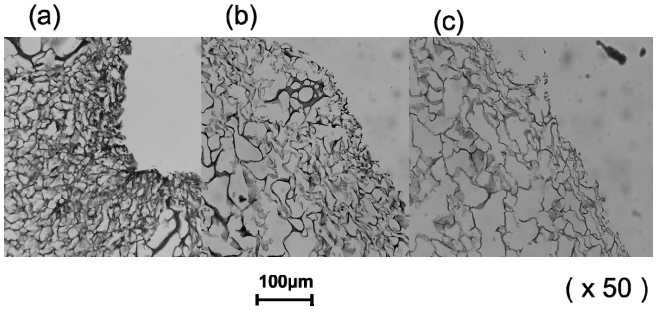


Fig-3. The histological appearances of the MSCs seeded collagen-matrix specimens in different strain groups. (H-E stain, × 50)

(a) non-stress group (b) 5 % strain group (c) 15 % strain group

3.2 Immunohistochemical observation for α-SMA

Fig-4 shows the immunohistochemical staining for α-SMA of cell-seeded matrices stimulated by each mechanical strains after 7 days in culture. Immunohistochemical staining revealed the presence of cells containing α-SMA in all cells in matrices in H-E staining, the occupation of α-SMA positive cells was the highest in the mechanical stain 15 % group, in the contrast, the lowest in 5 % strain group.

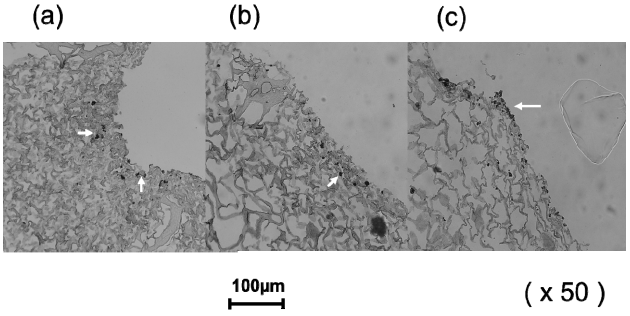


Fig-4. The immunohistochemical staining for α-SMA of the MSCs seeded collagen-matrix specimen in each mechanical strain group. (a) non-stress group (b) 5% strain group (c) 15% strain group. (× 50)
The arrows indicate α-SMA positive cells.

3.3 The immunohistochemical observation for type – I and II collagen

Fig-5 shows the immunohistochemical staining for type-I collagen. This immunostaining indicated the presence of differentiated MSCs with type-I collagen positive at culturing 7 days.

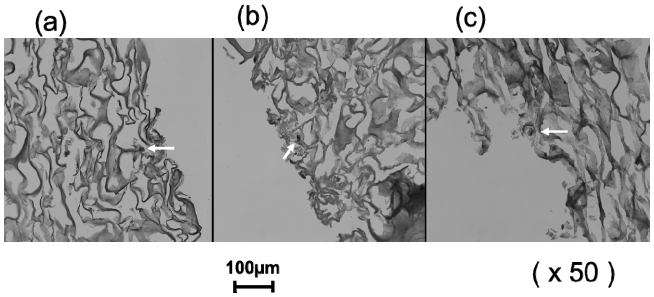


Fig-5. The immunohistochemical appearance for type-I collagen of the specimen in each mechanical strain group. (a) non-stress group (b) 5% strain group (c) 15% strain group. (× 50) The arrows indicate type-I collagen expression cells.

These MSCs with type-I collagen positive were observed in the area with α-SMA positive cells (Fig-6). However, no excellularmatix by type-I collagen synthesis and deposit was observed in the collagen-GAG scaffold.

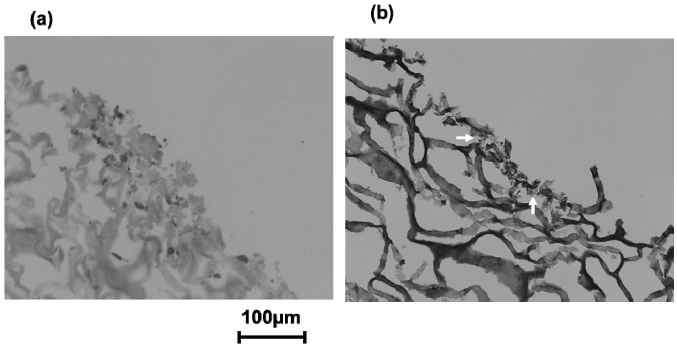


Fig-6. The immunohistochemical appearance of same area of the specimen in 15 % strain group. (a) the staining for α-SMA (b) the staining for type-I collagen. (× 50).

On the other hand, no MSCs stained positive in immunohistochemical staining for type-II collagen (Fig-7).

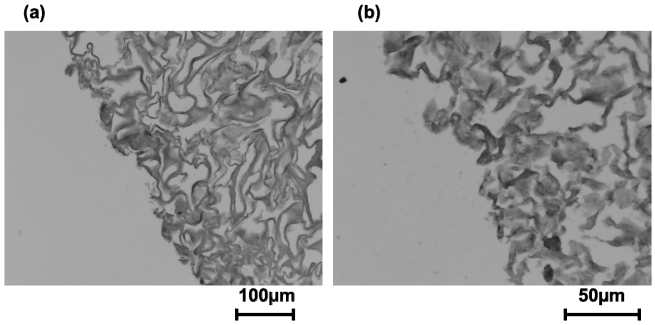


Fig-7. The immunohistochemical appearance for type-II collagen of the specimen in non-strain group. Neither positive cells nor extracellular matrices for type-II collagen were observed. (a) × 50, (b) × 100.

4. Discussion

In the regenerative tissue engineering using MSCs, the controls of the differentiation in vitro of stem cells and the tissue regeneration in vivo are significant problems to be solved. Many parts of these knowledge about the differentiation, tissue regeneration of stem cells have been attributed to the biochemical approach involved the molecular biology, however, recent studies on the regenerative tissue engineering have demonstrated that the other physical factors from outside but the biochemical factors can also contribute to the differentiation of MSCs by other signal transmission system.

Especially, as for the interaction between stem cells and scaffolds, it has demonstrated that the cell adhere molecular involved integrin can transmit not only the inside-out signals about the adhesion, contracture and moving of cells but the outside-in signals about the differentiation, bio-synthesis of extracellular matrix from matrix.

The studies for tissue engineering using the collagen-GAG developed in our laboratory have demonstrated that the amount and type of the extracellular matrix and the proliferation, differentiation of seeded cells depended on the type of collagen (type I or II). The chondrocyte seeded in type II collagen-GAG matrix synthesized the type II collagen, while the chondrocyte in type I collagen-GAG matrix displayed less type II collagen expression in previous study[12,13]. This facts has indicated that this collagen-GAG matrix might also send some outside-in signals concerning the differentiation and biosynthesis to the populating cells.

In this experiment, in order to evaluate the presence of the transmission channels in cell's adhere site to matrix, we used two factors regarding the interaction between the cell and scaffold matrix, which were the immunohistochemical staining for α-SMA and the cyclic mechanical stress stimulation.

Previous studies about α-SMA expression of MSCs have represented that they express the gene for a contractile action isoform, which can be demonstrated to be co-localized on the stress fibers and these α-SMA expressing

stem cells can actually contract a collagen-GAG analog of extracellular matrix in vitro[14]. This means that α-SMA expression can be evaluated as a index for activity of adhesion, contraction and migration of cells in the adhering site in collagen-GAG matrix.

On the other hand, the mechanical stimulation to MSCs on matrix is thought to be physical suffocating factor to adhering site between the cells and matrix. Presuming that the outside-in signal transmission system from collagen-GAG matrix to cells would exist in this adhering site, the type and amount of biosynthesized collagen must depend on the change of the mechanical stress to the specimens.

Fig-8 shows the α-SMA expression rate of total MSCs in each mechanical stress group. The more α-SMA positive cells displayed in survival cells according to the higher mechanical stimulating, Inferring from this result, in case the stem cell can not perform the sufficient attachment to matrix by only the usual adhesion mechanism through the integrins under high mechanical stimulation condition, extensive contraction through α-SMA expression appears to reinforce the cell attachment to the dynamic collagen-GAG scaffold matrix. Some studies have already stated that the α-SMA expression was activated by mechanical stress in cultured smooth muscle cells, myofibroblasts and osteoblasts[15-19]. Although the α-SMA expression mechanism could not be analyzed, this finding is remarkable as a early response of MSCs to the initial mechanical stress.

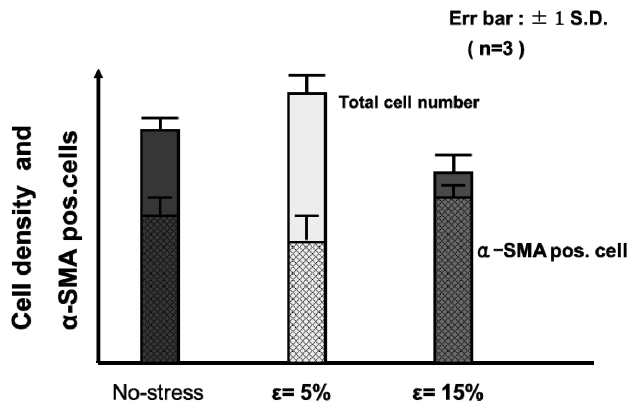


Fig-8. The occupational percentage of α-SMA expression in the proliferated total MSCs in each mechanical stress group.

Fig-9 shows the percentage of cells staining positive for the type-I collagen in each mechanical strain group. The rate appeared to change according to the magnitude of mechanical strain stimulation. The percentage of positive cell for the type-I collagen showed the highest value in 5 % strain and non-stress group, the lowest in 15 % ($p<0.05$). The fact that type-I collagen showed the higher value in mechanical strain than that in static group was coincided with the report by Altman G et al.[20]. This result was likely to attribute to signal transmission through the receptor for not the outside-in signals from matrix but the physical stimulation.

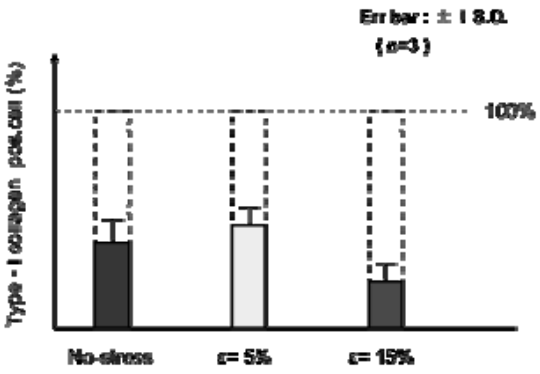


Fig-9. The occupational percentage of type-I collagen expression in the proliferated MSCs in each mechanical stress group.

On the other hand, as shown in Fig-6, the cells with type-I collagen positive were observed in α-SMA positive cells. Regarding the relation between the α-SMA and the type-I collagen expression rate of MSCs in each mechanical strain group (Fig-10), the type-I collagen expression correlated with the α-SMA expression in non-stress and lower mechanical stress group, while type-I collagen in high mechanical stress group expressed in irrelevance to α-SMA expression. Considering the α-SMA expression represented the adhering state of the cells to matrix, this result suggested us the speculation that the signal transmission for differentiation can not work efficiently under the unstable dynamic condition for adhering site of scaffold matrix by high mechanical stress. In other words, this result means that the type-I collagen-GAG matrix itself might transmit some outside-in signals for differentiation of the type-I expression cells via adhering site. Previous studies using other connective tissue cells and the type-I and II collagen-GAG matrices have also demonstrated the difference in the cell proliferation and extracellular matrix synthesis in the types I and II collagen matrices[9,21,22].

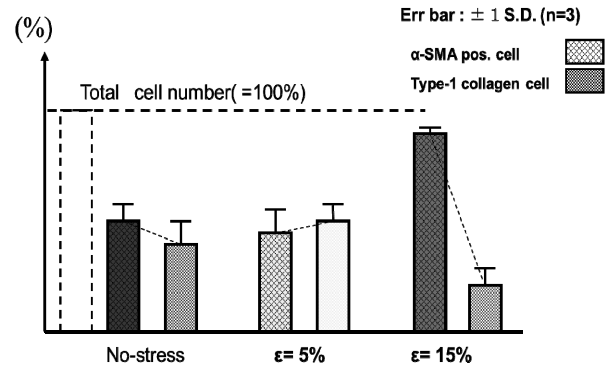


Fig-10. The relation between α-SMA positive cells and the type-I collagen expression cells in each mechanical stress group.

Present study suggested that our type-I collagen-GAG matrix might send the outside-in signal for differentiation of MSCs, which was transmitted through the adhering site of MSCs, which was different transmission channel from the biochemical stimulation factors to MSCs. This finding might contribute to the basic data for clinical application by this collagen-GAG. Henceforth, in order to develop this collagen-GAG matrix for regenerative tissue engineering, further study about the signal mechanism which this collagen-GAG may send for the differentiation and the other extracellular matrix biosynthesis of MSCs will be necessary.

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