骨髄間葉系幹細胞を播種された再生医療用タイプ１－コラーゲン－グルコサミノグルカン（Collagen-GAG）複合マトリックスの力学的環境下での細胞増殖と変形・劣化に関する研究

The study on the cell proliferation and the cell-mediated deformation, deterioration of bone marrow-derived mesenchymal stem cells-seeded type-I collagen -glycosaminoglycan scaffolds matrix under the mechanical stress application

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Summary

For the regenerative tissue engineering using pluripotent stem cell, many biomaterials have been developed as a scaffold. However, these scaffold materials have the potential problem concerning the cell-mediated deformation and deterioration of matrix, which may make the repairing tissue failure due to the contact loss between the implanted scaffold matrix and the host tissue. The mechanical stress might be effective for the preventing the deformation of these scaffolds, however, the effects on the cell proliferation and differentiation of stem cells and the degradation of scaffold matrix are still unclear.

Thus, in order to investigate these subjects, the bone-derived mesenchymal stem cells (MSCs) seeded type-I collagen-glycosaminoglycan (GAG) were prepared as a specimens, and provided an initial load-bearing condition by mechanical stretching stimulator, grown in culture and examined in macro- and microscopy.

As a results, all kinds of the mechanical strained specimens showed the prevention of the shrinkage of matrices macroscopically. However, the microscopic appearance indicated that the high mechanical stress engendered the destruction of the matrix structure with the decrease of total cell number density. While, the cell number of 5 % strain stressed group was increasing.

This study suggested that adequate mechanical stretching condition is commend as a promising treatment in vitro for the prevention of deformation of scaffold matrix with proliferated stem cells.

Keywords：Mesenchymal stem cell (MSC),　Type-I collagen-glycosaminoglycan (GAG)
Mechanical stress,　Cell-mediated contracture

1. Introduction

Resent strong interest in the regenerative tissue engineering using Embryonic stem cell (ES cell), Mesenchymal stem cell (MSC) or Induced pluripotent stem cells (iPS cells) can be attributed to their high potential of multi-differentiation.

The regenerative medical therapy by implanting stem cell seeded scaffolds in vivo to accelerate the regeneration process for injured or defective tissue is very popular approach for tissue engineering, and has already been investigated vigorously [1-7].

In this approach, the role of biomaterial as a scaffold is very important for successful tissue regeneration by stem cells. Up to now, many bio-degradable biomaterials have already been developed as a scaffold for tissue engineering

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[8-10]. However, because these bio-degradable materials are replaced by regenerative tissue after implantation, few knowledge and technique concerning the regulation of structural mechanical strength of scaffold involving the differentiation, proliferation of the seeded stem cells have been found,

The type-I collagen-GAG matrix used in this study has also been investigated in vitro experiments for tissue engineering using MSCs and connective tissue cells such as chondrocyte, meniscus cell, and anterior cruciate ligament cell etc. and has been estimated the potential biological functionality as a scaffold [11-14]. However, at the same time, these previous studies have indicated that the cell-mediated contraction of this collagen-GAG matrix was the potential problem for application [15,16]. The contraction of scaffold matrix may result in the restriction of cell proliferation by the reduction of pore volume, and what is worse clinically, the shrinkage of matrix by contraction could fail in the repairing tissue because of the contact loss between the implanted scaffold matrix and the host tissue. The prevention of the deformation of matrix and the control of the cell-mediated contraction could be the key point for clinical application of this scaffold type. To prevent the shrinkage of this scaffold matrix by the cell-mediated contraction, the investigations about increasing the stiffness and resistance of matrix using the cross-linking treatment have performed [17], the mechanical stretching stress is also expected as one of treatments for the prevention of the shrinkage [18]. While, this treatment may lead to anther fear that the mechanical stress might obstruct the cell's proliferation in scaffold through the destruction of matrix structure.

The purpose of this study is to investigate the effect of mechanical stretching stress for the preventing the contraction of bio-degradable scaffold by using bone-derived mesenchymal stem cell seeded collagen -glycosaminoglycan (GAG) .

2. Materials

2.1 Type-I Collagen-glycosaminoglycan matrx

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 suspention was frozen, collagen-GAG matrices with an average pore size of 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 hr. The gained matrix sheets were all sterilized, and cut out as a 10 mm × 50 mm × 3 mm sheets as a scaffold for cell culture.

2.2 Mesenchymal stem cell isolation and culture

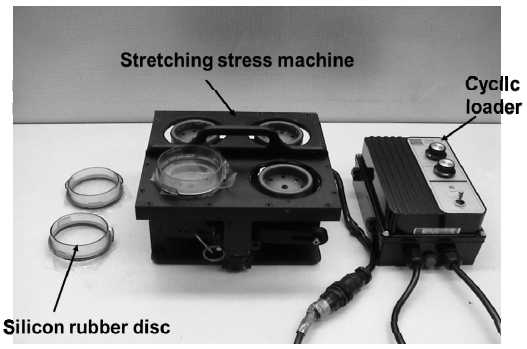
Canine bone marrow was aspirated from the tibia by a aseptic surgical procedure. An incision was made over the proximal medial anterior aspect of the tibia of dog, periosteum was exposed, and a small hole was drilled through the cortex. Several milliliters of bone marrow was drawn up using a injection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

Mesenchymal stem cells 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 % CO_2 and 95 % humidity under the change of medium in 3 times in a week.

2.3 Mechanical stimulation instrument

The laboratory mechanical strain apparatus were designed for mechanical stress examination on cultured stem cell-seeded matrix (Fig-1(a)). The mechanical strain is produced by pushing the centre of silicon rubber disc in culture chamber up with an actuator driven by cyclic load cell, as shown in Fig-1(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)



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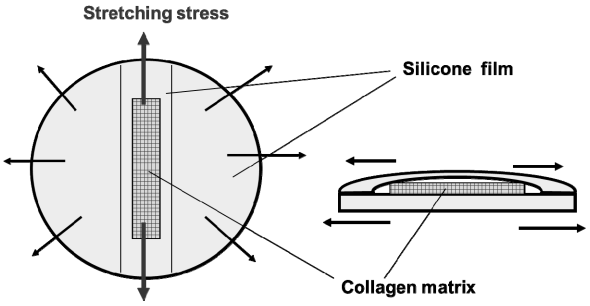

Fig-1. (a) The appearance of mechanical stretching apparatus. (b) The schema of producing of mechanical stretching strain on the collagen-GAG matrix.

3. Method

In this method, the stem cell-seeded matrix were provided an initial load-bearing condition by mechanical stretching stimulator, grown in culture and examined the shrinkage of the matrix by the cell mediated-contraction in macro- and microscopy.

3.1 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 were added to each matrix sheet specimen to prepare the cell density = 2.0×10^6 cell / sheet for mechanical stress examination.

3.2 Culture and mechanical strain application

The mechanical stimulation machine was sterilized by the washing by 70% ethanol and the exposure to ultraviolet radiation (UV) for 24 hrs prior to the setting of cell-seeded matrix. The matrix sheets were set between two silicon rubbers of cell chamber of the loading system as shown in Fig-3, 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 % CO_2 , 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.

3.3 Measurement of cell-mediated matrix contraction

According to previous studies, these cell-seeded matrix specimens were undergoing the shrinkage mediated by cells during the course of the culture experiment. To quantify the effects of mechanical stretching stress on the cell-mediated contraction, the longitudinal lengths of the seeded and unseeded matrices were measured 1, 3, 5, and 7 days post operation of mechanical stimulation apparatus.

3.4 Microscopic and Histological analysis

At cell culture 7 days under the mechanical stimulation, cell-seeded matrices and unseeded-control matrices were fixed in 10 % neutral buffered formalin, dehydrated in grated ethanol, and embedded in paraffin. 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 stain for α-smooth muscle actin (α-SMA).

The structure of collagen-GAG matrix and the cell number density of MSCs in matrix were investigated by H-E stained specimens, the contractility of cells was checked by the immunohistochemical stain for α-SMA.

The mouse monoclonal antibody for α-SMA was used in the immunohistochemical procedure in this study. 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.

4. Results

4.1 Measurement of cell-mediated matrix contraction

The cell-mediated contraction were determined as a percentage of the change of longitudinal length of matrix specimens in normalization to that of non-seeded matrix. Fig-2 shows the time dependence of the contraction of cell-seeded in the each mechanical strain.

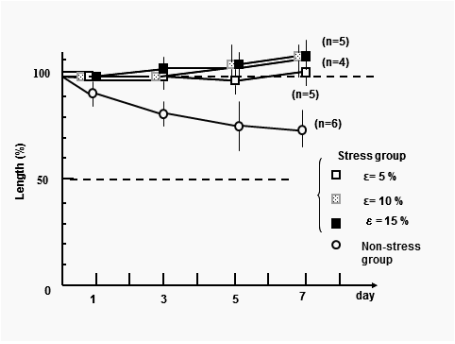

Fig-2. The time dependence of the cell-mediated contracture of collagen-GAG matrix.

In the non-stress loading group, the matrix specimens

shrunk more than 25 % during the culture period 7 days, while, none of specimens in the mechanical stress group showed significant shrinkage but rather some displayed the elongation in high stress stimulation.

The pattern of deformation of matrix by the cell-mediated contraction were marked in initial 3-5 days, and gradually changed to the steady state in non-stress group, while the curves of mechanical stress groups showed the elongation at the early stage an increased with time according to the degree of strains.

4.2 Microscopic and Histological observation

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

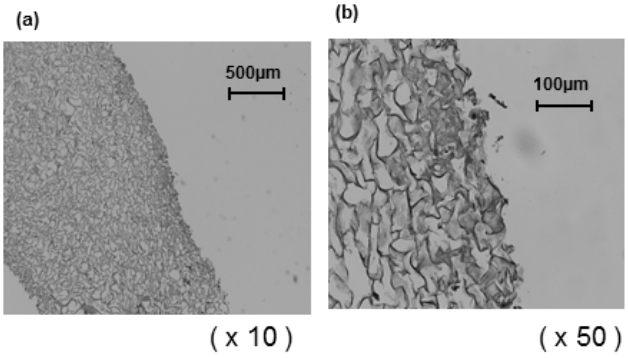

Fig-3. The histological appearance of the MSCs seeded collagen-matrix specimen in non- stress group. (H-E stain, (a) × 10, (b) × 50) The structure of matrix showed thick and right order. The many cells present in the scaffold matrix stimulated mechanical strain.

stimulated mechanical strain. As a sample data, Fig-3,4 show the H-E histology of cell-seeded matrices by each mechanical strains and cell densities. Many cells were round or spindle shape attached to the pore walls, the stem cells in high strain group often exhibited a flattened morphology. The collapse or disintegration of structure of collagen-GAG matrix tended to increase microscopically according to the increasing of mechanical strain although no shrinkage were observed in gross appearance in mechanical strain group.

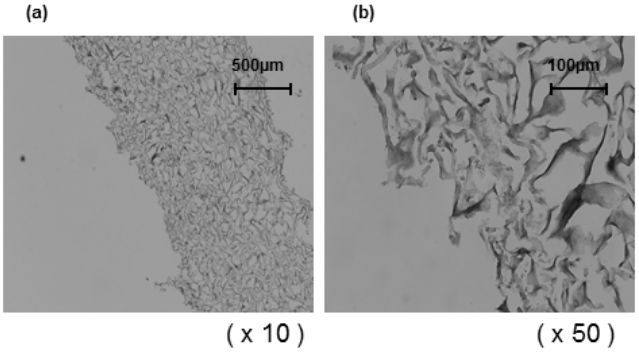

Fig-4. The histological appearance of the MSCs seeded collagen-matrix specimen in 15 % strain group. (H-E stain, (a) × 10, (b) × 50)
The degradation of matrix structure was obvious as compared with non-stress group.

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

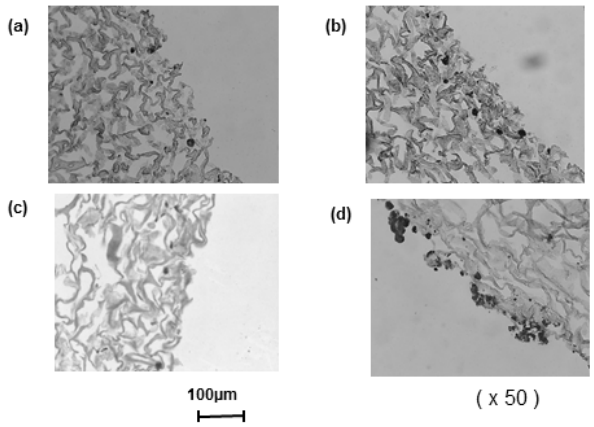

Fig-5. The immunohistochemical appearances of the MSCs seeded collagen-matrix specimens. (a) non-stress group (b) 5% strain group (c) 10 % strain group (d) 15 % strain group (× 50)

4.3 The relation between the cell density and mechanical stress

Fig-6 shows the relation between the mechanical strains and the survival stem cell density. The density of cells in each group were calculated by the area of matrix specimens

and the numbers of cells counted from the H-E histological sections. The density of high mechanical strain groups of 10 % and 15 % displayed lower value than that of the control group, while the density of 5 % strain group was the highest.

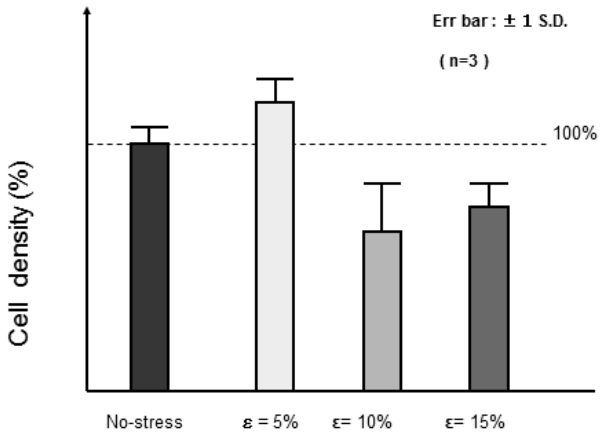

Fig-6. The relation between the each loading mechanical stress and the cell density of MSCs.

5. Discussion

The regenerative tissue engineering using the pluripotent stem cell such as mesenchymal stem cell, ES cell and the scaffolds will be promoted to new medical application hereafter. In this approach, the cell-mediated contraction, deformation or mechanical deterioration of the scaffold matrix is the potential problem for application because of the risk of contact loss between the implanted scaffold matrix and the host tissue.

Our previous studies for tissue engineering using the type -I collagen-GAG matrix and some connective tissue cells showed that this collagen-GAG matrix also had the serious problem concerning the cell-mediated contraction of matrix in spite of the potential biological functionality as a scaffold. Therefore, the prevention and control of the deformation of type-I collagen-GAG matrix by the cell-mediated contraction may be one of the key point for clinical application of this material.

Premised on the development of the mechanical properties of this collagen-GAG, the mechanical stretching stimulation is also expected as one of treatments for the prevention of the deformation. However, even if mechanical stretching stress could prevent the contraction of collagen-GAG matrix, this may lead to anther problems. The mechanical stress might induce the destruction of matrix structure and the cell-detachment to matrix, and result in the failure of cell's proliferation. To investigate the appropriate mechanical stress for preventing the cell-mediate contraction of this collagen-GAG, it was important to evaluate not only the structure of matrix but the alive cell number on matrix.

As a results in this experiment, all kinds of the mechanical strained groups of collagen-GAG matrix, the compression stiffness of which was estimated to be about 1.45×10^{-4} MPa in our preliminary study about the mechanical strength [17], showed the prevention of the shrinkage of matrices macroscopically. However, the microscopic appearance indicated that the high mechanical stress engendered the destruction of the matrix structure with the severe cell-mediated contraction which is demonstrated by extensive α-SMA expression[19]. As for the α-SMA expression of stem cell, high mechanical stimulation such as 15 % strain appeared to promote the contraction through α-SMA expression and this seemed to result in the breakage of matrix structure.

On the other hand, the number of survived stem cells against the mechanical stimulation was depend on not only the cell's adhere ability to matrix but also the structural conditions of scaffold matrix itself. As shown in Fig-5, high mechanical such as 10 %, 15 % strain groups destroyed and degraded the structure of matrix, the total cell number of these group were also reduced accordingly with the strength of mechanical stress. Considering the previous study that fibroblast proliferation were down-regulated after contraction, the α-SMA expression-mediated contraction in high stress might affect this cell number of MSCs in scaffold [20, 21].

Whereas, the cell number of 5 % strain group was increasing. This fact indicated that the adequate mechanical stress, the degree of which did not lead to the degradation of scaffold matrix and the obstruction to cell adhesion to matrix, might assist cells in migration into matrix and promote the proliferation of cells by appropriate driving force.

These results suggested that mechanical stretching method is commend as a promising treatment in vitro for the prevention of shrinkage of matrix with stem cells. According to Fig-2, the deformation of matrix by the cell-mediated contraction were marked in initial 3-5 days, and gradually changed to the steady state, the cell culture in vitro on this scaffold matrix could have efficiency of mechanical stress by the 7 days after the cell seeding.

On the other hand, regarding the differentiation of MSCs, the role of mechanical stress has not been yet clear. Further attentions about the correlation between the differentiation of stem cell and the mechanical stress should be drawn in future.

In summary, this work showed the initial cell-mediated contraction by stem cells seeded in collagen-GAG matrix might be prevented under the proper mechanical stress condition including the capability for proliferation. The findings of this study are important as a groundwork for clinical application using bio-degradable scaffold such as a collagen-GAG matrix for tissue engineering.

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References

1) Angele P, Kujat R, Nerlich M, Yoo J, Goldberg V, Johnstone B. : Engineering of osteochondral tissue with bone marrow mesenchymal progenitor cells in a derivatized hyaluronan-gelatin composite sponge. Tissue.Eng. 1999 ; 5(6) : 545-554.

2) Gao J, Dennis J.E, Solchaga L.A, Awadallah A.S, Goldberg V M, Caplan A.I. : Tissue-engineered fabrication of an osteochondral composite graft using rat bone marrow-derived mesenchymal stem cell. Tissue.Eng. 2001 ; 7(4) : 363-371

3) Noth U, Tuli R, Osyczka A.M, Danielson K.G, Tuan R.S. : Invitro engineered cartilage constructions produced by press-coating biodegradable polymer with human mesenchymal stem cells. Tissue.Eng. 2002 ; 8(1) : 131-144.

4) Hori Y, Nakamura T, Kimura D, Kaino K, Kurokawa Y, Satomi S, Shimizu Y. : Experimental study on tissue engineering of the small intestine by mesenchymal stem cell seeding. J.Surg.Res. 2002 ; 102(2) : 156-60.

5) Boo Y.S, Yamada Y, Okazaki Y, Hibano Y, Okada K, Hata K, Yoshikawa T, Sugiura Y, Ueda M. : Tissue-engineered bone using mesenchymal stem cells and a biodegradable scaffold. J.Cranifac.Surg. 2002 ; 13 (2) : 231-239.

6) Perry T.E, Kaushal S, Sutherland F.W, Guleserian K.J, Bischoff J, Sacks M, Mayer J.E. : Thoracic surgery directors assosciation award. Bone marrow as a cell source for tissue engineering heart valves. Ann.Thorac.Surg. 2003 ; 75 : 761-767.

7) Warren S.M, Nacamuli R.K, Song H.M, Longaker M.T. : Tissue-engineered bone using mesenchymal stem cells and a biodegradable scaffold. J.Cranifac.Surg. 2004 ; 15 : 34-37.

8) Zhang J, Doll B.A, Beckman E.J, Hollinger J.O. : A biodegradable polyurethane-ascorbic acid scaffold for bone tissue engineering. J.Biomed.Mater.Res. 2003 ; 67A : 389-400.

9) Yang J, Shi G, Bei J, Wang S, Cao Y, Shang Q, Yang G, Wang W. : Fabrication and surface modification of macroporous poly (L-lactic acid) and poly (L-lactic-co-glycolic acid) (70/30) cell scaffolds for human skin fibroblast cell culture. J.Biomed.Mater.Res. 2002 ; 62 : 438-46.

10) Kwon I.K, Park K.D, Choi S.W, Lee S.H, Lee E.B, Na J.S, Kim S.H, Kim Y.H. : Fibroblast culture on surface-modified poly (glycolide-co-epsilon-caprolactone) scaffold for tissue regeneration. J.Biomater.Sci.Polym.Ed. 2001 ; 12 : 1147-60.

11) Mueller S.M, Shortkoff S, Schneider T.O, Breiman H.A, Yannas I.V, Spector M. : Meniscus cells seeded in type I and type II collagen-GAG matrices in vitro. Biomaterials. 1999 ; 20 : 701-709.

12) Murray M. M, Spector M. : The migration of cells from the ruptured human anterior cruciate ligamen into collagen-glycosaminoglycan regeneration templates in vitro. Biomaterials. 2001 ; 22 : 2393-2402.

13) Spilker M.H, Asano K, Yannas I.V, Spector M. : Contarction of collagen-glycosaminoglycan matrices by peripheral nerve cells in vitro. Biomaterialas. 2001 ; 22 : 1085-1093.

14) Kinner B, Zaleskas J.M, Spector M. : Regulation of smooth muscle actin expression and contraction in adult human mesenchymal stem cells. Exp. Cell. Res. 2002 ; 278 : 72-83.

15) Nehrer S, Breinan H.A, Ramappa A, Shortkoff S, Young G, Minas T, Sledge C, Yannas I. V, Spector M. : Canine chondrocytes seeded in type I and type II collagen implants investigated in votro. J. Biomed. Mater.Res. 1997 ; 38 : 95-104.

16) Lee C.R, Breinan H.A, Nehrer S, Spector M. : Articular cartilage chondrocytes in type I and type II collagen-GAG matrices exhibiting contractile behavior in vitro. Tissue. Eng. 2000 ; 6 : 555-65.
17) Lee C.R, Grodzinsky A.J, Spector M. : The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chnodrocyte-mediated contraction, proliferation and biosyntheses. Biomaterials. 2001 ; 22 : 3145-3154.
18) Langelier E, Randcourt D, Bouchard S, Lord C, Stevens P.P, Germain L, Auger F.A. : Cyclic traction machine for long-term culture of fibroblast-populated collagen gels. Ann.Biomed.Eng. 1999 ; 27 : 67-72.
19) Kinner B,Zaleskas J.M, Spector M. : Reguration of smooth muscle actin and contraction in adult human mesenchymal stem cells. Exp.Cell.Res. 2002 ; 278 : 72-83.
20) Paye M, Nusgenes B.V, Lapiere C.M. : Modulation of cellular biosynthetic activity in the retracting collagen lattice. Eur. J.Cell. Biol. 1987 ; 45 : 44-50
21) Nakagawa S, Pawelek P, Grinnell F. : Long-term culture of fibroblasts in contracted collagen gels : effects on cell growth and biosynthetic activity. J.Invest.Dermatol. 1989 ; 93 : 792-98.