(2002)
8. C. H. Tang, R. S. Yang, H. C. Liou and W. M. Fu, J. Biomed. Mater. Res., **63**, 577 (2002)
9. P. H. Weigel, V. C. Hascall and M. Tammi, J. Biol. Chem., **272**, 13997 (1997)
10. W. Zhang, C. E. Watson, C. Liu, K. J. Williams and V. P. Werth, Biochem. J., **349**, 91 (2000)
11. L. S. Liu, C. K. Ng, A. Y. Thompson, J. W. Poser and R. C. Spiro, J. Biomed. Mater. Res., **62**, 128 (2002)
12. L. Sherma, J. Sleeman, P. Herrlich and H. Ponta, Curr. Opin. Cell. Biol., **6**,726 (1994)
13. P. W. Kincade,Z. Zheng, S. Katoh and L. Hanson, Curr. Opin. Cell. Biol., **9**, 635 (1997)
14. J. Entwistle, C. L. Hall and E. A. Turley, J. Cell Biochem., **61**, 569 (1996)
15. A. Pilloni and G. W. Bernard, Cell Tissue. Res., **294**, 323 (1998)
16. M. Nagahata, T. Tsuchiya, T. Ishiguro, N. Matsuda, Y. Nakatsuchi, A. Teramoto, A. Hachimori and K. Abe, Biochem. Biophys. Res. Commun.., **315**, 603-611 (2004)
17. T. Hamano, D. Chiba, K. Nakatsuka, M. Nagahata, A. Teramoto, Y. Kondo, A. Hachimori and K. Abe, Polym. Adv. Technol., **13**, 46 (2002)
18. E. Ruoslahti, Ann. Rev. Cell. Biol., **4**, 229 (1988)

Key Engineering Materials Vols. 288-289 (2005) pp. 409-412
online at http://www.scientific.net

Osteoblast Differentiation and Apatite Formation on Gamma-Irradiated PLLA Sheets

Kazuo Isama[a] and Toshie Tsuchiya[b]

Division of Medical Devices, National Institute of Health Sciences,

Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan

[a]isama@nihs.go.jp, [b]tsuchiya@nihs.go.jp

Keywords: Poly(L-lactide), gamma-ray irradiation, osteoblast differentiation, apatite formation.

Abstract. The effects of the γ-irradiatted PLLA on the osteoblasts and apatite formation were investigated *in vitro*. The PLLA sheet was γ-ray irradiated at the dose of 10, 25 or 50 kGy. The mouse osteoblast-like MC3T3-E1 cells and normal human osteoblast NHOst cells were micromass cultured on the PLLA sheet for 2 weeks, and then the proliferation and differentiation of the cells were determined. The proliferations of MC3T3-E1 and NHOst cells hardly changed with increasing irradiation dose. However, the differentiations of MC3T3-E1 and NHOst cells increased with irradiation dose. On the other hand, the surface of the PLLA sheet after soaking in the medium without the cells was characterized by SEM, EDX, FT-IR and XPS. The hydroxyapatite was formed on the surface of the PLLA sheet after soaking, and the amount of hydroxyapatite increased with irradiation dose. In summary, the γ-irradiated PLLA increased the differentiation of osteoblasts and also increased apatite-forming ability even without the osteoblasts. The osteoblast differentiation was enhanced well in the apatite formation on the surface of PLLA after the γ-irradiation.

Introduction

Poly(L-lactide) (PLLA) has been well reported on a good osteocompatibility *in vivo* and *in vitro*. The γ-ray sterilized PLLA sample was implanted *in vivo*, and newly bone was formed around the PLLA implant [1]. It was not clear whether there was the effect of γ-irradiation on the formation of newly bone in this result. However, it was the fact that γ-irradiation decreased the molecular weight and mechanical strength of PLLA [2]. On the other hand, PLLA fibers formed bone-like apatite in a simulated body fluid [3]. It was reported that the apatite layer formed on the bioactive glass increased the attachment and initial proliferation of osteoblasts [4]. If the apatite-forming ability of PLLA is increased by γ-irradiation, there may be a good influence on osteoblasts cultured on the irradiated PLLA. Therefore, we clarified the effects of the γ-irradiatted PLLA sheet on the osteoblasts and apatite formation *in vitro*.

Materials and Methods

Materials. PLLA sheet with 0.3 mm thickness (Shimadzu Co., Japan) was γ-ray irradiated at the dose of 10, 25 or 50 kGy using ^{60}Co as the radiation source. The weight average molecular weight (Mw) of the unirradiated PLLA was 271,000 and the Mw's of the irradiated PLLA's at the dose

of 10, 25 and 50 kGy were respectively 195,000, 142,000 and 95,000 by GPC.

Micromass Culture of Osteoblasts. Mouse osteoblast-like MC3T3-E1 cells (RIKEN Cell Bank, Japan) and normal human osteoblast NHOst cells (Clonetics Corporation, MD, USA) were grown in alpha minimum essential medium (α-MEM) supplemented with 20% fetal bovine serum. The PLLA sheet was cut into 14.0 mm diameter disk and laid in a 24-well dish. The 20 μl of cell suspension (2×10^6 cells/ml) was delivered on the disk. After the cells were attached on the disk, 1 ml of the complete medium that contained 10 mM disodium β-glycerophosphate in the culture medium was added. The complete medium was changed three times a week, and the cells cultured for 2 weeks in a 37°C humidified atmosphere of 5% CO_2.

Proliferation Assay. The number of the cells cultured on the PLLA sheet was determined by WST-8 assay [5]. Moreover, the protein and DNA contents of the cell lysate were measured by the Lowry method and the fluorescence assay using Hoechst 33258 dye, respectively [5].

Differentiation Assay. The calcium depositions of the cell cultures were stained by alizarin red S, and the areas stained dark-red were measured using the program Scion Image (Scion Co., MD, USA) [5]. The calcification was calculated as the normalized area in the cell number. Moreover, the collagen synthesis was evaluated by the hydroxyproline content of the cell lysate, and ALP activity of the cells was measured using *p*-nitrophenylphosphate as a substrate [5].

Soaking in the Medium. The PLLA sheet was cut into 14.0 mm diameter disk and laid in a 24-well dish. The complete medium of 1 ml was added without the cells. Then, the dish was stored in a 37°C humidified atmosphere of 5% CO_2, and the complete medium was changed three times a week. After soaking for 2 weeks, the PLLA disk was washed in deionized water five times quickly and dried in a silica gel desiccator.

Surface Analysis. The surface of the PLLA sheet after soaking in the complete medium without the cells was characterized by SEM, EDX, FT-IR and XPS according to the conventional methods.

Results

Proliferation of Osteoblasts Cultured on the PLLA Sheet. The cell number of MC3T3-E1 cells cultured on the PLLA sheet did not change with increasing irradiation dose (Fig. 1a). The protein and DNA contents of the cells also did not change. The other side, the cell number (Fig. 1b),

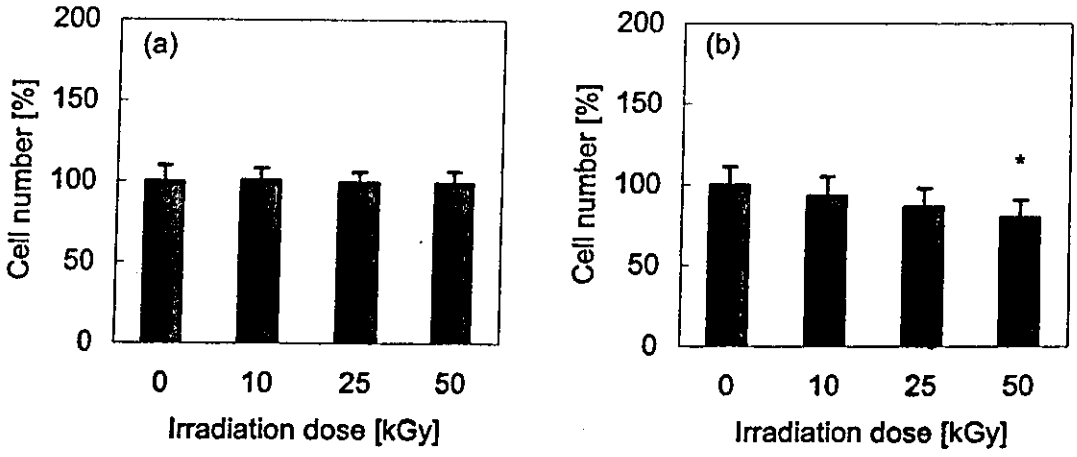


Fig. 1. The cell numbers of (a) MC3T3-E1 and (b) NHOst cells cultured on the γ-irradiated PLLA sheet.

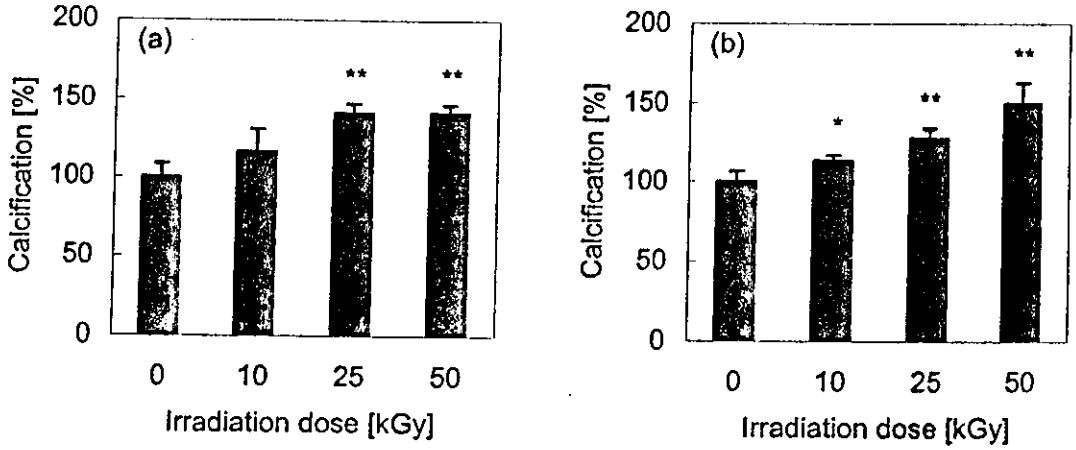


Fig. 2. The calcifications of (a) MC3T3-E1 and (b) NHOst cells cultured on the γ-irradiated PLLA sheet.

protein and DNA contents of NHOst cells cultured on the PLLA sheet slightly decreased with irradiation dose.

Differentiation of Osteoblasts Cultured on the PLLA Sheet. The calcification of MC3T3-E1 cells (Fig. 2a) and NHOst cells (Fig. 2b) remarkably increased with irradiation dose. The collagen synthesis and ALP activity of MC3T3-E1 and NHOst cells also increased as same as the calcification, respectively. The γ-irradiated PLLA remarkably promoted the differentiation of osteoblasts.

Apatite Formation on the PLLA Sheet. The SEM micrograph exhibited crystal particles on the surface of the PLLA sheet after soaking in the complete medium without the cells. The crystal particles were identified with hydroxyapatite by EDX, FT-IR and XPS spectra. The phosphate band in ATR/FT-IR spectra became strong with irradiation dose (Fig. 3). Moreover, the element rations of calcium and phosphorus increased but that of carbon decreased with irradiation dose, in XPS analysis (Fig. 4). The amount of hydroxyapatite formed on the γ-irradiated PLLA sheet increased with irradiation dose.

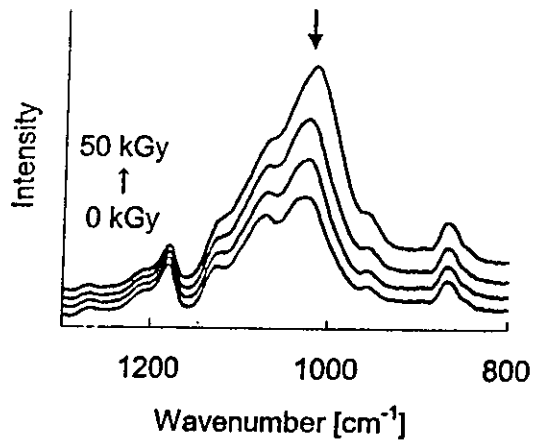


Fig. 3. The phosphate band of the γ-irradiated PLLA sheet after soaking in the medium.

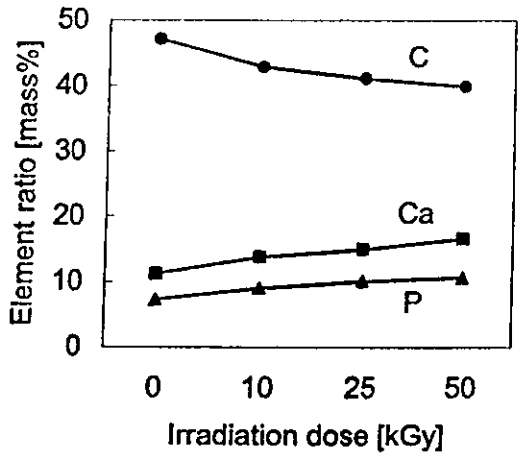


Fig. 4. The element ratios of calcium, phosphorus and carbon of the γ-irradiated PLLA sheet after soaking in the medium.

Discussion

In the present study, the γ-irradiated PLLA hardly affected the proliferation but remarkably promoted the differentiation of osteoblasts. It was expected that the low molecular weight PLLA eluted to the medium, because the molecular weight of PLLA decreased by γ-irradiation. In our recent studies, the low molecular weight PLLA enhanced the differentiation of MC3T3-E1 cells but inhibited that of NHOst cells [6, 7]. The present results, which the differentiations of MC3T3-E1 and NHOst cells both increased on the γ-irradiated PLLA sheet, would not be caused by the low molecular weight PLLA. The surface of the γ-irradiated PLLA should good influence on the differentiation of osteoblasts.

On the other hand, the γ-irradiation increased the apatite-forming ability of the PLLA sheet. Tanahashi and Matsuda reported that some negatively charged groups such as phosphate and carboxyl group strongly induced apatite formation in a simulated body fluid. They described that the apatite formation was initiated via calcium ion-absorption upon complexation with a negative surface-charged group [8]. In our study, the molecular weight of PLLA decreased with hydrolysis of ester bonds by γ-irradiation [2]. Therefore, the amount of carboxyl group of the γ-irradiated PLLA would increase with irradiation dose, and the carboxyl group would promote the apatite-forming ability of the PLLA sheet.

Fujibayashi *et al.* compared *in vivo* bone ingrowth and *in vitro* apatite formation on Na_2O-CaO-SiO_2 glasses. The quantities of newly bone formed on the glasses correlated with their apatite-forming abilities in simulated body fluid. They propose to evaluate the apatite-forming ability in order to confirm the *in vivo* bioactibity of biomaterials [9]. In our present study, the γ-irradiation enhanced the apatite-forming ability of the PLLA sheet, and then the γ-irradiated PLLA sheet promoted the differentiation of osteoblasts. The osteoblast differentiation should connect with the apatite formation on the γ-irradiated PLLA sheet.

In conclusion, the γ-irradiated PLLA hardly affected the proliferation but promoted the differentiation of osteoblasts with increasing irradiation dose. On the other hand, the hydroxyapatite was formed on the PLLA sheet in the medium, and the γ-irradiation enhanced apatite-forming ability of the PLLA. It was suggested that the connection between the osteoblast differentiation and apatite formation on the γ-irradiated PLLA sheets.

References

[1] T.E. Otto, P. Patka, H.J.Th.M. Haarman, C.P.A.T. Klein and R. Vriesde: J. Mater. Sci. Mater. Med. 5 (1994), 407-410.

[2] K. Isama and T. Tsuchiya: Bull. Natl. Inst. Health Sci. 119 (2001), 61-64.

[3] X. Yuan, A.F.T. Mak and J. Li: J. Biomed. Mater. Res. 57 (2001), 140-150.

[4] N. Olmo, A.I. Martin, A.J. Salinas, J. Turnay, M. Vallet-Regi and M.A. Lizarbe: Biomaterials 24 (2003), 3383-3393.

[5] K. Isama and T. Tsuchiya: J. Biomater. Sci. Polymer Edn. 13 (2002), 153-166.

[6] K. Isama and T. Tsuchiya: Biomaterials 24 (2003), 3303-3309.

[7] K. Isama, Y. Ikarashi and T. Tsuchiya: BIO INDUSTRY 19 (2002), 21-29.

[8] M. Tanahashi and T. Matsuda: J. Biomed. Mater. Res. 34 (1997), 305-315.

[9] S. Fujibayashi, M. Neo, H.M. Kim, T. Kokubo and T. Nakamura: Biomaterials 24 (2003), 1349-1356.

Toshie Tsuchiya[1]

A Useful Marker for Evaluating the Safety and Efficacy of Tissue Engineered Products

Reference: Tsuchiya, T., "A Useful Marker for Evaluating the Safety and Efficacy of Tissue Engineered Products," *Tissue Engineered Medical Products (TEMPS), ASTM STP 1452*, E. Schutte, G. L. Picciolo, and D. S. Kaplan, Eds., ASTM International, West Conshohocken, PA, 2004.

Abstract: We propose a survey of the function of the gap-junctional intercellular communication (GJIC) as a useful marker for evaluating the safety and efficacy of tissue-engineered products.

Keywords: Gap junction, Connexin43, normal human dermal fibroblast, bFGF, KGF, cell differentiation, tumor promotion.

Introduction

An in vitro system for evaluating the safety of tissue engineered products is convenient because of its rapidity and low cost consumption. On the basis of recent studies, intercellular channels called gap-junctions are considered to play an important role on the tumor-promotion stage during the tumorigenesis induced by biomaterials [1]. We demonstrate the significance of the intercellular communication during the neuronal cell differentiation and cytokine productions. From these results, we propose a survey of the function of the gap-junctional communication as a potentially useful marker for evaluating the safety and efficacy of tissue engineered products.

Increase in Gap-Junctional Intercellular Communications (GJIC) of Normal Human Dermal Fibroblasts (NHDF) on Surfaces Coated with High-Molecular-Weight Hyaluronic Acid

Normal cells and tissues have functional gap-junctions. Gap-junction are hydrophilic intercellular channels that allow intercellular passage of small molecules (up to 1 kDa). They are constructed from connexin proteins that form structures called connexons. Moreover, many substances, such as ions, sugars, nucleotides, amino acids, drugs, carcinogens, and so on, are small enough to move between cells though gap-junction channels. Gap junctions are important for coordinating the activities of electrically active cells, and they are thought to play many regulatory roles, such as growth control, developmental and differentiation processes, synchronization, and metabolic regulation. The aim of the study was to clarify the effect of hyaluronic acid (HA) on intercellular communication *via* gap junction.
Many investigators have demonstrated increases in cell migration, invasion, and

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

proliferation by exposure to HA. It also has been reported [2] that HA in collagen gel culture enhances the proliferation and chondroitin 6-sulfate synthesis of chondrocytes while maintaining their phenotype. HA is involved in these process *via* cell-surface receptors, such as the receptor for HA-mediated motility (RHAMM) and CD44 glycoproteins [3]. Additionally, cellular behaviors such as adhesion, differentiation, and proliferation are greatly affected by surface properties such as receptor-ligand binding, hydrophilicity, roughness, charge, and morphology of the materials.

In this study, safety evaluation for HA was investigated using NHDF cells, which are known to express CD44 glycoproteins on their surface.

Normal human dermal fibroblast (NHDF) cells were used to detect differences in gap-junctional intercellular communication (GJIC) by hyaluronic acid (HA). HA is a linear polymer built from repeating disaccharide units that consists of N-acetyl-D-glucosamine and D-glucuronic acid linked by a β 1-4 glycosidic acid. The NHDF cells were cultured with different molecular weights (MW) of HA for four days. The rate of cell attachment in dishes coated with high-molecular-weight (HMW; 310 kDa or 800 kDa) HA at 2mg/dish were significantly reduced at an early time point compared with low-molecular-weight (LMW; 4.8 kDa or 48 kDa) HA with the same coating amounts. HA-coated surfaces were observed by atomic force microscopy (AFM) under air and showed that HA molecules ran parallel in the dish coated with LMW HA and had an aggregated island structure in the dish coated with HMW HA surfaces. The cell functions of GJIC were assayed by a scrape-loading dye transfer (SLDT) method using a dye solution of Lucifer yellow. Promotion of the dye transfer was clearly obtained in the cell monolayer grown on the surface coated with HMW HA. These results suggest that HMW HA promotes the function of GJIC in NHDF cells. In contrast, when HMW HA was added to the monolayer of NHDF cells, the functions of GJIC clearly were lowered in comparison with the cells grown in the control dish or with those grown on the surface of HMW HA. LMW HA showed tumor-promoting activities in two-stage Balb 3T3 cell transformation assay [4]. Therefore it is suggested that the MW size of HA and its application method may be important factors for generating biocompatible tissue-engineered products because of the manner in which the GJIC participates in cell differentiation and cell growth rate.

In this study [5], the cell adhesion and cell function of GJIC were compared on dishes treated with various molecular weights of HA. The extent of attachment of NHDF cells on the HA-coated surfaces decreased linearly proportional to the MW size of HA at an early time point in the culture.

The SLDT method was used to study the effects of HA on the GJIC of NHDF cells. The results indicate that NHDF cells on dishes coated with HA of HMW (800 kDa) promote GJIC. However, the addition of HMW HA showed the opposite effect. Nutrients that promote the formation of the gap junction, such as bFGF, EGF, and TGF-β, could be enveloped in the molecule of the coated HMW HA. In the case either of addition or coating of LMW HA, the inhibitory effect of gap formation was not observed. In conclusion, it is suggested that the selection of the MW of HA is an important factor in designing biocompatible artificial skins that retain and enhance the function of GJIC in NHDF cells.

Increase in Gap-Junctional Intercellular Communication (GJIC) by High Molecular Weight Hyaluronic Acid Associated with Fibroblast Growth Factor 2 and Keratinocyte Growth Factor Production in Normal Human Dermal Fibroblasts

Most normal cells within tissues have functional gap-junctional intercellular communication (GJIC).

The importance of GJIC makes it desirable to estimate gap-junction channel function by a rapid and reliable quantitative method. The scrape-loading dye transfer (SLDT) method [6], involving use of the fluorescent dye Lucifer yellow and published [6] as a rapid and relatively uncomplicated method for measuring intercellular communication, has by now become a routine assay for analyzing the effects of various reagents on GJIC.

Hyaluronic acid (HA) is a negatively charged glycosaminoglycan composed of repeated disaccarides of D-glucuronic acid and N-acetylglucosamine and is found in most types of extracellular in the mammalian body. By interaction with other matrix molecules, HA provides stability and elasticity to the extracellular matrix. HA has been implicated in biological processes such as cell adhesion and proliferation. HA-binding proteins such as CD44, and aggrecan, and versican have been implicated in structuring the extracellular matrix (ECM) by stabilizing large macromolecular aggregates. More importantly, it was suggested that the receptor for HA-mediated motility (RHAMM) regulates gap junction channel and connexin43 expression, possibly through its actions on focal adhesions and the associated cytoskeleton [7]. It also reported that HA can both promote [8] or inhibit cytokine expression depending on its molecular mass [9].

Fibroblast growth factors (FGFs) play multiple roles during development and in adult tissues as paracrine regulators of growth and differentiation. Furthermore, it was reported that stimulation of neurite growth correlated strongly with the amount of FGF-2 bound to surfaces coated with heparin, heparan sulfate, or HA [10]. Keratinocyte growth factor (KGF) is a member of the FGF family and is expressed almost exclusively by stromal cells from a variety of tissues, including the lung, skin, mammary gland, and prostate. KGF in vivo is a member of the heparin-binding FGF family and is a paracrine mediator of proliferation and differentiation of a wide variety of epithelial cells. KGF has also been shown to cause angiogenesis and repair after major damage.

The aim of the study was to explore the relationship between gap junction channel function and production of growth factors [8]. We investigate the effect of LMW HA and HMW HA on FGF-2 and KGF expression by NHDF cells in vitro, and gap-junctional channel function as estimated by the SLDT method, simultaneously.

The effects of different molecular weights of hyaluronic acid (HA), a major component of extracellular matrix, on gap-junctional intercellular communication (GJIC) in normal human dermal fibroblasts (NHDF cells) were investigated. NHDF cells were cultured for four days with different fibroblasts molecular weights of HA and then the extent of GJIC was assessed by the scrape-loading dye transfer method, using Lucifer yellow. The area of dye transfer [8], a quantitative indicator more than the dye migration distance [4,6], was greater in the dishes coated with HA than in those to which HA was added. Thus, NHDF cells cultured on surface coated with high molecular weight (HMW) HA (MW, 800 kDa) showed greatly enhanced GJIC.

Furthermore, another aim of this study was to evaluate the effects of different molecular weights of HA on the production of FGF-2 and KGF because both are important cytokines produced by NHDF cells. When FGF-2 and KGF cultured levels of cell extracts and media were determined by ELIZA, both levels were significantly enhanced when cells were grown on plates coated with HMW HA. This finding indicated that the function of gap-junction channels in NHDF cells grown on plates coated with HMW HA may promote the biosynthesis of growth factors such as FGF-2 and KGF [8].

A Useful Marker for Evaluating Tissue-Engineered Products: Gap-Junctional Communication for Assessment of the Tumor Promoting Action and Disruption of Cell Differentiation in Tissue Engineered Products

Disruption of gap-junctional communication has been implicated in tumor promotion as well as abnormal development. Tumor promoting chemicals, such as phorbolesters, have been reported to inhibit gap-junctional intercellular communication in vitro and in vivo. It has been assumed that tumor promoters induce suppression of initial cells from surrounding communicating normal cells, resulting in a clonal expansion of the initiated cells.

Many tumor cells exhibit aberrant cell contact-mediated intercellular communication, i.e., loss of gap-junctional communication among themselves or with surrounding normal cells. In tissue engineering areas, various kinds of chemicals and metallic ions are used as scaffolds and catalysts. One of the representative degradable materials is polylactic acid, which has been widely used in biomaterials. However, the tumorigenicity of poly-L-lactide (PLLA) film was reported via a rat two-year-implantation study [11]. PLLA has been applied in surgical devices, such as a bone plates, pins, and screw, for which a longer duration of mechanical toughness is required and supported by a slow degradation of high molecular weights of PLLA. Usually, it takes several years for the complete absorption of such kinds of PLLA into the tissues. However, the mechanisms of PLLA tumorigenicity in rats are unclear. We investigated the effects of poly-D, L-lactic acid (PDLA, MW. 5000), a commercial product and its monomers of L- and D-lactic acid on the gap-junctional intercellular communication (GJIC). GJIC is considered to be important in the neuronal functions, because neurons have the equipment of connexins, and germ-line mutations of the Cx32 gene have been reported to be responsible for X-linked Charcot-Marie-Tooth disease of the peripheral nervous system. Therefore, the effects of neuronal toxic metal ions such as Cd^{5+}, V^{5+}, and Zn^{2+} ions were surveyed using two assay systems, namely, rat neuronal cell differentiation and V79 metabolic cooperation to clarify the inhibitory action of these ions on the GJIC of rat midbrain cells and V79 cells, both cells have gap-junctions composed of connexin 43 (Cx43). Whether the connexins play an important role in neuronal cell differentiation or not, three kinds of metal ions were assayed and compared with its inhibitory potentials between cell differentiation and metabolic cooperation.

Metabolic cooperation is a form of intercellular communication in which the mutant phenotype of enzyme-deficient cells is corrected by normal cells or by different mutant cells. Thus, wild-type Chinese hamster V79 cells (6-thioguanin-sensitive (6-TGs)) reduce the recovery of 6-thioguanine-resistant cells (6-TGr) when they are cultured together at high densities through intercellular

communication (metabolic cooperation). Cooperation is inhibited by 12-O-tetradecanoyl phorbol-13-acetate (TPA) rescuing the 6-thioguanine-resistance cells. These results may be useful in the study of an aspect of the mechanism of tumor-promotion and in assaying for promoters.

Three metal ions induced inhibitory action on embryonic midbrain differentiation, and the disruption of the cell-cell communication by these metal ions possibly led to the inhibition of the differentiation of the midbrain cells. Next, some polymers and their oligomers used in the tissue engineering field have inhibitory activities on GJIC. One cause may be related to the different manufacturing process using various kinds of metallic catalysts. From the present results, the in vitro short-term test for estimating the function of GJIC is important and useful for the safety of tissue-engineered products, especially with regard to dedifferentiation [10].

A Strategy for the Suppression of Tumorigenesis Induced by Biomaterials: Restoration of Transformed Phenotype of Polyetherurethane-Induced Tumor Cells by Cx43 Transfection

Polyetherurethanes (PEUs) are used for implant applications because of their useful elastomeric properties and high tensile strength, lubricity, good abrasion resistance, and ease of handling. However, some kinds of PEUs are known to be unstable in the body and can induce tumors in rats. We have reported the tumorigenic potentials of these PEUs in vivo and in vitro [13]. For many years, foreign body carcinogenesis in rodent species has been recognized as a classic model of multistage endogenous tumorigenesis that requires half to two-thirds of the life span for tumor development. A number of studies have demonstrated that physical, not chemical, characteristics are responsible for this phenomenon and that a dose-dependent relationship is evident with respect to implant size and tumor frequency [14]. We have examined the potency of tumor-initiating and tumor-promoting activities of the PEUs. The promoting activities were considered to be stronger than the initiating activities [15]. Furthermore, inhibitory action of polyethylene's surface on gap-junctional intercellular communication (GJIC) has been detected [16,17]. Thus, different inhibitory potential of GJIC on the surface of the biomaterials, including PEUs, may likely be a key step in determining the tumorigenic potentials. PEU-components had inhibited GJIC in cultures of Balb/c 3T3 A31-1-1 cells and Chinese V79 fibroblasts. GJIC has long been postulated to play an important role in the maintenance of cell homeostasis and in the control of cell growth. Therefore, the loss of GJIC has been considered to cause aberrant development and tumor formation. Gap- junctions are formed by jux taposition of two hemichannels known as connexons, located on the surface of adjacent cells [18]. Each connexon consists of six connexin (Cx) molecules. Cx43 is the most widely expressed Cx type. Accumulating evidence indicates that loss or reduction of GJIC in malignant transformed cells or tumor cells closely correlates to transcriptional down-regulation of Cx genes and /or to aberrant localization of Cx proteins. Restoration of GJIC by transfection of Cx genes has been demonstrated to reverse the transformed phenotype of cancer cells [19].

To clarify a tumor promoting activity of PEU, we have established an U41 rat tumor cell line from malignant fibrous histiocytoma (MFHC) developed in the PEU film implanted sites. Function of GJIC in our cell line was assayed by the scrape-loading dye transfer method and the expression of Cx43 connexin was

examined either by western blotting or by reverse transcription-PCR (RT-PCR). Since the introduction of Cx43 gene overcame the GJIC function, poor GJIC development in U41 cells might be caused by a suppressed connexin expression. The Cx43 up-regulated stable transfectant showed rather controlled cell growth on contact inhibition, and revealed poor anchorage independent growth in soft agar [20].

Biomaterials such as polyetherurethanes (PEUs) are the scaffolding, which is indispensable for the development of the bio-artificial organs. However, PEUs can induce tumors in subcutaneous implantation sites in rats. We have shown that the different inhibitory potential of gap-junctional intercellular communication (GJIC) on the surface of the biomaterial, including PEUs, is a key step in determining the tumorigenic potentiality. Here we show that suppression of a gap-junctional protein connexin 43 (Cx43) plays an important role *in vivo* tumorigenesis induced by PEUs for the first time and that Cx43 transfection may be an effective strategy for preventing tumorigenesis induced by biomaterials [20]. The rat tumor cell line U41 is derived from tumors in the subcutaneous implantation of PEU films. The GJIC and the expression of Cx43 were suppressed in U41. The restoration of normal phenotype, such as reduction of growth rate, recovery of contact inhibition and loss of colony formation ability in soft agar, was achieved by Cx43 transfection. These results strongly suggest that suppression of Cx43 expression plays an important role in the development of rat malignant fibrous hisitiocytome (MFHC) caused by PEUs and that Cx43 transfection is effective for prevention of tumorigenesis induced by PEUs [20].

References

[1] Tsuchiya, T., and Nakamura, A., "A New Hypothesis of Tumorigenesis Induced by Biomaterials: Inhibitory Potentials of Intercellular Communication ·Play An Important Role on the Tumor-Promotion Stage," *J. Long. Term. Effects Med. Implants*, Vol. 5, 1995, pp.233-242.

[2] Kawasaki, K., Ochi, M., Uchio, Y., Adachi, N., and Matsusaki, M., "Hyaluronic Acid Enhances Proliferation and Chondroitin Sulfate Synthesis in Cultured Chondrocytes Embedded in Collagen Gels," *J Cell Physiol*, Vol. 179, 1999, pp. 142-148.

[3] Alaish, S. M., Yager, D. R., Diegelmann, R. F., and Cohen, I. K., "Hyaluronic Acid Metabolism in Keloid Fibroblasts," *J Pediatric Surg*, Vol. 30, 1995, pp. 949-952.

[4] Park, J., and Tsuchiya, T., "Tumor-Promoting Actibity of 48 kDa Molecular Mass Hyaluronic Acid," *Materials Transactions*, Vol. 43, 2002, pp. 3128-3130.

[5] Park, J. U., and Tsuchiya, T., "Increase in Gap-Junctional Intercellular Communications (GJIC) of Normal Human Dermal Fibroblasts (NHDF) on Surfaces Coated with High-Molecular-Weight Hyaluronic Acid (HMW HA)," *J Biomed Mater Res*, Vol. 60(4), 2002 pp.541-547.

[6] Trosko, J. E., Madhukar, B. V., and Chang, C. C., "Endogenous and Exogenous Modulation of Gap Junctional Intercellular communication: Toxicological and Pharmacological Implication," *Life Sci*,Vol. 53(1), 1993, pp. 1-19.

[7] Nagy, J. I., Hossain, M. Z., Lynn, B.D., Curpen, G. E., Yang S., and Turley, E. A., "Increased Connexin-43 and Gap junctional Communication Correlate

with Altered Phenotypic Characteristics of Cells Overexpressing the Receptor for Hyaluronic Acid-Mediated Motility," *Cell Growth Differ.*, Vol. 7(6), 1996, pp. 745-751.

[8] Park, J.U., and Tsuchiya, T., "Increase in Gap Junctional Intercellular Communication by High Molecular Weight Hyaluronic Acid Associated with Fibroblast Growth Factor 2 and Keratinocyte Growth Factor Production in Noemal Human Dermal Fibroblasts," *Tissue Engineering*. Vol. 8 No. 3, 2002, pp. 419-427.

[9] Neumann, A., Schinzel, R., Riederer, P. D., and Munch, G., "High Molecular Weight Hyaluronic Acid Inhibits Advanced Glycation End Product-Induced NF-kappaB Activation and Cytokine Expression," *FEBS Lett,* Vol. 25, 453(3), 1999, pp. 283-287.

[10] Walicke, P.A., "Interactions between Basic Fibroblast Growth Factor (FGF) and Glycosoaminoglycans in Promoting Neurite Outgrowth," *Exp Neurol*, Vol. 102(1), 1988, pp.144-8.

[11] Nakamura, T., Shimizu, Y., Okumura, N., Matsui, T., Hyon, S. H., and Shimamoto, T., "Tumorigenicity of Poly-L-lactide (PLLA) Plates Compared with Medical-Grade Polyethylene," *J Biomed Mater Res*, Vol. 28(1), 1994, pp.17-25.

[12] Tsuchiya, T., "A Useful Marker for Evaluating Tissue-Engineered Products: Gap-Junctional Communication for Assessment of the Tumor-Promoting Action and Disruption of Cell Differentiation in Tissue-Engineered Products," *J Biomater Sci Polym Ed*, Vol. 11(9), 2000, pp. 947-959.

[13] Tsuchiya, T., Hata, H., and Nakamura, A., "Studies on the Tumor-Promoting Activity of Biomaterials: Inhibition of Metabolic Cooperation by Polyetherurethane and Silicone," *J Biomed Mater Res*, Vol. 29(1), 1995, pp. 113-119.

[14] Schoen, F. J., "Biomaterial-Associated Infection, Neoplasia, and Calcification Clinicopathologic Features and Pathophysiologic concepts." *ASAIO Trans*, Vol. 33(1), 1987, pp. 8-18.

[15] Tsuchiya, T., Nakaoka, R., Degawa, H., and Nakamura, A., "Studies on the Mechanisms of Tumorigenesis Induced by Polyetherurethanes in Rats: Leachable and Biodegradable Oligomers Involving the Diphenyl Carbamate Structure Acted as an Initiator on the Transformation of Balb 3T3 Cells," *J Biomed Mater Res*, Vol. 31(3), 1996, pp.299-303.

[16] Nakaoka, R., Tsuchiya, T., Kato, K., Ikada, Y., and Nakamura, A., "Studies on Tumor-Promoting Activity of Polyethylene: Inhibitory Activity of Metabolic Cooperation on Polyethylene Surfaces is Markedly Decreased by Surface Modification with Collagen but not with RGDS Peptide," *J Biomed Mater Res*, Vol. 35(3), 1997, pp.391-397.

[17] Nakaoka, R., Tsuchiya, T., and Nakamura, A., "The Inhibitory Mechanism of Gap Junctional Intercellular Communication Induced by Polyethylene and the Restorative Effects by Surface Modification with Various Proteins," *J Biomed Mater Res*, Vol. 57(4), 2001, pp.567-574.

[18] Bennett, M. V., Barrio, L. C., Bargiello, T. A., Spray, D. C., Hertzberg, E., and Saez, J. C., "Gap Junctions: New Tools, New Answers, New Questions," *Neuron*, Vol. 6(3), 1991, pp.305-320.

[19] Huang, R. P., Fan, Y., Hossain, M. Z., Peng, A., Zeng, Z. L., and

Boynton, A. L., "Reversion of the Neoplastic Phenotype of Human Glioblastoma Cells by Connexin 43 (cx43),"*Cancer Res*, Vol. 58(22), 1998, pp.5089-5096.

[20] Ichikawa, A., and Tsuchiya, T., "A Strategy for the Suppression of Tumorigenesis Induced by Biomaterials: Restoration of Transformed Phenotype of Polyetherurethane-Induced Tumor Cells by Cx43 Transfection," *Cytotechnology*, Vol. 39, 2002, pp.1-8.

Novel mechanism of tumorigenesis: Increased transforming growth factor-β1 suppresses the expression of connexin 43 in BALB/cJ mice after 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

Received 15 December 2003; revised 24 March 2004; accepted 6 April 2004
Published online 4 June 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30090

Abstract: Poly-L-lactic acid (PLLA) is a widely used promising material for surgical implants such as tissue-engineered scaffolds. In this study, we aimed to determine the *in vivo* effect of PLLA plates on the cellular function of subcutaneous tissue in the two mouse strains, BALB/cJ and SJL/J, higher and lower tumorigenic strains, respectively. Gap-junctional intercellular communication (GJIC) and the expression of connexin 43 (Cx43) protein were significantly suppressed, whereas the secretion of transforming growth factor-β1 (TGF-β1) level was significantly increased in PLLA-implanted BALB/cJ mice compared with BALB/cJ controls. However, no significant difference in TGF-β1 secretion was observed between the SJL/J-implanted and SJL/J control mice. We found for the first time that a significant difference was observed between the two strains; thus, the PLLA increased the secretion of TGF-β1 and suppressed the mRNA expression of Cx43 at the earlier stage after implantation into the higher-tumorigenic strain, BALB/cJ mice. This novel mechanism might have a vital role in the inhibition of GJIC and promote the tumorigenesis in BALB/cJ mice. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 70A: 335–340, 2004

Key words: poly-L-lactic acid; gap-junctional intercellular communication (GJIC); connexin 43; transforming growth factor (TGF)-β; tumorigenesis

INTRODUCTION

The implantation of a biomaterial always induces a host inflammatory response. The extent and resolution of these responses have a vital role in determining the long-term success of implanted medical devices.[1–3] Poly-L-lactic acid (PLLA) is a widely used material for surgical implants and clinically as a bioabsorbable suture material.[4,5] Polyurethanes (PUs) have also been used for implant applications because of their useful elastomeric properties and high tensile strength, lubricity, and good abrasion resistance. Some adverse effects of the biomaterials, such as PLLA and PUs, have been reported in animal experiments. Long-term implants of PLLA produced tumorigenicity in rats.[6] Different kinds of PUs induced various tumor incidences in rats.[7] All tumors have been generally viewed as the outcome of disruption of the homeostatic regulation of the cellular ability to respond to extracellular signals, which trigger intracellular signal transduction abnormalities.[8] During the evolutionary transition from the single-cell organism to the multicellular organism, many genes appeared to accompany these cellular functions. One of these genes was the gene coding for a membrane-associated protein channel (the gap junction).[9] Gap-junctional intercellular communications (GJIC) 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 a cell- and development-specific manner.[10,11] GJIC also has an important role in the maintenance of cell homeostasis and in the control of cell growth.[12] So, the loss of GJIC has been considered to cause abnormal development and tumor formation.[13–15] Several tumor promoters have been shown to restrict GJIC by phosphorylation of connexin proteins, such as connexin 43 (Cx43), which is an essential

Correspondence to: T. Tsuchiya; e-mail: tsuchiya@nihs.go.jp
Contract grant sponsor: Health and Labour Sciences Research Grants
Contract grant sponsor: Research on Advanced Medical Technology, Ministry of Health, Labour and Welfare
Contract grant sponsor: Japan Health Sciences Foundation

protein to form the gap-junction channel.[16,17] We have hypothesized that the different tumorigenic potentials of PLLA and PUs are caused mainly by the different tumor-promoting activities of these biomaterials. Therefore, we investigated the effects of PLLA on the subcutaneous tissue between the two strains of female mice, BALB/cJ and SJL/J.

MATERIALS AND METHODS

Animals

Five-week-old female BALB/cJ and SJL/J mice were purchased from Charles River (Japan) and maintained in the animal center according to the animal welfare National Institute of Health Sciences guidance. All mice were fed with standard pellet diets and water *ad libitum*, before and after the implantation.

Implantation of PLLA

PLLA was obtained from Shimadzu Co. Ltd. as uniform plates. Implants (size: 20 × 10 × 1 mm, weight-average molecular weight 200,000) were sterilized using ethylene oxide gas before use. Sodium pentobarbital (4 mg/kg) was intraperitoneally administered to the mice. The dorsal skin was shaved and scrubbed with 70% alcohol. Using an aseptic technique, an incision of approximately 2 cm was made; away from the incision, a subcutaneous pocket was formed by blunt dissection, and one piece of PLLA was placed in the pocket. The incision was closed with silk threads. In both strains, controls were obtained by sham operation and subsequent subcutaneous pocket formation. After surgery, the mice were housed in individual cages. After 30 days, mice from the implanted group were sacrificed, implanted materials were excised out, and subcutaneous tissues from the adjacent sites were collected for culture. At the same time, subcutaneous tissues were removed from the sites in the sham-operated controls that correlated with the implant sites.

Cell culture of subcutaneous tissues

The subcutaneous tissues were maintained in minimum essential medium supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C.

Scrape-loading and dye transfer (SLDT) assay

SLDT technique was performed by the method of El-Fouly et al.[18] Confluent monolayer cells in 35-mm culture dishes were used. After rinsing with Ca^{2+} Mg^{2+} phosphate-buffered saline [PBS (+)], cell dishes were loaded with 0.1% Lucifer Yellow (Molecular Probes, Eugene, OR) in PBS (+) solution and were 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 transfer was monitored using a fluorescence microscope, equipped with a type UFX-DXII CCD camera and super high-pressure mercury lamp power supply (Nikon, Tokyo, Japan).

Western blot analysis

When cells grew confluently in 60-mm tissue culture dishes, all cells were lysed directly in 100 μL of 2% sodium dodecyl sulfate (SDS) gel loading buffer (50 m*M* Tris-HCl, pH 6.8, 100 m*M* 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The protein concentration of the cleared lysate was measured using the microplate BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL). Equivalent protein samples were analyzed by 7.5% SDS–polyacrylamide gel electrophoresis. The proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK). Cx43 protein was detected by anti-Cx43 polyclonal antibodies (ZYMED Laboratories, Inc., San Francisco, CA). The membrane was soaked with Block Ace (Yukijirusi Nyugyo, Sappro, Japan), reacted with the anti-Cx43 polyclonal antibodies for 1 h, and after washes with PBS containing 0.1% Tween20, reacted with the secondary anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase for 1 h. After several washes with PBS–Tween20, the membrane was detected with the ECL detection system (Amersham Pharmacia Biotech UK Ltd.).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Cx43 mRNA expression was verified by RT-PCR. Total cellular RNA was isolated from cultured cells in Trizol reagent (Life Technologies, Inc., Frederick, MD) following the manufacturer's instructions. The concentration of total RNA was determined using a UV spectrophotometer (Gene Quant; Pharmacia Biotech, Piscataway, NJ). cDNA was synthesized from 1 μg of total RNA by RT using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). 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) and 0.2 m*M* of each deoxynucleotide triphosphate. The sequence of the primer pairs were as follows: forward 5′-ACAGTCTGCCTTTCGCTGTAAC-3′ and reverse 5′-GTAAGGATCGCTTCTTCCCTTC-3′. The PCR cycle was as follows: initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with final extension at 72°C for 7 min. The amplified product was separated on 1.5% agarose gel and visualized with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME). For relative quantitation, the signal intensity of each lane was standardized to that of a housekeeping gene,

GAPDH. To amplify this gene, the following primer pairs were used: forward 5′-CCCATCACCATCTTCCAGGAGC-GAGA-3′ and reverse 5′-TGGCCAAGGTCATCCATGA-CAACTTTGG-3′.

Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded onto 60-mm dishes. The conditioned medium was collected and obtained after the centrifugation at 1000 rpm for 2 min. The transforming growth factor (TGF)-β levels of the media were measured with commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN).

Cytokine treatment

Here, we used sham-operated BALB/cJ mice cells as a control. One hundred thousand cells were seeded onto 35-mm tissue culture dishes and cultured. After 4 h seeding in a 5% CO_2 atmosphere at 37°C, cells were treated with TGF-β1 (0, 2, and 10 ng/mL). Thereafter, SLDT and RT-PCR were performed. Purified human TGF-β1 was purchased from R&D Systems.

Statistical analysis

Student *t* test was used to compare the implanted samples with the controls. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± standard deviation.

RESULTS AND DISCUSSION

There are many known tumorigenesis-inducing factors. It was reported that many plastics induce malignant tumors when implanted subcutaneously into rats and mice.[19–22] PLLA shows slow degradation, and therefore has been applied as a biomaterial for surgical devices such as bone plates, pins, and screws. It was reported in different studies that polyetherurethane, polyethylene, and PLLA produced tumors in rats.[6,7,23–25] In our study, tumors were induced by PLLA plates in BALB/cJ mice at 100% incidence but not in SJL/J mice at the surrounding tissues of PLLA plates during a 10-month *in vivo* study. To understand the mechanisms of tumorigenesis induced by PLLA, we focused on the inhibitory effects on GJIC at the early stage of tumorigenesis. To assess functional GJIC, the SLDT assay was performed. Brand et al.[26] reported that BALB/cJ mice are a higher and SJL/J mice are a lower tumorigenic strain. Our present results showed that the GJIC was significantly inhibited in 1-month PLLA-implanted BALB/cJ mice cells compared with BALB/cJ controls (Fig. 1). In contrast, no significant difference was observed between the 1-month PLLA-implanted SJL/J mice and SJL/J controls (Fig. 1). The data also revealed that the dye migration was higher in control BALB/cJ mice than control SJL/J mice (Fig. 1). High responder to the tumorigenicity may be classified as animals that are easily suppressed in both GJIC function and the connexins expression. This perturbed gap junction is likely to have a major role in the PLLA-induced tumorigenesis. Gap junctions are also regulated by the posttranslational phosphorylation of the carboxy-terminal tail region on the connexin molecule. Phosphorylation of connexin molecules is closely related with the inhibition of GJIC.[27,28] Phosphorylation has been involved in controlling a broad variety of connexin processes that include trafficking, gathering/nongathering, degradation, and also the gating of gap channels. It was also reported that communication-deficient cells did not express the Cx43–biphosphorylated (P_2) isoform but cells with low gap-junction permeability showed detectable amounts of the Cx43–monophosphorylated (P_1) isoform.[16] To survey the cause, we examined the mRNA and protein expression of the Cx43 gene. Here, mRNA expression was suppressed in PLLA-implanted BALB/cJ mice compared with BALB/cJ controls [Fig. 2(A)]. No significant difference was observed between the PLLA-implanted SJL/J mice and SJL/J controls [Fig. 2(B)]. We also found that the total level of protein expression such as unphos-

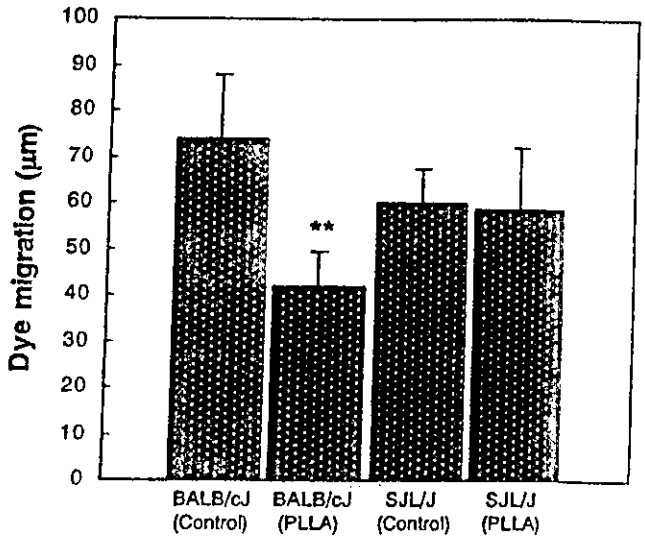


Figure 1. Statistical analysis of the SLDT assay. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. GJIC was significantly inhibited in PLLA-implanted BALB/cJ mice cells compared with BALB/cJ controls. $^{**}p < 0.01$.

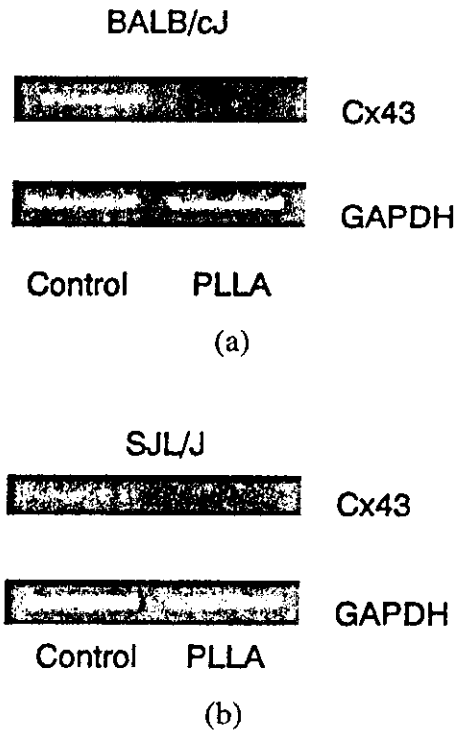


Figure 2. mRNA expression of Cx43 by RT-PCR analysis. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. SYBR Green I stained PCR products after agarose gel electrophoresis showed that (A) mRNA expression was suppressed in PLLA-implanted BALB/cJ mice compared with BALB/cJ controls, and (B) no significant difference was observed between the PLLA-implanted SJL/J mice and SJL/J controls.

phorylated (P_0), P_1, and P_2 levels were significantly decreased in PLLA-implanted BALB/cJ mice compared with the control (Fig. 3). Asamoto et al.[29] reported that tumorigenicity was enhanced when the expression of Cx43 protein was suppressed by the anti-sense RNA of Cx43. A similar tendency was also observed in our study where the protein expression might be inhibited via down-regulation of the mRNA level. The genetic alteration and posttranslational

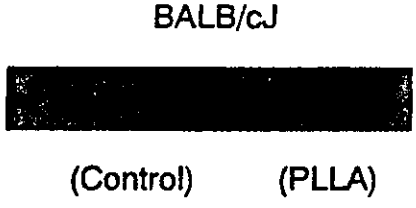


Figure 3. Protein expression of Cx43 by Western blot analysis. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. Total level of protein expression such P_0, P_1, and P_2 levels were significantly decreased in PLLA-implanted BALB/cJ mice compared with the controls.

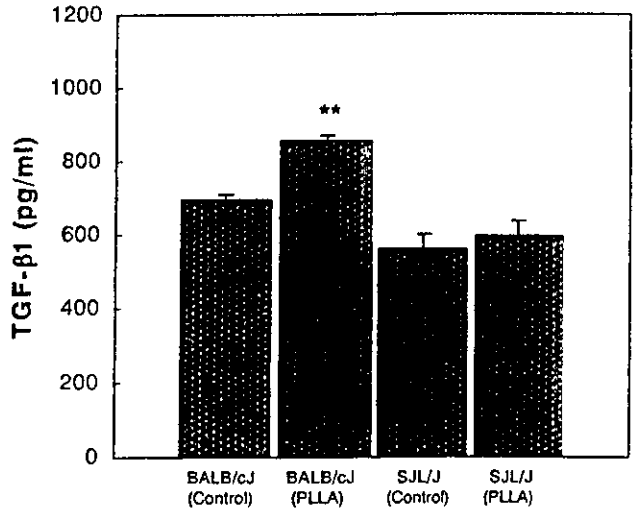
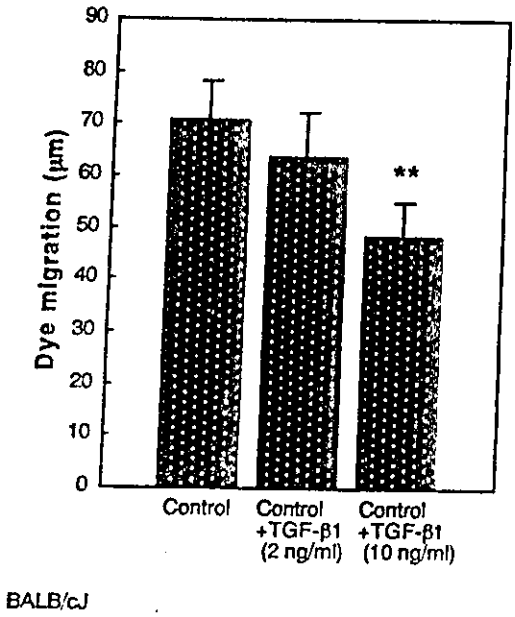


Figure 4. Statistical analysis of TGF-β1 cytokine assay by ELISA. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. Secretion of the TGF-β1 level was significantly increased in PLLA-implanted BALB/cJ mice compared with BALB/cJ controls. $^{**}p < 0.01$.

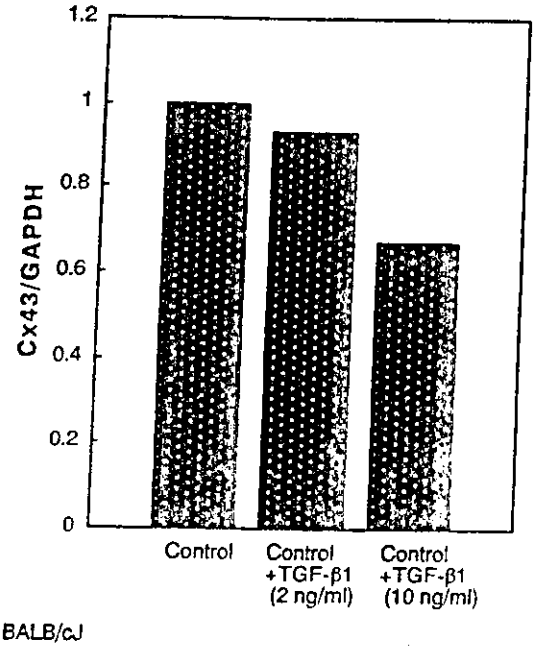
modification in the Cx43 protein was shown to be involved in impaired GJIC and could be associated with tumorigenesis. Therefore, it is suggested that the inhibitory effect of PLLA on GJIC might be caused by the alteration in the Cx43 protein, causing enhancement of tumorigenesis. Moreover, Moorby and Patel[30] reported a direct action of the Cx43 protein on cell growth that was mediated via the cytoplasmic carboxyl domain.

Because TGF-β1 inhibits GJIC by decreasing the phosphorylated form of Cx43[31] and the phosphorylation of Cx43 has been implicated in gap-junction assembly and gating events,[16,27,32] we hypothesized that TGF-β1 might have an important role on PLLA-implanted BALB/cJ mice. Figure 4 clearly demonstrates that the secretion of the TGF-β1 level was significantly increased in PLLA-implanted BALB/cJ subcutaneous tissue in comparison with those from BALB/cJ control mice. No significant difference was found in the secretion of TGF-β1 between the SJL/J implanted and SJL/J control mice. TGF-β2 and TGF-β3 cytokine assay revealed no significant difference in TGF-β2 secretion and TGF-β3 was below the detection level (data not shown). So we performed an *in vitro* study, which showed that the intercellular communication and the mRNA expression of Cx43 were significantly suppressed in BALB/cJ control cells when treated with TGF-β1 [Fig. 5(A,B)].

In conclusion, we suggest that increased secretion of TGF-β1 (Fig. 4) suppressed expression of the gap-junctional protein Cx43 (Fig. 3) at the earlier stage after implantation of PLLA in BALB/cJ mice, resulting in



(a)



(b)

Figure 5. (A) SLDT assay. (B) National Institutes of Health image analysis quantitation of RT-PCR bands. In both figures, BALB/cJ control cells were treated with 2 and 10 ng/mL TGF-β1. GJIC was significantly inhibited and mRNA expression was significantly suppressed in BALB/cJ control cells treated with 10 ng/mL TGF-β1 compared with BALB/cJ controls. $^{**}p < 0.01$. Three dishes were used for one data point (bar) as one experiment. Results shown are representative of two independent experiments.

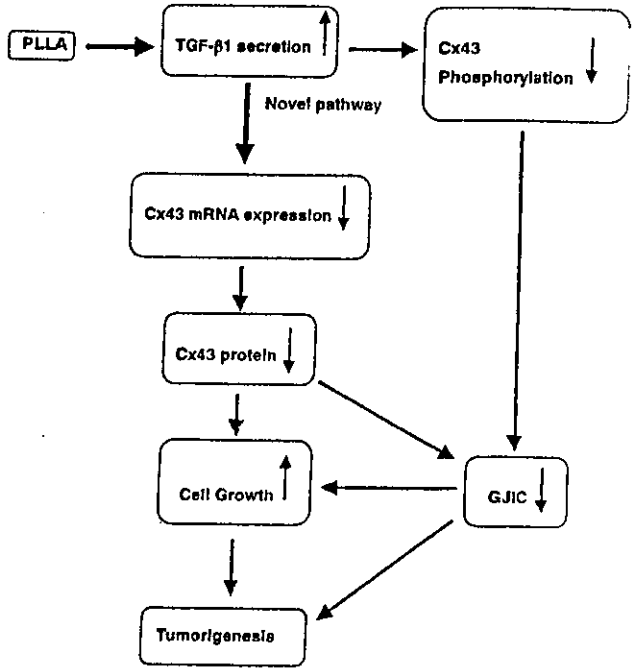


Figure 6. Schematic representation of the pathway of tumorigenesis induced by PLLA in BALB/cJ mice.

the suppression of the function of GJIC (Fig. 1) and at the same time, mRNA expression of Cx43 was suppressed in BALB/cJ mice (higher tumorigenic) but not in SJL/J mice (lower tumorigenic) [Fig. 2(A,B)]. TGF-β1 also suppressed the expression of mRNA of Cx43 and the function of GJIC in the BALB/cJ mouse cells *in vitro* [Fig. 5(A,B)]. These results indicated the novel mechanism of tumorigenesis induced by PLLA (Fig. 6).

References

1. Tang L, Eaton JW. Inflammatory responses to biomaterials. Am J Clin Pathol 1995;103:466–471.
2. Rames A, Williams DF. Immune response in biocompatibility. Biomaterials 1992;13:731–743.
3. Anderson JM. Mechanisms of inflammation and infection with implanted devices. Cardiovasc Pathol 1993;2:33s–41s.
4. Kulkarni RK, Pani KC, Neuman C, Leonard F. Polylactic acid for surgical implants. Arch Surg 1966;93:839–843.
5. Craig PH, Williams JA, Davis KW, Magoun AD, Levy AJ, Bogdansky S, Jones JP Jr. A biological comparison of polyglactin 910 and polyglycolic acid synthetic absorbable sutures. Surg Gynecol Obstet 1995;141:1–10.
6. Nakamura T, Shimizu Y, Okumura N, Matsui T, Hyon SH, Shimamoto T. Tumorigenicity of poly-L-lactide (PLLA) plates compared with medical-grade polyethylene. J Biomed Mater Res 1994;28:17–25.
7. Nakamura A, Kawasaki Y, Takada K, Aida Y, Kurokama Y, Kojima S, Shintani H, Matsui M, Nohmi T, Matsuoka A, Sofuni T, Kurihara M, Miyata N, Uchima T, Fujimaki M. Difference in tumor incidence and other tissue responses to polyetherurethanes and polydimethylsiloxane in long-term subcutaneous implantation into rats. J Biomed Mater Res 1992;26:631–650.

8. Trosko JE, Madhukar BV, Chang CC. Endogenous and exogenous modulation of gap junctional intercellular communication: toxicological and pharmacological implications. Life Sci 1993;53:1–19.
9. Trosko JE, Ruch RJ. Cell–cell communication in carcinogenesis. Front Biosci 1998;3:D208–236.
10. Eiberger J, Degen J, Romualdi A, Deutsch U, Willecke K, Sohl G. Connexin genes in the mouse and human genome. Cell Commun Adhes 2001;8:163–165.
11. Bruzzone R, White TW, Paul DL. Connections with connexins: the molecular basis of direct intercellular signaling. Eur J Biochem 1996;238:1–27.
12. Loewenstein WR. Junctional intercellular communication and the control of growth. Biochim Biophys Acta 1979;560:1–65.
13. Guthrie SC, Gilula NB. Gap junctional communication and development. Trends Neurosci 1989;12:12–16.
14. Klaunig JE, Ruch RJ. Role of inhibition of intercellular communication in carcinogenesis. Lab Invest 1990;62:135–146.
15. Mesnil M, Yamasaki H. Cell–cell communication and growth control of normal and cancer cells: evidence and hypothesis. Mol Carcinog 1993;7:14–17.
16. Musil LS, Goodenough DA. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. J Cell Biol 1991;115:1357–1374.
17. Musil LS, Goodenough DA. Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. Cell 1993;74:1065–1077.
18. El-Fouly MH, Trosko JE, Chang CC. Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. Exp Cell Res 1987;168:422–430.
19. Turner FC. Sarcomas at sites of subcutaneously implanted bakelite disks in rats. J Natl Cancer Inst 1941;2:81–83.
20. Oppenheimer BS, Oppenheimer ET, Danishefsky I, Stout AP, Eirich FR. Further studies of polymers as carcinogenic agents in animals. Cancer Res 1955;15:333–340.
21. Bischoff F, Bryson G. Carcinogenesis through solid state surface. Prog Exp Tumor Res 1964;5:85–133.
22. Karp RD. Tumorigenesis by Millipore filters in mice: histology and ultrastructure of tissue reactions as related to pore size. J Natl Cancer Inst 1973;51:1275–1285.
23. Tsuchiya T, Hata H, Nakamura A. Studies on the tumor-promoting activity of biomaterials: inhibition of metabolic cooperation by polyetherurethane and silicone. J Biomed Mater Res 1995;29:113–119.
24. Tsuchiya T. A useful marker for evaluating tissue-engineered products: gap-junctional communication for assessment of the tumor-promoting action and disruption of cell differentiation in tissue-engineered products. J Biomater Sci Polym Ed 2000; 11:947–959.
25. Nakaoka R, Tsuchiya T, Kato K, Ikada Y, Nakamura A. Studies on tumor-promoting activity of polyethylene: inhibitory activity of metabolic cooperation on polyethylene surfaces is markedly decreased by surface modification with collagen but not with RGDS peptide. J Biomed Mater Res 1997;35:391–397.
26. Brand I, Buoen LC, Brand KG. Foreign-body tumors of mice: strain and sex differences in latency and incidence. J Natl Cancer Inst 1977;58:1443–1447.
27. Musil LS, Cunningham BA, Edelman GM, Goodenough DA. Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. J Cell Biol 1990;111:2077–2088.
28. Lampe PD, Lau AF. Regulation of gap junctions by phosphorylation of connexins. Arch Biochem Biophys 2000;384:205–215.
29. Asamoto M, Toriyama-Baba T, Krutovskikh V, Cohen SM, Tsuda H. Enhanced tumorigenicity of rat bladder squamous cell carcinoma cells after abrogation of gap junctional intercellular communication. Jpn J Cancer Res 1998;89:481–486.
30. Moorby C, Patel M. Dual functions for connexins: Cx43 regulates growth independently of gap junction formation. Exp Cell Res 2001;271:238–248.
31. Wyatt LE, Chung CY, Carlsen B, Iida-Klein A, Rudkin GH, Ishida K, Yamaguchi DT, Miller TA. Bone morphogenetic protein-2 (BMP-2) and transforming growth factor-beta1 (TGF-beta1) alter connexin 43 phosphorylation in MC3T3-E1 cells. BMC Cell Biol 2001;2:14.
32. Laird DW, Castillo M, Kasprzak L. Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. J Cell Biol 1995; 131:1193–1203.

Gene expression changes in BALB/3T3 transformants induced by poly(L-lactic acid) or polyurethane films

Atsuko Matsuoka, Toshie Tsuchiya
Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Received 26 March 2003; revised 9 July 2003; accepted 25 August 2003
Published online 19 December 2003 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.20068

Abstract: We performed DNA microarray analysis on two BALB/3T3 transformants (A5 and A6) induced by polyurethane (PU) film, two (L11 and L21) induced by biodegradable poly(L-lactic acid) (PLLA) film, and the parental cells. The transforming ability of the cells was in the order A5 < A6 < L21 < L11. In all, 1176 cancer-related genes were up- or down-regulated in at least one transformant. Those that were markedly up-regulated were c-*fos* protooncogene, FBJ osteosarcoma oncogene B, and Jun oncogene; those markedly down-regulated were pleiotrophin, histidine triad nucleotide-binding protein, protein kinase C iota, and large multifunctional protease 7. A common function of proteins encoded by genes that underwent marked expression changes was bone formation. The genes were c-*fos*, FBJ osteosarcoma, Jun, pleiotrophin, a disintegrin-like and metalloprotease with TS-1 motif protein 1. This finding was consistent with the tumor formation in the 2-year PLLA or PU subcutaneous implantation into rats. The number of genes that underwent marked expression change in each transformant was consistent with its malignancy. PLLA induced more malignant transformants than PU, especially in relation to osteosarcoma-like gene expression. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 68A: 376–382, 2004

Key words: BALB/3T3; transformation; PU; PLLA; DNA microarray analysis

INTRODUCTION

Polyurethanes (PUs) are widely used in medical devices because of their elasticity, high tensile strength, biocompatibility, and ease of handling. Poly(L-lactic acid) (PLLA) is used for bone screws and bone fixing plates because of its biodegradability. Some PUs, however, are unstable *in vivo* and induce tumors in rats.[1] Although there have been a number of *in vitro* studies on chemically induced transformation, few have analyzed the transformant DNA.

In the present study, we used DNA microarrays to analyze gene expression in transformants induced on high-molecular-weight polymer materials and related altered expression to the malignancy of the transformants, focusing on the consequences of transformation rather than on the process.

MATERIALS AND METHODS

Cells

Mouse Balb/3T3 clone A31-1-1 cells provided by Dr. T. Kuroki[2] (University of Tokyo) were maintained in minimum essential medium supplemented with 10% heat-inactivated fetal calf serum in 5% CO_2 in air at 37°C.

Correspondence to: A. Matsuoka; e-mail: matsuoka@nihs.go.jp

Materials

PU [MDI/PTMO 1000/BD, weight-averaged molecular weight (M_w) 220,000] was obtained from Sanyo-kasei Co. Ltd. and PLLA (M_w 200,000) films (thickness 0.3 mm) were obtained from Shimadzu Corporation.

Coating of materials on the glass dishes

PU was dissolved in tetrahydrofuran. Half the surface area of glass dishes (diameter 6 cm) was coated with 320 mg of PU. After the dishes dried, they were sterilized by autoclaving at 121°C for 15 min. In the case of PLLA, the film was cut to fit the bottom of plastic dishes (diameter 6 cm) and attached with a small amount of acetone. The acetone was evaporated completely and the dishes were sterilized under UV-irradiation for 2 h.

The transformation assay

Cells were seeded at a density of 1×10^4 /plate (diameter 6 cm) on a coating and cultured in medium that was changed twice per week. After 6 weeks, transformants were isolated and stored at −80°C.