Review Article

Importance of inflammation reaction of scaffold for the application of regenerative medicine

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It has been widely accepted that regenerative medicine including stem cell therapy and tissue engineering offers a huge potential technique to whole organ and tissue transplantation for diseased, failed or malfunctioned organs. There were already launched eighteen products in market after the approval of KFDA. However, these products were more developed cell therapy products rather than tissue engineered products. To reconstruct a new tissue by tissue engineering, three basic components such as (1) primary/stem cells, (2) biomaterials as scaffold substrates and (3) growth factors must be needed.

Among of these three key components, scaffolds as biomaterials might be played a very critical role in tissue engineered products. The function of scaffolds is to direct the growth of cells seeded within the porous structure of the scaffold or of cells migrating from surrounding tissue, eventually mimicking a natural extracellular matrix. For the scaffold materials, the family of poly (α-hydroxy acid)s are extensively clinically used or tested due to good biocompatibility, controllable biodegradabilitiy, and relatively good processability. The main reasons of the retard of FDA approval for tissue engineered products might be the induction of inflammation reaction in terms of safety. In our laboratory, we have been investigated the reduction of inflammation reaction of poly (α-hydroxy acid)s by the hybridization with natural biomaterials. In this review, poly (α-hydroxy acid)s/natural hybrid scaffold biomaterials have been introduced in order to reduce the host response resulting in approaching to a more natural three dimensional environment and supporting biological signals for tissue growth and reorganization of organ.

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Introduction

In Korea, there are launching in market as eighteen products related with regenerative medicinal products including cell therapy products and tissue engineered products (TEMPs) from 2001 up to now as listed in Table 1[1]. First approved one on 2001, Chondron™ by SewonCellon Tech Co Ltd as autologous chondrocytes for the treatment chondyle defects have been sold steadily around last 10

Table 1 List of approved cell therapy products by KFDA in Korea (Feb 2012, 18 items)

Order	Trade name	Company	Category	Characteristic	Target disease	Clinical approval date	Condition of approval
1	Chondron	SewonCellontech	Autologous	Chondrocyte	Knee cartilage deletion	Jan. 30, 2001.	Conditional approval of phase 3 (Completion in 2008)
2	Holoderm	Tegoscience	Autologous	Skin keratinocyte	Skin burn therapy	Dec. 10, 2002.	Conditional approval of phase 3 (Completion in 2008)
3	Kaloderm	Tegoscience	Allogeneic	Skin keratinocyte	Skin burn therapy	March 21, 2005.	Approval after completion of phase 3
4	Keraheal	MCTT	Autologous	Skin keratinocyte	Skin burn therapy	May. 03, 2006.	Conditional approval of phase 3
5	Inno-Lak	Innomedisys	Autologous	Activated lymphocyte	Non-small cell lung cancer	Feb. 14, 2007.	Conditional approval of phase 3
6	CreaVax-RCC Inj.	Creagene	Autologous	Dendritic cell	Metastatic renal cell carcinoma	May. 15, 2007.	Conditional approval of phase 3
7	Adipose	Antrogen	Autologous	Immature fat cell	Depressed scar	Aug. 03, 2007.	Approval after completion of phase 3
8	Immuncell-LC	Innocell	Autologous	Activated lymphocyte	Hepatoma	Aug. 06, 2007.	Conditional approval of phase 3
9	NKM	NKBIO	Autologous	Activated lymphocyte	Malignant lymphoma	Aug. 07, 2007.	Conditional approval of phase 3
10	Hyalograft 3D™	Cha Bio & Diostech	Autologous	Skin fibroblast	Diabetogenous tinea ulcer	Sept. 14, 2007.	Conditional approval of phase 3
11	RMS Ossron	SewonCellontech	Autologous	Osteocyte	Local osteoblastic palpation	Aug. 26, 2007.	Approval after completion of phase 3
12	AutoStem	Cha Bio& Diostech	Autologous	Minimum operation fat cell	Remediation of hypodermic fat defective location	Feb. 01, 2010.	-
13	Queen cell	Antrogen	Autologous	Minimum operation fat cell	Remediation of hypodermic fat defective location	March.26, 2010.	-
14	Cure skin	Sbiomedics	Autologous	Fibroblast	Remediation of depressed scar location with acne	May. 11, 2010.	-
15	LSK Autograft	Cha Bio & Diostech	Autologous	Skin keratinocyte	Skin burn therapy	Sept. 17, 2010.	-
16	Hearticellgram	Pharmicell	Autologous	Bone marrow stromal cells.	Patients with acute myocardial infarction	July. 01, 2011.	-
17	Cartistem	Medipost	Allogeneic	Mesenchymal stem cells derived from human umbilical cord blood	Osteoarthrisis	Jan. 18, 2012.	-
18	Cupistem	Antrogen	Autologous	Mesenchymal-like stem cells derived from the adipose	Crohn's disease	Jan. 18, 2012.	-

years. Also, keratinocyte from autologous or allogenous as Haloderm™ and Kaloderm™ by TegoScience Co Ltd have been developed the treatment of burn patients resulting in relatively success treatment for patients during last 7～10 years, respectively. Very recently, Hearticellgram-AMI™ developed by FCB-Pharmicell Co Ltd was approved by the Korea Food & Drug Administration (KFDA) as the world's first stem cell treatment for clinical use for heart attack patients in July 2011. KFDA approved two treatments such as Medipost's Cartistem™ for the treatment of osteoarthrisis using allogenic adult bone marrow derived stem cell (BMSC) and Anterogen's Cupistem™ for the treatment of Crohn's disease using autologous adult adipose derived stem cell (ADSC), registering both as the world's second and third authorized stem cell procedures in Jan 2012. Among these, the Cartistem treatment is the world's first allogenic stem cell treatment which is advantageous because it has a higher mass-production potential and consistent treatment efficacy. Even though these activities might be still infancy for the industrialization for the regenerative medicinal products in Korea, it looks like a good sign in terms of the treatment for patients[2].

As listed in Table 1, commercialized products have been limited in cell therapy products rather than TEMPs since cell therapy can be only the injection of stem cell via vein or aorta to injury site. In order to replace malfunctioned organ, tissue engineering technique might be more effective rather than cell therapy techniques as simple injection of cells. To reconstruct a new tissue/organ by tissue engineering technique, three basic components such as (1) primary/stem cells, (2) biomaterials as scaffold substrates and (3) growth factors must be needed. Among of these three key components, scaffolds as biomaterials might be played a very critical role in TEMPs. The function of scaffolds is to direct the growth of cells seeded within the porous structure of the scaffold or of cells migrating from surrounding tissue, eventually mimicking a natural extracellular matrix (ECM)[3-8].

Main hurdle of commercialization for TEMPs

Around 1992 as 20 years ago, Advance Tissue Science Co (USA), now merged to Smith & Nephew Co., USA, had been submitted to approve to USA FDA for first cartilage TEMPs as autologous chondrocyte seeded in polyglycolic acid (PGA) nonwoven scaffold. At that time, no one had doubted to approve cartilage TEMPs since PGA was already approved by FDA in human clinical trial and chondrocyte was used autologous primary cell. At last, this product has been still retard up to approve FDA. Main reason might be in terms of safety. Implanted TEMPs have been reported to induce sequential events of immunologic reactions in response to injury caused by implantation procedures and result in acute inflammation marked by a dense infiltration of inflammation-mediating cells at the materials-tissue interface[3-8]. Prolonged irritations provoked by implanted biomaterials advance acute inflammation into chronic adverse tissue response characterized by the accumulation of dense fibrotic tissue encapsulating the implants as shown Figure 1[9].

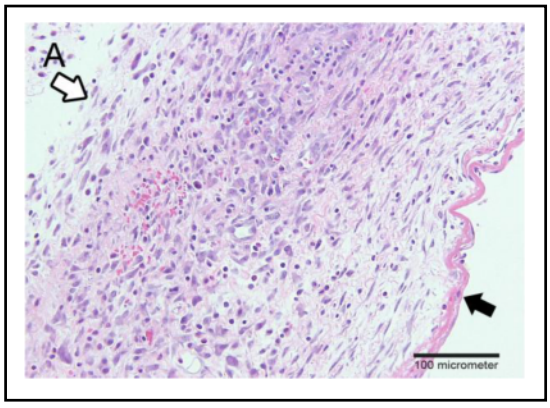

Fig.1 Photomicrographs of HE stain sections of the directly bordering tissue after PLGA [bar length=100 μm (x100)]

Note the 10,000 cell/cm^2 number of inflammatory cell and 500 μm of fibrous band thickness in vicinity to tissue implanted samples. Polymer-tissue interface surfaces are indicated by white arrow. The fibrous wall thickness was represented by black and white arrow.

PGA and poly(lactide-*co*-glycolide) (PLGA) are a member of a group of poly(α-hydroxy acid) that is among the few synthetic polymers approved for human clinical use by FDA. Consequently, it has been extensively used and tested for scaffold materials as a bioerodible material due to good biocompatibility, relatively good mechanical property, lower toxicity and controllable biodegradability. It has been clinically utilized for three decades as sutures, bone plates, screws and drug delivery vehicles and its safety has been proved in many medical applications. PLGA degrades by nonspecific hydrolytic scission of their ester bonds into their original monomer, lactic acid and glycolic acid. During these processes, there is very minimal sys-

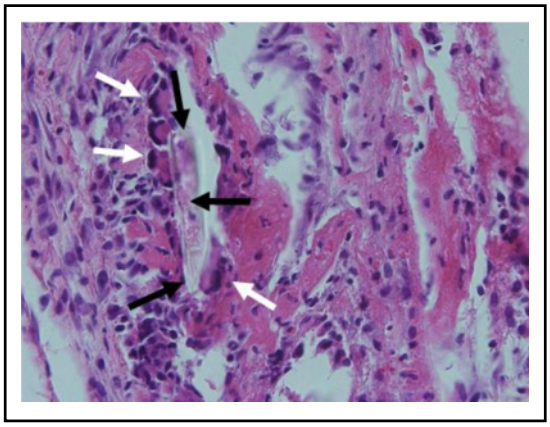

Fig.2 Foreign body granuloma
Small PLGA debris (black arrows) broken off from the PLGA film and is surrounded by macrophages and multinucleated giant cells (white arrows). These induced macrophages and multinucleated giant cells were remaining over 2 months.

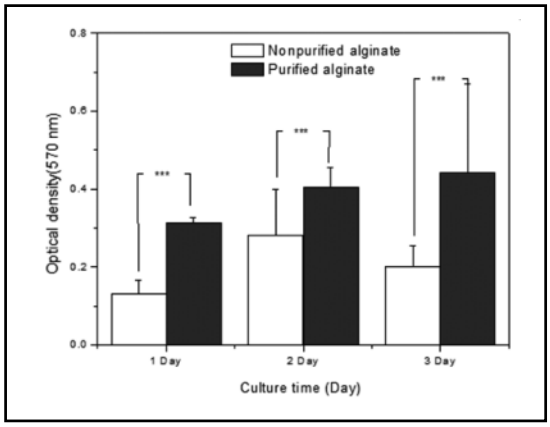

Fig.3 NIH/3T3 fibroblast cell proliferation in purified and non-purified alginate film after 1, 2 and 3 days *in vitro*

temic toxicity, however, in some cases, their acidic degradation products can decrease the pH in the surrounding tissue that result in local inflammatory reaction and potentially poor tissue development as shown in Figure 2[10]. Also, its poor mechanical strength, small pore size and hydrophobic surface properties for cell seeding have limited its usage.

Currently, biomaterials are endowed with biocompatibility through three different methods which are: (i) coating with hydrophilic molecules, (ii) modifying surface characteristics using physiochemical methods and (iii) impregnating bioactive substances. Previous reports showed that application of mineral layer or localized delivery of anti-inflammatory agent such as corticosteroid with cytokine could be effectively suppressed inflammation and fibrosis of implant[11]. Although the methods of such studies are experimentally available, it is usually complicate to prepare the implants, and adverse effects of a specific growth factor have not been clearly defined. In addition, the mechanisms by which PLGA induces local inflammatory responses have not been discussed sufficiently[12].

In our laboratory, the natural/synthetic hybrid scaffolds have been investigated during the last 15 years such as small intestine submucosa (SIS)[12,13], demineralized bone particles (DBP)[10,14], DBP gel[15], fibrin[16], keratin[17], hyaluronic acid[18], collagen gel[6], silk[19] and a 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer (PMEH)[20,21], alginate[22] with PLGA to reduce cellular inflammatory response of PLGA. In this review, we introduced the effect of purification for alginate to inflammation reaction, DBP/PLGA and SIS/PLGA hybrid scaffold in terms of scaffold design for the reduction of host response and the augmentation of tissue formation.

The effect of purification for alginate to inflammation reaction[22]

Alginate, a polysaccharide extracted from brown seaweed, remains the most widely used biomaterial for immobilizing cells to be transplanted, because of the good viability of encapsulated cell and the relatively ease processing for cell encapsulation. However, the main drawback might be the immune rejection *in vivo*. To overcome this problem, we have developed using modified *Korbutt's* method for alginate purification. NIH/3T3 fibroblast and RAW 264.7 macrophage cells were seeded in purified and non-purified alginate film, and then cell viability were analyzed by MTT assay in Figure 3. Cell proliferation on each group of alginate films increased gradually from 1 day to 3 days. At all culture periods, there was significant higher cell proliferation on purified alginate films than that of non-purified alginate films.

Figure 4 shows the inhibitory effect of alginate films on the NO production in activated cells. The results of purification process were not shown the significant difference between the endotoxin content within purified alginate and non-purified alginate. NO production was slightly higher non-purified alginate film than purified alginate film. The production of NO by macrophages in response to alginate

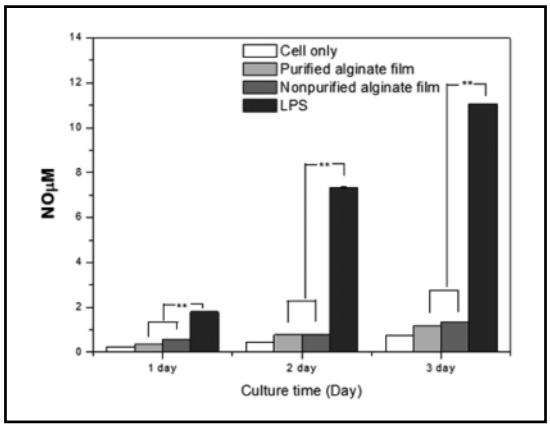

Fig.4 Reduction of NO production by purified and non-purified alginate film after 1, 2 and 3 days *in vitro*

film has revealed an indication of an inflammatory immune response. NO production of macrophage cells were not affected by stimulation of the alginate film.

RT-PCR was performed to assess mRNA expression for inflammation cytokines such as IL-1β and 1L-6 in Figure 5. The level of IL-6 mRNA expression was more highly expressed on non-purified alginate film than on purified alginate film on 1 and 2 days. At day 3, the band of the level for expression of IL-6, was observed similar between non-purified and purified alginate film. However, the expression of IL-6 was observed differently between two groups, the expression of IL-6 showed up higher in the non-purified alginate film than purified alginate film at day 4. Also, from the results of the expression of IL-1β, it showed up significantly in the non-purified film only at day 1 and 2. The reason of these results might be the increase of guluronic acid content and decrease of mannuronic acid content through the purification process. Mannuronic acids stimulate monocytes to produce TNF-α, IL-1β and IL-6 as well as lipopolysaccharide (LPS) was stimulate cytokine secretion in macrophages. Because L-glycero-D-manno-heptose and 3-keto-3-deoxy-D-mannooctulsonic acid was similar to structure of mannuronic acid, non-purified alginate film showed more strong IL-1β and IL-6 expression than purified alginate film.

Purified and non-purified alginate films were implanted into a Wistar rat and the implanted alginate film were extracted after 1 and 2 weeks. Tissues surrounding implants were harvested and evaluated by histology through H&E staining and ED-1 immunohistochemistry staining in Figure 6. The recruitment of inflammatory cells was assessed by

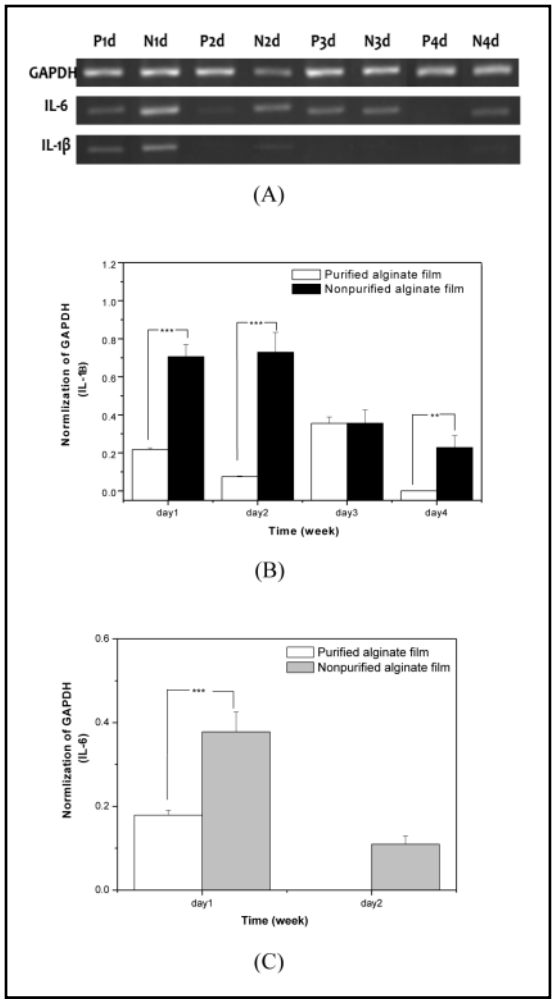

Fig.5 Gene expression profiles of GAPDH, IL-6 and IL-1β as analyzed by RT-PCR on day 1, 2, 3 and 4

(A) The result of agarose gel electrophoresis normalization of by (B) IL-1β and (C) IL-6 by GAPDH.

counting the number of neutrophils, macrophages and foreign body giant cells infiltrated into surrounding tissue of the implant. ED-1 immunohistological staining showed that numerous macrophages were present in the host tissue as well as at the edges of the non-purified alginate films. Non-purified alginate film revealed more inflammatory cells and more thick the fibrotic wall thickness from H&E staining. And from the result of ED-1 staining, we confirmed that non-purified alginate film had more inflammatory cells and fibrotic thickness surrounded the alginate film. Thus, inflammation was most likely caused by specific graft rejection of the non-purified alginate film, whereas the immunoreactivity of the purified alginate film was much lower. In this result, contaminants in alginate were removed by

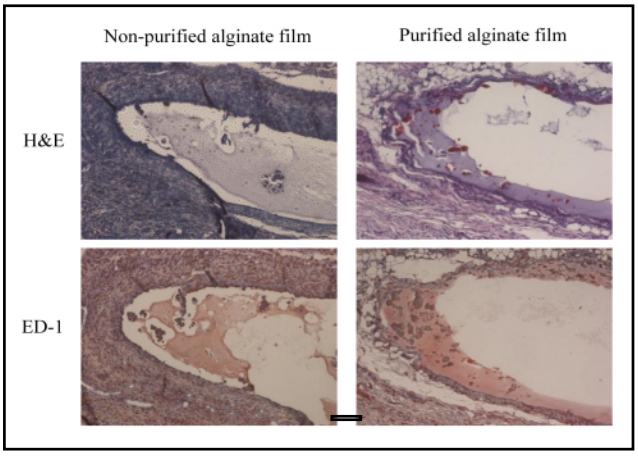

Fig.6 H&E and ED-1 immunohistochemistry staining in non-purified and purified alginate film after 2 weeks of implantation *in vivo* (magnification, x100)

the purification process resulting in the reduction inflammation reaction.

Hybridization of DBP decreased the inflammation reaction of PLGA[9,14,15]

DBP have long been recognized as a powerful inducer of new bone growth. Many authors reported that this osteoinductive property is mainly due to bone morphogenetic proteins (BMPs). We demonstrated that DBP enhanced hydrophilicity of PLGA scaffold with an increase of content and reduced adverse cellular response associated with inflammation. We focused our attention on the early stages of inflammatory reaction and used histological and molecular analyses to assess how cells and tissue responded to DBP-PLGA hybrid materials *in vivo* and *in vitro*. We evaluated the effect of five different ratios DBP/PLGA hybrid materials on cellular inflammatory response and tissue reaction induced by PLGA.

To elucidate the cellular responses associated with inflammation on PLGA/DBP films, we measured the level of mRNA expression of TNF-α and IL-1β from HL-60 cell in 48 hours after culture with PLGA or DBP/PLGA films as shown in Figure 7. TNF-α mRNA in HL-60 highly expressed following PLGA film, as compared to DBP/PLGA films; it was significantly lower following DBP/PLGA films than PLGA film with increases in contents of DBP, 10, 20, 40 and 80% of DBP. The intensity of TNF-α expression of PLGA film was significantly ten times higher or more than that of 40% DBP/PLGA films. HL-60 cell with 80% DBP/

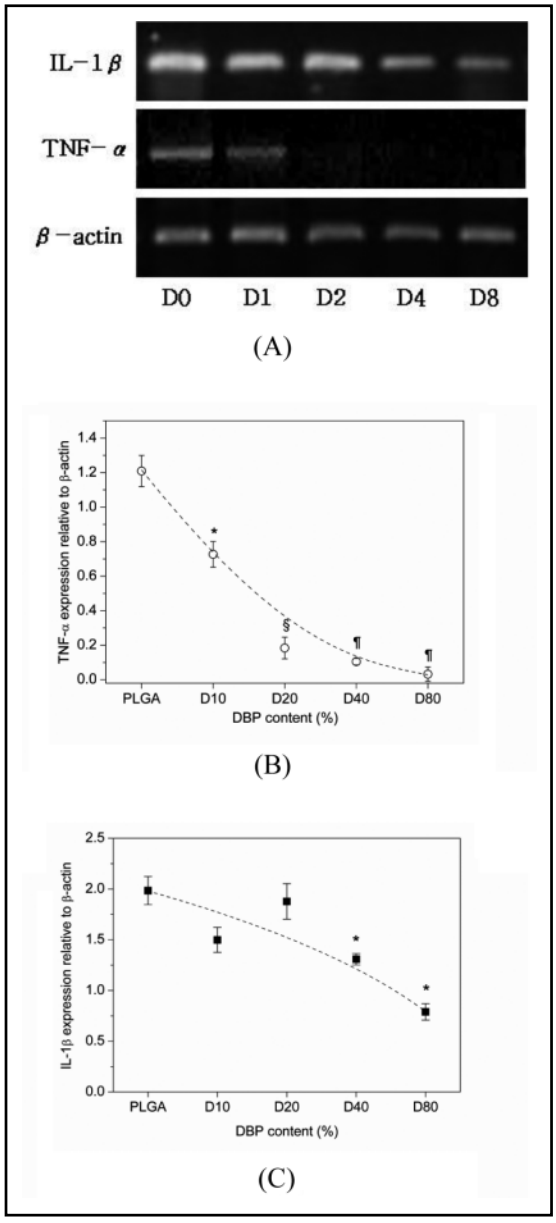

Fig.7 Gene expression profiles of IL-1β and TNF-α as analyzed by RT-PCR

(A) Representative gene expression band as (B) TNF-α, (C) IL-1β. The expression of IL-1β and TNF-α mRNA on HL-60 cells fell continuously with an increase in content of DBP in PLGA.

PLGA film rarely expressed TNF-α mRNA. Similarly, IL-1β mRNA expression decreased markedly with 40% and 80% DBP/PLGA film compared to PLGA film. No significant differences of IL-1β mRNA expression were observed between PLGA, 10% DBP/PLGA and 20% DBP/PLGA.

To further characterize *in vivo* inflammatory response surrounding the implants, histological examination was

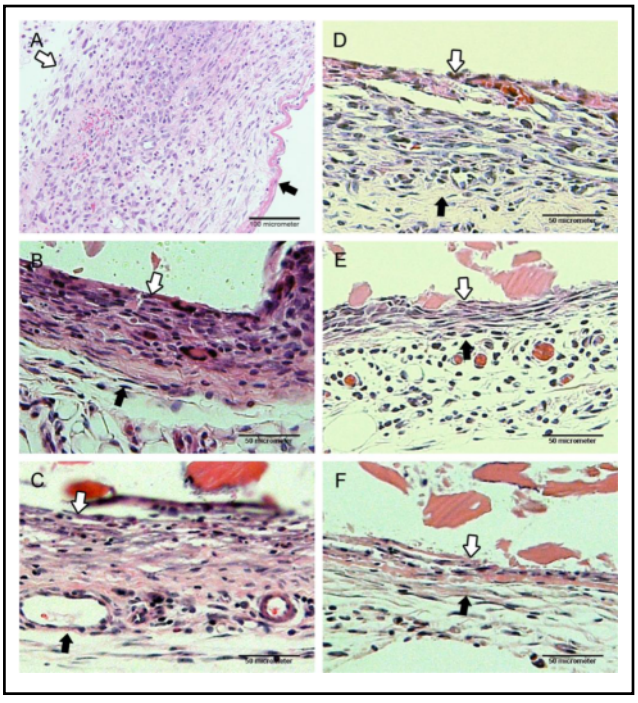

Fig.8 Photomicrographs of HE stain sections of the directly bordering tissue after PLGA, DBP hybrid PLGA films and DBP

(A) shows the tissue implanted with PLGA, bar length = 100 μm (x100), (B)-(F) show the tissue implanted with 10% DBP/PLGA, 20% DBP/PLGA, 40% DBP/PLGA, 80% DBP/PLGA and DBP, respectively, bar length = 50 μm(x400). Note that the number of inflammatory cell and fibrous band thickness in vicinity to tissue implanted samples was decreased as DBP content in PLGA film was increased. Polymer-tissue interface surfaces are indicated by white arrow. The fibrous wall thickness was represented by black and white arrow.

performed at day 5 after implantation. Remarkable inflammation was observed in tissue surrounding the PLGA film; however this inflammatory reaction was progressively diminished with an increase in contents of DBP in PLGA film. We observed numerous recruited neutrophils infiltrates with a large number of multinucleated giant cells (MNGCs) adjacent tissue after PLGA film implantation. However, this inflammatory cellular response decreased as content of DBP continuously increased in PLGA film. The density of inflammatory cell following PLGA film implantation was approximately two times higher than that following 40% DBP/PLGA film. The DBP film had fewer inflammatory cells relative to the PLGA film. The fibrotic thickness was significantly decreased in DBP/PLGA hybrid and DBP film. PLGA film had five times or more broad fibrotic band than the other samples. In 40% or 80% DBP/PLGA hybrid film,

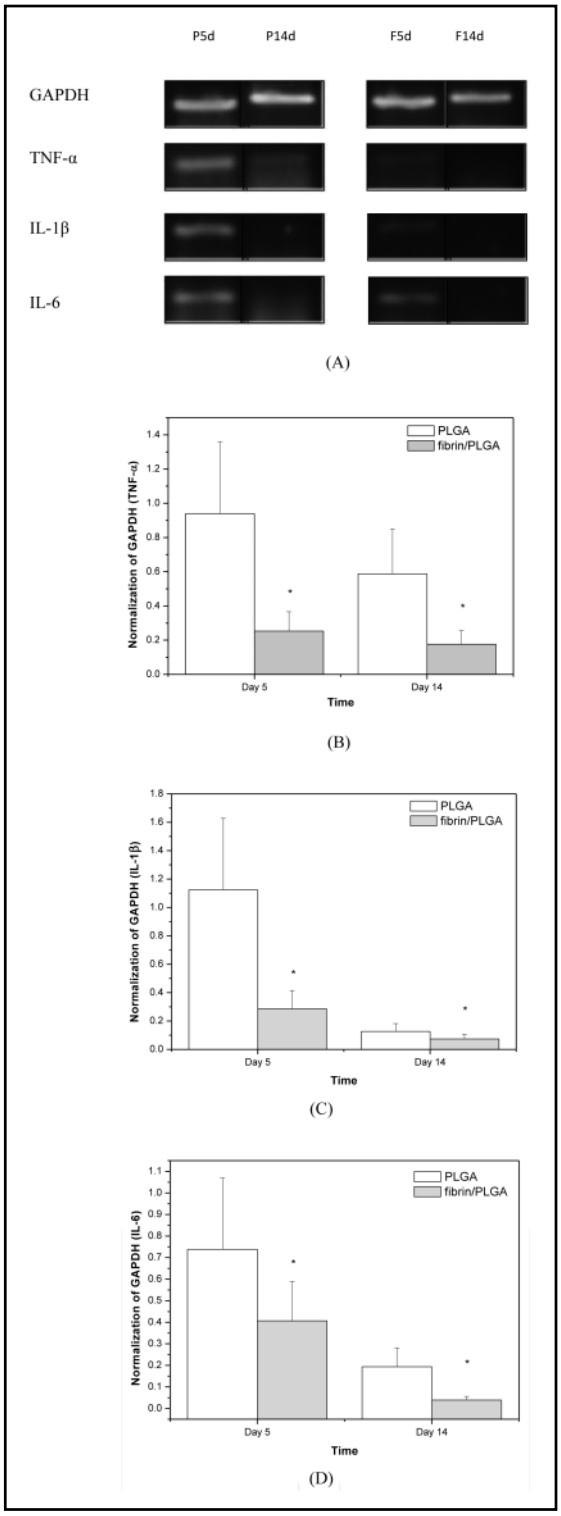

Fig.9 Gene expression profiles of GAPDH, TNF-α, IL-1β and IL-6 as analyzed by RT-PCR on day 5 and 14

(A) The result of agarose gel electrophoresis Normalization of by (B) TNF-α, (C) IL-1β and (D) IL-6 by GAPDH.

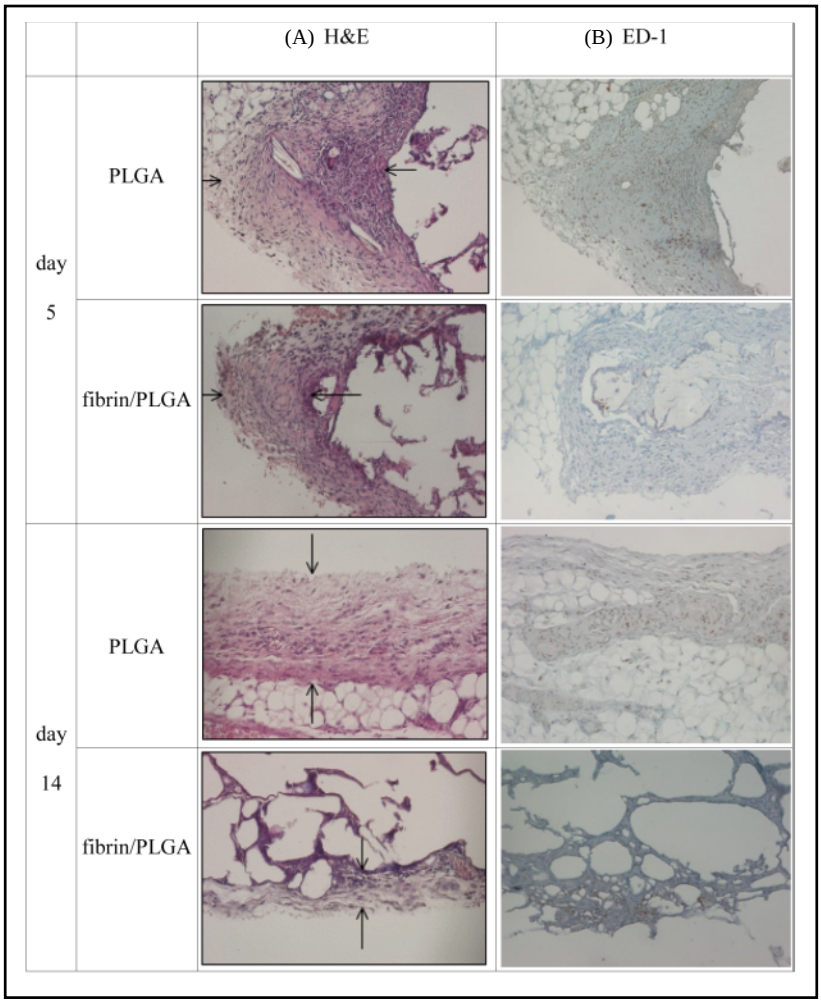

Fig.10 H&E and ED-1 immunohistochemistry staining in scaffold after 5 and 14 days of implantation

(A) The staining of H&E image (magnification, x40), (B) ED-1 immunohistochemistry staining in PLGA and fibrin/PLGA scaffolds after 5 and 14 day of implantation *in vivo*. (magnification, x200)

macrophages or foreign body giant cells were rarely observed in immediate contact with DBP fragment surface and a thin collagenous fibrous band surrounded the samples. The DBP film seldom recruited MNGCs compared to PLGA or DBP/PLGA hybrid film (Fig.8).

In this study, we observed that DBP hybrid PLGA materials did not significantly affect the viability of HL-60 cells during 3D *in vitro* culture, however, their inflammatory response to DBP hybrid polymeric materials was obviously reduced. The probable cause for these results is that TNF-α expression may be strongly suppressed by bioactive substances released from DBP; this pattern was enhanced with an increase in content of DBP in PLGA film, which can result in suppression of the IL-1β expression that reduces proliferation and fibrous capsular formation of fibroblasts.

In this study, we have shown that by impregnating DBP in the PLGA materials, the inflammatory reaction could be effectively reduced *in vivo* and *in vitro*. This result suggests that hybridization of natural materials such as DBP is suitable for control of an adverse tissue reaction of polymeric materials shown *in vivo* application.

Hybridization of fibrin decreased the inflammation reaction of PLGA[16,23)]

To complete disadvantage on implantation of PLGA, we used fibrin as natural material. Fibrin has many advantages such as the abundance of fibrinogen, their relatively ease of its purification. This study was designed to investigate the effect of fibrin as a natural material on the local inflammatory reaction of PLGA *in vivo*. To evaluate the influence of fibrin content in PLGA materials on inflammatory cytokine expression, RT-PCR used for TNF-α, IL-1β and IL-6 (Fig.9). As shown in Figure 9, inflammatory cytokine expressions as TNF-α, IL-1β and IL-6 were significantly decreased. Fibrotic wall thickness and macrophage were evaluated by H&E and ED-1 immunohistochemical stain-

ing, respectively (Fig.10). It also shows that fibrotic wall thickness and number of macrophage were significantly decreased after coating of fibrin into PLGA scaffold. In this study, we showed that fibrin/PLGA scaffolds reduction of inflammatory reaction were superior to PLGA scaffold in terms of reduction of inflammatory reaction. We concluded that fibrin could reduce inflammatory response of PLGA.

The phenomena of decreasing of inflammatory reaction of PLGA also have been found the hybridization with natural polymer as silk, keratin, small intestine submucosa (SIS), DBP gel, fibrin, keratin, hyaluronic acid and collagen gel from our previous studies.

Conclusion

Tissue engineering including regenerative medicine shows tremendous potential as a revolutionary research push. Also, many successful results have been reported the potential for regenerating tissues and organs such as skin, bone, cartilage, nerve of peripheral and central, tendon, muscle, corneal, bladder and urethra, and liver as well as composite systems like a human phalanx and joint on the basis of scaffold biomaterials from polymers, ceramic, metal, composites and its hybrids. The prerequisite physicochemical properties of scaffolds are (i) to support and deliver for cells, (ii) to induce, differentiate and conduit tissue growth, (iii) to target cell-adhesion substrate, (iv) to stimulate cellular response, (v) wound healing barrier, (vi) biocompatible and biodegradable, (vii) relatively easy processability and malleability into desired shapes, (viii) highly porous with large surface/volume, (ix) mechanical strength and dimensional stability, (x) sterilizability, (xi) do not induce inflammatory reaction and fibrotic capsule and so on.

From this point of view, the design and control for the suppression of inflammatory reaction to host tissue must be needed by the combination of scaffold matrix and bioactive molecules including genes, peptide molecules and cytokines for the application of patients. Moreover, the combination of the cells and redesigned bioactive scaffolds has attempted to expand to a tissue level of hierarchy. In order to achieve this goal, the novel hybrid scaffold biomaterials, the novel scaffolds fabrication methods and the novel characterization methods must be developed.

Acknowledgement

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Conflict of interests

None

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