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EVALUATION OF THE IMMUNO-PROTECTIVE EFFECTS OF THE NEW-TYPE OF BAGS USING ELISA- AND FACS-ANALYSIS.

RAHMAN MS, YASMIN BANU, ATSUKO MATSUOKA, AKIRA ICHIKAWA, MASAMUNE SAKAI*, HIROYUKI IKEDA* AND TOSHIE TSUCHIYA.
National Institute of Health Sciences, Division of Medical Devices, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.
**UBE Industries, LTD. Polymer Laboratory (Chiba), 8-1, Goi Minamikaigan, Ichihara City, Chiba 290-0045, Japan*

Abstract

This study investigated the usefulness of modified polyurethane (MPU) coating micropore membrane bags for diminishing the immunological responses following organ or tissue transplantation in allogeneic setting. Spleen from Brown Norway (BN) rats (donor) were placed into the peritoneal cavity of Lewis rat (recipient) either directly or inside of MPU coated bags. Lewis rat with Sham's operation served as control. After 12 and 24 weeks, cytokines of IL –4, IL –13, TNF –α and IFN -γ, and flow cytometric evaluations for $CD4^+$ and $CD8^+$ cells of the recipient blood were carried out. TNF –α levels proved polyurethane coating effective in reducing inflammatory reaction at 12 weeks. Twelve week IFN -γ and, $CD4^+$ and $CD8^+$ cells indicated that graft-versus-host-reaction (GVHR) took place but polyurethane coated bag did not prevent or reduced this reaction. Thus, this study shows that MPU coating might be functional in preventing inflammatory reaction but is not useful for preventing GVHR.

1. Introduction

Polyurethanes form a versatile and useful class of polymers. As biomaterials, their uses have included the artificial heart, catheters, and synthetic blood conduits.[1] Rejection and graft-versus-host disease are common as the solid organ transplantation and potential immunosuppressive drugs are routinely used after organ transplantation. Unfortunately, the risks of opportunistic infections, lymphoblastic malignancy and metabolic complications are frequently associated with immunosuppressive therapy.[2-4] However, following most of the allotransplants, cell traffic seems to be a striking event with all transplants.[5] Donor cells leaving the solid organ and recipient cell entering it include passenger leucocytes that were shown to be the main cause of allograft immunogenicity. TNF -α and IFN -γ are pro-inflammatory Th1-type cytokines, mediate cellular immune responses and have been shown to be involved in allograft rejection. On the other hand, Th2-cytokine IL –4 and IL -13 played their role in promoting graft survival has been suggested in animal models.[11] Donor $CD4^+$ and $CD8^+$ cells that traffic to recipient, play an important role in the immunogeneic outcome of the host tissue. The objective of this examination was to evaluate the effect of modified polyurethane (MPU) coated micropore membrane bag to eliminate or reduce the immunogenicity in an *in vivo* study using rat model.

2. Materials and Methods

2.1. Polyurethane coated bag

Bags with a size of 2 x 1.5 cm were made from MPU coated micropore membrane. The outer surface of the bag were coated by MPU. Bags were sterilized and turned into the hydrophilic characteristic bags by gradually dipping in 100 %, 90 %, 80 %, 70 %, 50 % and 25 % ethanol aquous solution. Each step lasted for a half day and finally washed by distilled water and phosphate buffer saline.

2.2. Animals and Experimental Groups

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Eight-week-old Lewis and Brown-Norway (BN) female rats were obtained from Charles River Japan Inc. Kanagawa, Japan. Rats were maintained in an air-conditioned animal facility at the national Institute of Health Sciences. The principles of laboratory animal care (according to NIH Publication No. 85-23, revised 1985) were carefully followed in this study. The rats were fed with a commercial diet and water *ad libitum*, pre- and post-implantation periods. Spleens from BN rats (donor) were placed into the peritoneal cavity of Lewis rats (recipient) either directly or inside of MPU coated bags. Lewis rat with Sham's operation served as control. In another group, only MPU coated bag was placed into the peritoneal cavity.

2.3. Operative Procedures

The donor, under sedation with ether was anesthetized by intraperitoneal administration of pentobarbital sodium (20 mg/ kg body weight). After proper sterilization, peritoneal cavity was opened through a ventral midline incision. From the BN rats spleens were removed and washed with PBS and immediately placed into the peritoneal cavity of Lewis rats, either directly or inside of a MPU coated bag containing 1 ml of RPMI 1640 medium (Dutch modification, Gibco BRL, Life Technologies Ltd., Paisley, Scotland). In bag group, only bag containing 1 ml of the medium was placed into the Lewis peritoneal cavity and in control group, Sham's operation was performed. After the designated experimental period, rats were anesthetized in the same fashion as the initial operation. On opening the abdomen, spleens were collected (12 weeks post implantation only) and blood was collected from the descending abdominal aorta of recipient and then the rats were sacrificed.

2.4. Cytokines Assay

After 12 and 24 weeks of implantatiohn, blood collected from the recipient was centrifuged and supernatant were stored at –80°C until cytokines were measured. Cytokines levels of IL- 4, IL – 13, TNF- α and IFN- γ were measured using conventional ELISA assay (Biosource International, Inc., CA, USA) according to the manufacturer's instruction. Cytokine concentrations were calculated using manufacturer supplied cytokine standards and expressed in pg/ml.

2.5. Flow Cytometry

The analysis for $CD4^+$ and $CD8^+$ cells was carried out by flow cytometry. Anti-coagulant treated venous blood samples were analyzed with two-color flow cytometry to determine the percentages of $CD4^+$ and $CD8^+$ T cells. Briefly, 100 µl of heparin treated venous blood were incubated with 20 µl of the indicated FITC-labeled anti-CD4 and PE-labeled anti-CD8 mAb, vortexed vigorously and incubated at room temperature for 45 min. Following washing with 4 ml PBS two times, and futher washing with 1 ml of immuno-lyse working solution and 250 µl of fixative was added within 30 sec to 2 min. After washing by PBS solution two times, the pelleted cells were analyzed with an EPICS XL II cytometer (Beckman-Coulter, Margency, France). The analysis was focused on lymphocytes, identified by their forward and right angle scatter features. At least 10000 events were collected in the lymphocyte gate and analyzed.

2.6. Antibodies

Rat mAb FITC –conjugated anti CD4 (IM3056) and PE –conjugated anti CD8 were used in this study. These mAb and their isotype-matched negative control mAb [mouse IgG1 –FITC (IM0639) and IgG1 –PE (IM0670)] were purchased from Beckman Coulter Immunotech (Marseille, France).

2.7. Statistical analysis

Data are presented as mean ± SD. Values of different experimental groups were analyzed with a paired t test. Differences were considered statistically significant at $p < 0.05$).

3. Results

3.1. Macroscopic findings

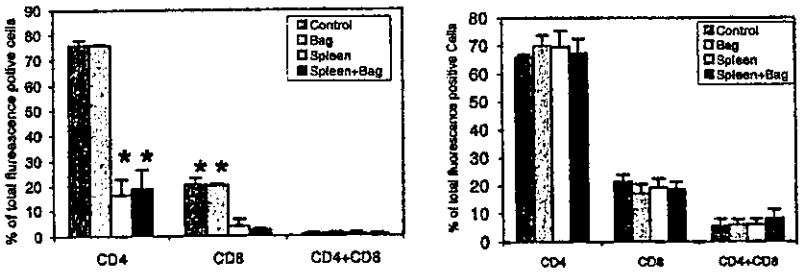
At 12 weeks, spleens implanted either directly into the peritoneal cavity or inside of MPU coated bags were collected and observed. The spleens shrinked and weight of the spleens that were directly implanted into the peritoneal cavity was more reduced as compared with the weight of the spleens that were implanted inside the polyurethane coated bags.

3.2. Cytokines Expression

	12 week				24 week			
	C	B	S	BS	C	B	S	BS
TNF-	-	↑	↑↑	↑	-	-	±	±
IL-4	-	-	-	-	-	-	-	-
IL-13	-	-	-	-		ND		
IFN-γ	-	↓	↓	↓		ND		

Table 1. C, control; B, bag; S, spleen; BS, spleen inside bag. N.D., not done; ↑ , increase; ↓ , decrease; -, no increase than control; ± , no or almost no increase than control..

Expressions of various cytokines are summarized in Table 1. Expressions of TNF -α at 12 post-implantation weeks were increased in all groups as compared to control group. Highest expression of TNF -α was detected in spleen group, followed by the group of spleen inside of the bag. There was no or almost no increase in TNF -α expression among various groups as compared to control at 24 post-implantation weeks. IL -4 expression at 12 and 24 post-implantation weeks and IL -13 expression at 12 post-implantation weeks were similar among all experimental groups. Expression IFN -γ at 12 post-implantation weeks were decreased in all experimental groups when compared with that in the control group.



(a). 12 weeks implantation (b). 24 weeks implantation

Figure 1. FACS analysis for $CD4^+$ and $CD8^+$ lymphocytes of recipient blood. (a) 12 weeks implantation, (b) 24 weeks implantation. Significantly different from the controls at $^*p < 0.05$.

3.3. Flow Cytometry

At 12 post-implantation weeks, number of $CD4^+$ cells were significantly decreased in spleen-group and the group of the spleen involved inside of the bag (Spleen+Bag) and the maximum decrease was found in spleen group. $CD8^+$, and both $CD4^+$ and $CD8^+$ cells were also decreased in spleen gruoup and the group of spleen involved inside of the bag [Fig. 1(a)]. There was no difference in $CD4^+$, $CD8^+$, and both $CD4^+$ and $CD8^+$ cell numbers among various groups at 24 post-implantation weeks [Fig. 1(b)].

4. Discussion

In allorecognition, the specific activation of T cells is initiated by the binding of foreign alloantigen and/or host MHC molecule complex to T-cell receptors. The activated helper T cells secrete lymphokines, which induce the proliferation and maturation of activated cytotoxic T cells. In addition, helper T cells secrete IFN -γ, which induces cell surface expression of MHC antigens and activates macrophages. At 12 weeks following spleen implantation, inflammation was noted in all groups except control group and the polyurethane bags exhibited some protective effect for the implanted organ. The donor spleen T cells were cytotoxic to recipient T cells and resulted a decrease in secretion of IFN -γ in 12 week post-implantation recipient serum and decreased the number of recipient $CD4^+$ and $CD8^+$ T cells. At 24 weeks of post transplantation the spleens placed directly into the peritoneal cavity were absorbed by phagocytosis and those inside the bags were completely necrosed. Therefore, there was no effective source of donor cytotoxic T cells to act on recipient T cells and no difference of $CD4^+$ and $CD8^+$ T cells number was observed among different groups at 24 weeks of post implantation. No difference in cytokines TNF -α and IL -4 among different groups of implantation were consistent with the fact that there was no effect of donor T-cells. Also, no inflammatory reaction was observed at 24 weeks of post-implantation. As to the best of our knowledge this study is the first to investigate the role of MPU coating in preventing inflammatory/immunological responses in allogeneic setting, we could not compare our data with other study. However, our data are in agreement of our in vitro study where polyurethane coated bag increased the viability of donor bone-marrow lymphocytes and diminished immunological responses by recipient blood lymphotytes (unpublished data).

This study suggests that MPU coated bag is helpful in diminishing the inflammatory response but played no part in preventing the immunological-reaction in allogeneic settings. Further, this analysis serves as an early indicator in diminishing inflammatory reactions and more experiments are necessary to improve our knowledge of the potential role of MPU coating as anti-inflammatory therapeutic agent or as a coating agents of biomaterials.

5.Acknowledgements

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DIFFERENT EXPRESSION OF GAP JUNCTIONAL PROTEIN CONNEXIN43 IN TWO STRAINS OF MICE AFTER ONE-MONTH IMPLANTATION OF POLY-L-LACTIC ACID

SAIFUDDIN AHMED, TOSHIE TSUCHIYA.

Division of Medical Devices, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya ku, Tokyo 158-8501, Japan.

Abstract. The implantation of a biomaterial often induces host inflammatory responses. Some adverse effects by the biomaterials, such as poly-L-lactic acid (PLLA) and polyurethanes (PUs) were reported in animal experiments. PLLA produced tumorigenicity in rats after long-term implantation. The purpose of this study was to determine the in vitro effect of PLAO3 (high-molecular weights of PLLA) and PU8 (PTMO/MDI/BD) on the function of the normal human dermal fibroblasts (NHDF) and the in vivo effect of PLAO3 on the function of the cells originated from the subcutaneous tissue in the two female mouse strains, BALB/cJ and SJL/J. The results with Scrape-loading and dye transfer (SLDT) assay, Western Blot and RT-PCR analysis clearly demonstrated that gap-junctional intercellular communication (GJIC) and the expression of Cx43 were significantly suppressed in PLAO3-implanted group of BALB/cJ mice in compared to the control mice. While, no significant difference was found in GJIC and the expression of mRNA level but a little bit difference was observed in the Cx43 protein expression between the SJL/J implanted and the control mice. We considered that the PLAO3 suppressed irreversibly gap junctional protein connexin43 at the earlier stage after implantation and the suppression of connexin43 gene-expression might play a vital role in the inhibition of GJIC and thus promotes the tumorigenesis.

Keywords: Poly-L-lactic acid, GJIC, Connexin43.

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1. INTRODUCTION

Some adverse effects caused by the biomaterials, such as poly-L-lactic acid (PLLA) and polyurethanes (PUs) were reported in animal experiments [1]. PLLA produced tumorigenicity in rats after long-term implantation. PUs were also used for implant applications because of their useful elastomeric properties and high tensile strength, lubricity, and good abrasion resistance. However, different kinds of PUs induced various tumor incidences in rats [2]. All tumors have been generally viewed as the outcome of disruption of the homeostatic regulation of cellular ability to response to extra-cellular signals, which trigger intra-cellular signal transduction abnormally [3]. We have hypothesized that the different tumorigenic potentials of PLLA and PUs are caused mainly by the different tumor-promoting activities of these biomaterials. In the present study, we investigated the effect of PLAO3 (a high-molecular weight PLLA) and PU8 on the normal human dermal fibroblast (NHDF). Our present results showed that the PLAO3 inhibited GJIC, whereas PU8 did not inhibit GJIC after 17 days culture on these materials. These findings inspired us to investigate the role of PLAO3 on the subcutaneous tissue of the two different responder strains of BALB/cJ and SJL/J mice.

2. MATERIALS AND METHODS

2.1. ***NHDF Cell culture:*** The NHDF cells were obtained from Asahi Techno Glass (Tokyo, Japan), and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5 % CO_2 atmosphere at 37°C.

2.2. ***Animals:*** Five-week-old female BALB/cJ and SJL/J mice were obtained from Charles River (Japan).

2.3. ***Implantation of PLAO3:*** PLAO3 (20 X 10 X 1 mm) was obtained from Shimadzu Co. Ltd., and sterilized by ethylene oxide gas prior to use. Sodium pentobarbital (4 mg/kg) was intraperitoneally administered to the mice. A dorsal incision of approximately 2 cm was made, opposite sites from the incision a subcutaneous pocket was formed by blunt dissection, and one piece of PLAO3 was placed in the pocket. The incision was closed with silk thread. In both strains, Sham's operation group served as controls. After 30 days, mice were sacrificed and subcutaneous tissues were obtained for subsequent culture.

2.4. ***Cell culture of subcutaneous tissues:*** The subcutaneous tissues were maintained in

minimum essential medium (MEM) supplemented with 10% FBS in a 5 % CO_2 atmosphere at 37°C. Cells were collected by trypsinization after adequate growth.

2.5. ***Giemsa staining:*** When cells reached confluence in tissue culture dishes, cells were fixed and stained with giemsa solution. Cells morphology was determined under an inverted light microscope.

2.6. ***Scrape-loading and dye transfer (SLDT) assay for detection of GJIC:*** Confluent monolayer cells, after rinsing with Ca^{2+} Mg^{2+} phosphate-buffered saline [PBS (+)] were loaded with 0.05% Lucifer Yellow (Molecular Probes, Eugene, OR, USA)/PBS (+) solution and scraped immediately with a sharp blade. After incubation for 5 min at 37°C, cells were washed three times with PBS (+) and the extent of dye migration length was measured using fluorescence microscope.

2.7. ***Western Blot analysis:*** Cells were lysed directly in 100 µl of lysis buffer (50 mM Tris-Hcl, pH 6.8, 2% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride). Equivalent protein samples were then prepared in 7.5 % SDS-PAGE sample buffer containing 2-ME and loaded on 7% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Cx43 protein was detected by anti-Cx43 polyclonal antibodies and ECL system.

2.8. ***RT-PCR analysis:*** Total cellular RNA was isolated from cultured cells in Trizol reagent (Life Technologies, Inc.) following the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA by reverse transcript (RT) using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech). Amplification was performed in a volume of 25 µl containing 1 µl of cDNA, 10 pmol of each primer, 0.625 unit of *Taq* polymerase (Promega, Madison, WI, USA) and 0.2 mM of each deoxynucleotide triphosphate. The amplified product was electrophoresis using 1.5% agarose gel and visualized with SYBR Green. GAPDH gene was amplified as internal control.

3. RESULTS

NHDF cells: Giemsa staining showed that the NHDF cell cultures predominantly formed a uniform monolayer of cells. All cultures maintained the elongated shape of NHDF cells. There was no difference in morphology among the control, PLAO3 and PU8. In SLDT, the GJIC was significantly inhibited in PLAO3-exposed NHDF cells in

compared to the controls. On the contrary, no difference was observed between the PU8 implanted and the control groups.

Mouse cells: Inverted light microscopy and Giemsa staining showed that the mouse cells in cultures formed a cis-cross pattern and caused decreased contact inhibition in BALB/cJ control group (Figure 1A). On the other hand, in SJL/J control group, cells were parallel and maintained the contact inhibition (Figure 1C). All cells in the implanted groups of both the strains, showed cis-cross pattern and the cells were piled up in BALB/cJ group more than in SJL/J group (Figure 1B and 1D).

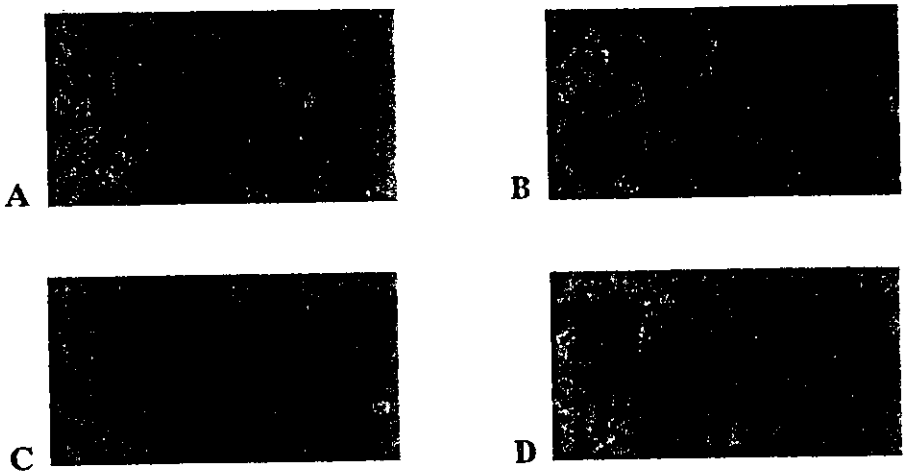

Figure 1 **Mice cells morphology. A: BAJB/cJ control, B: BALB/cJ implanted, C: SJL/J control and D: SJL/J implanted.**

In SLDT, the GJIC was significantly inhibited in PLAO3-implanted BALB/cJ cells in compared to BALB/cJ controls. No difference was observed between the PLAO3-implanted SJL/J and its controls. To clarify the cause, we also examined the mRNA and protein expression levels of connexin43 gene and found that the mRNA and protein expression were suppressed in PLAO3-implanted BALA/cJ mice in compared to BALA/cJ controls. No difference was observed between the PLAO3-implanted SJL/J and SJL/J controls.

4. DISCUSSION

Many factors, that caused tumorigenesis were known, we especially paid attention to the inhibition of the GJIC in the PLAO3-exposed cells. PLAO3 is a widely used

biomaterial for medical and surgical implants. Gap junctions are transmembrane channels that allow the cell-cell transfer of small molecules and are composed of protein subunits known as connexin; at least 19 connexins exist and they are expressed in various kinds of tissues of rodents. Several tumor promoters have been shown to inhibit GJIC by phosphorylation modification of connexin proteins. Connexins are essential proteins to maintain the gap junctional channel [4]. To understand the mechanisms of tumorigenesis induced by PLAO3, we paid attention to the inhibitory effects on GJIC. GJIC is important for normal differentiation of the cells such as neurons and osteoblasts. In the present study, the GJIC was inhibited in PLAO3-exposed NHDF and -implanted BALB/cJ mouse cells. This perturbed gap junction is most likely to play the major role in the PLAO3-induced tumorigenesis. Our results also showed that the mRNA and protein expression of connexin43 gene were suppressed in PLAO3-implanted BALB/cJ mice. Together with these results, we speculated that the inhibitory effect of PLAO3 on GJIC might be due to the alteration in the connexin43 protein. The post-translational modification and decrease in the connexin43 protein has been shown to be involved with impaired GJIC and could be associated in tumorigenesis mechanism. All experiments will be further analyzed at 6 and 12 months after PLAO3 implantation and these experimental data will give us the basic information that are useful for understanding the adverse event induced by medical and surgical implants.

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BIOCOMPATIBLE BIOMATERIALS FOR THE HUMAN CHONDROCYTE DIFFERENTIATION ESTIMATED BY RT-PCR METHOD.

TOSHIE TSUCHIYA, MASAMUNE SAKAI*, HIROYUKI IKEDA*, TADAHIKO MASHINO AND YASMIN BANU.**

National Institute of Health Sciences, Division of Medical Devices, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.

**UBE Industries, LTD. Polymer Laboratory (Chiba), 8-1, Goi Minamikaigan, Ichihara City, Chiba 290-0045, Japan*

***Kyoritsu College of Pharmacy,1-5-30 shibakouen, minato-ku, Tokyo 105-8512, Japan.*

Abstract

Biocompatibility of the biomaterials for the differentiation of the human articular chondrocytes were estimated by reverse transcription-polymerase chain reaction (RT-PCR). We used five biodegradable polymers for culturing with human articular chondrocytes. In addition to these five materials, we also estimated aquous type of fullerene, namely C60 dimalonic acid (C60DMA). Cultures were carried out using micromass culture method for 4 weeks. Collagen type II, aggrecan and connexin43 gene levels were estimated using RT-PCR methods. Among the biomaterials, Poly glycolic acid (PGA) showed the highest expression level of the collagen type II gene. On the contraty, C60DMA showed the lowest expression level among six kinds of test substances. In the case of the aggrecan gene, PGA also showed the highest levels, and C60DMA showed the lowest ones. However, the expression patterns of the connexin 43 gene were different from previous two genes. Using the multi regression analysis was carried out between differentiation and these three gene expression levels. There was a high correlation between cellular differentiation and three gene expression levels.

1. Introduction

Properties of degradation of scaffolds are the important character in the long-term success of a tissue-engineered cartilage construct. The biodegradable polymers hold the additional advantage that the cartilage tissue, with the biodegradation of the polymers, may gradually replace the space occupied by the scaffolds. Extensive studies have been carried out using bioreabsorbable materials. However, most of those studies used animal cells, whereas little information is available on the chondrogenic effects of these materials with human articular chondrocytes (HAC). The biocompatibility of the biodegradable polymers using human articular cartilage in a micromass culture system was studied. In the present in vitro micromass study, we investigated the biocompatibility of a synthetic biodegradable materials and a fullerene derivative of C60 dimalonic acid (C60DMA) as the indication of the cellular proliferation, differentiation and the expression level of 3 genes such as collagen type II, aggrecan and connexin43, estimated by RT-PCR method.

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2. Materials and Methods

Cell and Materials

Chondrocyte growth medium and HAC were commercially obtained from BioWhittaker, Inc. (Walkersville, MD, USA). Chondrocytes growth medium contains bovine insulin, basic fibroblast growth factor, insulin like growth factor-1, transferring, gentamicin sulfate and fetal bovine serum (5% v/v). PGA (Mw = 3,000) and PLGA (Mw = 5,000) were purchased from Nakalai Tesque Inc. (Kyoto, Japan) and, PGCL (Mw = 3,000) was from Taki Chemical Co (, Japan). P(LA-CL)25 Mw = (10,000) , PCL (Ti) (Mw = 130,000) and fullerene C60-dimalonic acid (C60 DMA) were synthesized in our laboratoty.

Cell culture

In vitro high-density micromass cultures of HAC were initiated by spotting $4x10^5$ cells in 20 µl of medium onto each well of 12-well microplates for tissue culture (Costar ® Type 3513, Corning Co. Ltd., NY, USA) and PCL(Ti) coated glass wells (diameter, 22mm). After two hours of cell spotting in a 5 % CO_2 incubator at 37°C, the wells were flooded with chondrocyte culture media (2 ml/well). Media were supplemented with DMSO (0.8 µl/ml), PGA (50 µg/ml), PGCL (50 µg/ml), PLGA (50 µg/ml), P(LA-CL)25 (50 µg/ml), and fullerene C60 DMA (60 µg/ml), respectively. HAC cultured on tissue culture polystyrene but not exposed to any biomaterials served as a control. The media were changed in every 3 days and the cultures were continued for 4 weeks.

Proliferation assay

Cell proliferation was quantitatively measured by alamar blue (Biosource, International, Inc, Camarillo, CA) assay after 4 weeks of culture as previously described.[1]

Differentiation assay

Proteoglycans are typical contents of the cartilage matrix. The extent of chondrogenesis was determined by staining the cartilage specific proteoglycans with alcian blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as previously described.[2]

RNA harvest

After the designated 4 weeks culture period, RNA was extracted from all matrices except PCL(Ti) matrix. For PCL(Ti) matrix, we did not have enough samples for RNA harvest as cells from 50 % of the cultured wells were detached over night following cell spotting. Total celluar RNA was extracted from cultured cells of four wells (for each material) in 0.5 ml Trizol reagent (Life Technologies, Inc., Frederick, MD, USA) according to manufacturer's instruction.

Reverse transcription (RT) and polymerase chain reaction (PCR)

The matrix molecules probed as part of this study was collagen type II and aggrecan. The gap junction protein gene of Cx43 was also studied. The single strand cDNA was prepared from 1 µg of total RNA by reverse transcription (RT) using a commercially available First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After proper optimization of PCR condition, subsequent PCR was performed with 4 µg of cDNA in a 20 µl reaction mixture (10 x PCR buffer 2 µl,

dNTP 1.6 μl, forward and reverse, each primer 0.4 μl, Taq DNA polymerase 0.1 μl and rest of the amount of distilled water). The codon sequence used for the primer sets was as follows:

Collagen type II: forward 5'-GGCAATAGCAGGTTCACGTACA-3'
reverse 5'-CGATAACAGTCTTGCCCCACTT-3'

Aggrecan: forward 5'-TCGAGGACAGCGAGGCC-3'
reverse 5'-TCGAGGGTGTAGCGTGTAGAGA-3'.

Connexin 43 (Homo Sapiens):
forward 5'-ATGGGTGACTGGAGCGCCTTAGGCAAACTC-3'
reverse 5'- GACCTCGGCCTGATGACCTGGAGATCTAG -3'

The polymerization of GAPDH was accomplished by 25 cycles with the corresponding PCR program. Electrophoresis of PCR products was done on 3% agarose gel for the visualization of collagen type II and aggrecan and, on 1% agarose gel for Cx43 after staining with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA). The relative intensity of signals from each lane was analyzed with a computerized scanner. For relative quantitation, the signal intensity of each lane was standardized to that of a housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH):

forward 5'-CCCATCACCATCTTCCAGGAGCGAGA-3'
reverse 5'-TGGCCAAGGTCATCCATGACAACTTTGG-3'.

3. Results

Cell proliferation assay

The cell proliferations of PGA, PGCL and PLGA were fairly parallel as that of control cell proliferation. The cell proliferation of P(LA-CL)25, PCL(Ti) and fullerene C-60 DMA were significantly inhibited as compared to control. The values of cell proliferation for the samples exposed to PGA, PGCL, PLGA, P(LA-CL)25, PCL(Ti) and fullerene C-60 were 101, 102, 104, 93, 84, and 93 %, respectively.

Proteoglycan synthesis

Intensity of alcian blue staining was found to be higher in PGA, PGCL and PLGA containing cultures than that was found with the control culture. Among the biomaterials, PGA caused a significant 3.1 fold increase of cell differentiation when compared to control ($p < 0.05$).

Extracellular matrix genes expression

RT-PCR analysis showed that all matrices consistently expressed collagen type II gene and PGA matrix had the strongest induction. Slight increase expressions of collagen type II gene were noted with PGCL and PLGA matrices. Expression of collagen type II gene in P(LA-CL)25 was faint and in fullerene C60 DMA was almost nil. PGA matrix showed the strongest induction of aggrecan gene. Aggrecan gene expressions were decreased in PLGA and P(LACL)25 matrices.

Expression of gap junction protein connexin 43 gene

PGA induced the highest level of Cx43 mRNA expression and moderate level of expression was noticed in PLGA treated culture. A faint expression in P(LA-CL)25 and almost nil expression in flurrene C60 DMA treated cultures were observed.

Multi-regression analysis

Using the multi regression analysis, correlation was investigated between the differentiation estimated by alcian blue method and three genes expression levels. There was a high correlation between the cellular differentiation and three gene expression (correlation coefficient is 0.96) (Fig.1). Especially, two kinds of expression levels of aggrecan, and connexin 43 genes, were found to be critical factors for estimating the extent of cellular differentiation of human articular chondrocytes(Fig. 1).

4. Discussion

During differentiation, chondrocytes secrete extracellular matrix (ECM) molecules characteristic of cartilage, such as type II collagen, aggrecan, and link protein, offering an environment that preserves the chondrocyte phenotype. Therefore, chondrocyte are defined both by their morphology and ability to produce these characteristic ECM. Collagen type II is regarded as the most important component among the ECM molecules. Previous study detected type II collagen as early as 7 days after beginning 3-D culture and at 21 days, the matrix of the entire aggregate contained type II collagen.[3] Among the ECM molecules, aggrecan is a major proteoglycan[4] and had been reported that in chick cartilage, aggrecan starts to be expressed at embryonic day 5 in limb rudiments, continues through the entire period of chondrocyte development, and remains a biochemical marker of the cartilage phenotype thereafter.[5] In this study, we have well demonstrated cell differentiation with the formation of cartilaginous nodules on culture plate, by alcian blue staining, which is commonly used for identification of cartilage, and by expression of ECM molecules collagen type II and aggrecan. The morphology after the designated culture period revealed that cells aggregated on the culture plate and resulted in the formation of cartilaginous nodules. The greatest cell differentiation, 3.1-fold increase of the controls was found in the sample treated with PGA. The potencies of cell differentiation after 4 weeks of culture from most to least were in the following order; PGA>> PLGA > PGCL > Cont. = DMSO > P(LA-CL)25 = PCL(Ti) >> fullerene C60 DMA. The increased cell differentiation with PGA and PLGA matrices are in agreement with our previous findings in micromass culture system[1], however, in this study we have included the matrix genes expression of these materials. Results of the present study confirmed PGA and PLGA as useful scaffolding matrices for cartilage tissue engineering, and knowledge with other matrices will further contribute to develop improved cartilaginous constructs for future clinical implants. In this study, RT-PCR analysis showed that the mRNA level of x43 gene expression was consistent with the chondrogenic differentiation in the presence of different biomaterials. Our findings of Cx43 expression by chondrocytes are in agreement of previous study that reported expression of functional gap junctions by chondrocytes isolated from adult articular cartilage[6]. Gap junction mediated intercellular communication is critically involved in the development of cartilage during differentiation[7].

In this study, the data of cell differentiation by alcian blue and, observed expression of collagen type II, aggrecan and Cx43 suggest that the process of cell differentiation might be due to the interconnection of cells by means of gap junction along with other molecular mechanism. However, the specific association of gap junction in the process of chondrogenic differentiation and the cell signaling processes remains unexplored. Future studies are required to analyze the specific role that the gap junction proteins have in chondrocyte differentiation.

5. Acknowledgement

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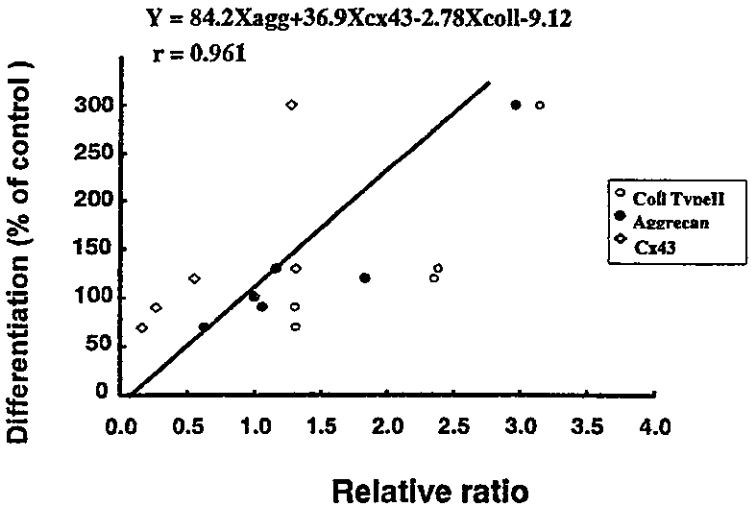

Fig.1.Relationship between the differentiation and the expression levels of three genes of collagen typeII, aggrecan and connexin43 using multi-regression analysis.

ティッシュエンジニアリング用マテリアルの製品化条件と国際標準化

Manufacturing of the biomaterial as the scaffold for tissue engineering and the international standardization in the field of tissue engineered medical products

Keywords
品質評価　製品化
国際標準化　安全性
有効性

土屋 利江
国立医薬品食品衛生研究所 療品部

Summary

Biodegradable polymers are often used as the scaffolds for the tissue engineering, and these polymers are classified in class IV under the revised Pharmaceutical Affairs Law.

A certain biodegradable polymer for tissue engineering did not show the toxicity in cartilage, but showed the severe toxicity in the other organs. If the constituted ingredients showed organ toxicity in design phase, we should avoid to use such constituents since the beginning. I introduce problems and the activities of the international standardization in the field of the tissue engineered medical products.

Tsuchiya, Toshie
Division of Medical Devices, National Institute of Health Sciences
E-mail : tsuchiya@nihs.go.jp

はじめに

ティッシュエンジニアリング用マテリアルは，すでに医療材料，医療機器として使用されているものが多い。たとえば，生分解性高分子材料や，通常細胞のよい基質となるコラーゲンに代表される天然材料がある。

ティッシュエンジニアリング用マテリアルとしての安全性は，医療材料としての前臨床評価試験と，さらにティッシュエンジニアリング用マテリアルとして臨床使用を考慮した評価が必要となる。

たとえば，神経再生用のティッシュエンジニアリング用マテリアルであれば，マテリアルを細胞・組織の足場として評価したとき，神経が目的とする正常な機能などを保っていることを確認しなければならない。また，その状態を維持できることも示す必要があると考える。

骨の分化に及ぼす影響を調べた結果，P(LA-CL) 25 10000では軟骨分化は抑制され，コントロールの90％程度の分化に留まった。ラット胎仔軟骨前駆細胞では，顕著な分化促進作用を認めたことより，細胞の採取組織部位や種の違いによりオリゴマーに対する反応は異なることが明らかになった。一方，P(LA-CL) 50 18000を試験した結果では，ヒト軟骨細胞はコントロールと同程度の分化レベルであった。しかし，細胞の増殖に及ぼす影響は，P(LA-CL) 25 10000とP(LA-CL) 50 18000は，ヒト軟骨細胞に対して，7～5％程度の増殖抑制が観察されたが，ラット胎仔軟骨前駆細胞ではP(LA-CL) 25 10000でコントロールの25％阻害，P(LA-CL) 50 18000では，コントロールの40％減少し，強く阻害した。

したがって，同じ濃度レベルの共重合体オリゴマーについて比較した結果，ラットおよびヒト細胞間では，細胞分化や増殖能に及ぼす影響が著しく異なることが明らかになった。

組織工学利用医療用具の評価を行う上で，動物モデルからヒト臨床使用するときに，分化・増殖機能が両種間で異なる可能性を考慮する必要がある。

一般的に，治療効果の高い動物モデルを使った論文や学会発表が多い。高齢の対象患者で治療効果があることを示す必要がある。海外で使用されているものの中には，有用性のあり・なしが論争になっているものもある。

国際標準化

現在，再生医療製品の国際標準化の動きがある団体として3団体があげられる。一番初めに活動を開始し，最も活発な作業を行っているのが，American Society for Testing and Materials (ASTM) のF04のセクションの中にあるTissue Engineered Medical Products (TEMPS) のグループである。最近では，ASTMはInternational standardであると，米国のメインパーソンがInternational Organization for Standardization (ISO) の会議でも主張している。しかし，欧州のISOメンバーはその主張に異議を唱えている状況にある。このような状況下，2004年の2月26～27日の二日間にわたり，スイス・ジュネーブにあるWorld Trade Organization (WHO) 本部で「The high-level workshop on International Standards for Medical Technologies」が開催された。ISO, International Electrotechnical Commission (IEC) と International Telecommunication union's Telecommunication Standardization (ITU-T) が主催したWorld Standard Cooperation (WSC) の第一回会議が開かれた。共催団体は，WHO, GHTF, Association for the Advancement of Medical Instrumentation (AAMI), the European Confederation of Medical Device Association (EUCOMED), the Japan Federation of Medical Devices Associations(JFMDA)の5つであった。日本からは，JFMDAの三浦氏 (GE横河メディカルシステム株式会社) と筆者が招待講演者として招かれた。参加者は約70名で，各TC (technical committee) の議長が参加していた。5つのセッション (1. Vision, 2. Links between regulators and standards developers, 3. Standard development practice, 4. New Technologies and standards[emerging technology], 5. Development dimension) が開かれ，三浦氏はsession 2で，筆者はsession 3で講演した。筆者は，standard practice developmentのセッションの目的・内容を考慮して，standard reference materialとわが国の医療機器・細胞組織医療機器分野の健全な発展のための規制環境の整備について紹介した。本セッションの他の講演者は，各TCで標準化された多くのタイトルの紹介に終始して時間切れの状態であった。一方，TC194 (医療用具の生物学的評価) でのわが国の活動過程の報告は，standard practice developmentの過程を実際に示した講演内容であると議長からコメントされた。今後もこのような横断的な標準化の会議が開かれるであろう。わが国では，現在さまざまな医療機器の標準化作業が行われているが，世界の人々に優れた医療機器を提供するためにも，有用な基準を提案し，国際的に理解され認知される標準化活動をする必要があると考える。

組織工学関連のISOでの標準化は，TC150が先行し，WG11では，「General

requirements for safety, marking and for information to be provided by the manufacturer」の文書作業が開始されている。

TC194では，新たにmedical devices utilizing tissuesに関するsub committeeを作り，その中に3つのワーキンググループ(WG1～3)が作られた。

「Animal tissue and their derivatives utilized in the manufacture of medical devices [Part1 : Analysis and management of risk (WG1), Part2 : Controls on sourcing, collection and handling (WG2), Part3 : Validation of the elimination and/or inactivation of viruses and transmissible agents (WG3)]」の文書化作業が開始され，2004年6月28日～7月2日にNorwayのTromsoで初めての会議が開催される。現在は，ENの文書が転用されているが，文書化後，時間も経過し，引用されている内容も古いことから，全面的に加筆修正が行われると考えられる。

再生医療製品の製品化までには，多くの知識・技術が必要である。医療機器同様，臨床家・研究者・企業人が連携し，お互いに補足しあう形で技術や材料を提供し，製品化に向けて努力すれば，世界の国々の患者さんに先端的医療製品を発信できる可能性がある。

民間の調査レポートによれば，ある再生医療関連の米国企業は，5億ドルの開発研究費をかけながら，製品化後の売り上げは0.2～0.3億ドルで，借金返済のめどがたたず倒産に追い込まれたと報告している。合理的なコストダウンと国際市場で治療効果があり，優良と認められる製品でないかぎり，企業ベースでの持続的な製品化はなかなか困難な状況であると思われる。したがって，材料開発研究者，臨床家，企業人などがばらばらに研究を進めるのではなく，相互によい連携をとり，力を合わせて着実に製品化に向けた努力をすべきであると考える。

日本再生歯科医学会誌

バイオマテリアルの安全性について 組織工学用材料を中心として

Safety evaluation of biomaterials for tissue engineering

国立医薬品食品衛生研究所　療品部

土屋 利江

Toshie TSUCHIYA

Division of Medical Devices, National Institute of Health Sciences

はじめに

医療用具(平成17年度4月から医療機器に名称変更)に使用されるバイオマテリアルには，金属，セラミックス，高分子，天然材料などがある．それぞれ医療用具として使用される目的，部位により，それらの単体あるいは複合材料が使用されている．近年は，ナノテクノロジー技術を応用した医療材料・医療機器開発が活発に行われており，米国の最近のニュースでは，がん治療分野で，大型の予算がついたとの報道がなされている．

医療材料・医療機器は，従来の承認された既存材料の組み合わせのみでは，限界があり，今後は，生物の仕組みを制御する機能を組み合わせた医療機器開発が盛んになるものと考えられる．薬と医療機器の組合されたステント，さらに，最近では，細胞・組織と医療機器がくみあわされたバイオ皮膚・バイオ軟骨・バイオ骨などがあげられる．細胞組織医療機器と日本語で表記されるものは，通常 tissue engineered medical products (TEPS)として海外では，標準化すべき課題として取り上げられ，米国規格協会(ASTM)では，すでにいくつかの関連文書ができている．一方，tissue engineering といわれる技術については，近年，複数の学会が立ち上げられ，社会的にも大きな関心事となった．しかし，事業としては，海外を含めて成功しているようにはおもえない．また，いくつかの問題点と課題も浮き彫りにされてきた．

テイッシュエンジニアリング用バイオマテリアルは，既に，医療材料，医療機器として使用されているものが多い．例えば，生分解性材料や,通常，細胞の良い基質となるコラーゲンに代表される天然材料がある．

テイッシュエンジニアリング用バイオマテリアルとしての安全性は，医療材料としての前

臨床評価試験と,更に,テイッシュエンジニアリング用マテリアルとして使用実態を考慮した評価が必要となる.

例えば,神経再生用のテイッシュエンジニアリング用マテリアルであれば,マテリアルを細胞・組織の足場として評価した時,神経が目的とする正常な機能等を保っていることを確認しなければならない.また,その状態を維持できることも示す必要があると考える.

ある種のテイッシュエンジニアリング用マテリアルが,軟骨では毒性を示さないが,神経では毒性を示すことは既にしられている.設計段階で,神経毒性を示す構成成分が原材料として使用されていれば,推測可能であり,はじめから,そのような合成方法はさけるべきである.使用範囲や製品としての価値を低くする設計をさけることがポイントとなる.当然のことだが意外とおろそかにされている感がする.

分解性材料は,やがては,生体内で分解・吸収・代謝・排泄される.通常は,生体内に残存し続ける医療用具の方が安全性上,リスクが高いと考えやすいが,薬事法改正による新クラス分類では,生分解性材料は,4つのクラスの中でリスクが最も高いクラスIVに分類されている.このクラス分類の考え方は,わが国だけでなく,Global Harmonization Task Force (GHTF) で示された国際レベルでのクラス分類とも整合している.医療材料は,その性質,使用方法により,ヒトへの安全性の確保について慎重に考慮すべきであると考える.

現在,考えられる問題点やこの分野の国際標準化の動向について紹介する.

1. テイッシュエンジニアリング用バイオマテリアル製品化のための課題

製品化の上で重要だと考えられるポイントについて以下に私見を列挙する.

(a) バイオマテリアルの選別が重要である.材料が承認されているからといって,テイッシュエンジニアリング用バイオマテリアルとして適切であるかどうかは,保証されていない.使用する組織・使用方法により,バイオマテリアルの細胞・組織との反応性が異なるからである.

(b) 天然材料では,エンドトキシン汚染があると考え,エンドトキシン汚染がないことを事前に確認して使用することが重要である.高分子でもエンドトキシン汚染に留意すべきである.

(c) 天然材料中に混在するエンドトキシンは,材料の特性に応じて吸着しやすく,材料から,通常の方法に従って溶液を調製し,測定しても正確な測定値を示しているとは限らない.エンドトキシン汚染濃度は,回収率を考慮して測定することが重要である.従来の試験溶液調製方法では,汚染されているエンドトキシンの1%程度以下しか検出されていない例も多い.

(d) テイッシュエンジニアリング用バイオマテリアルの原材料,製造工程などを明確にし,

安全性上問題はないか, 検討した後に使用すること. たまたま, 入手したものを出発物質として細胞を接着させ, 培養しても, その結果に対する解釈はあやふやなものとなり, 科学的な説明が困難となる. 同名の材料でも, 細胞の反応は, 阻害・亢進と対照的に著しく異なることに留意すべきである. 製造メーカーの異なる同名の材料を並べて試験した場合, 細胞との反応性は異なる. 学会でも材料の製造工程は, 不明のまま使用されている例が多い.

(e) モノマーやポリマーの種類, 触媒, 添加物などは, 安全性, 有効性に影響を与える因子となる. 設計の段階で, 十分考慮すべきである. 研究計画と同様, 製品化の上で十分な事前評価が重要である.

(f) 優れた医療機器開発と低コスト化をねらった精密な事前計画を練るキーパーソンがヒットメーカーでは活躍している. 材料のみでなく, 人材が活躍できる環境作りも重要と考える.

(g) 医療材料でありながら, 化学発ガン物質もある. 従って, 発ガン性を懸念する場合には, 最低限, 対象材料や対象物質の溶液での形質転換試験等によりチェックしておく必要がある. ガイドラインで記載されている遺伝毒性試験の中で, Ames Test や染色体異常試験では, 陽性とはならないが, 動物実験では発ガン性を示す材料が少なからずある.

(h) バイオ製品では, これから標準化すべき課題としては, バイオ製品の力学強度の評価指標と評価方法がある. 組織工学製品の標準化を進めている米国(ASTM F04)は, バイオ軟骨において, いくつかの力学指標を盛り込んだ文書(案)を作成しており, 今年の8月第1回目の投票が行われた. 11月のASTM 会議で投票結果と反対コメントなどが明らかになり, 今後の方針が決定される予定である.

(i) 国際的に掲げられているバイオ製品の課題は, 欠損部位など治療目的とする部位へのバイオ製品の固定, バイオ製品の生体内での機能維持, 目的外細胞・組織形成の否定,非侵襲的臨床評価による有効性評価指標など、若年者のみでなく, 対象患者である高齢者での治療効果の提示、バイオ製品の評価のための動物モデル(治癒効果に種差がある. 軟骨の動物モデルに関する文書(案)が ASTM でまとめられている.)感染因子の否定などである.

(j) 今までに報告されているリスク因子としては, 天然由来材料:未知の感染因子, アレルギー反応, エンドトキシン等の吸着、生分解性材料:炎症反応, ゲッシ類動物での高頻度腫瘍化, 最近の研究から, 腫瘍化の程度に系統差があること, 腫瘍化した細胞は, 軟寒天コロニー試験でコロニー形成能陰性であったが, ヌードマウス移植試験で早期に大きな腫瘍を形成した.

通常の医療材料としての前臨床試験は, 使用部位, 使用期間により, 確認すべき試