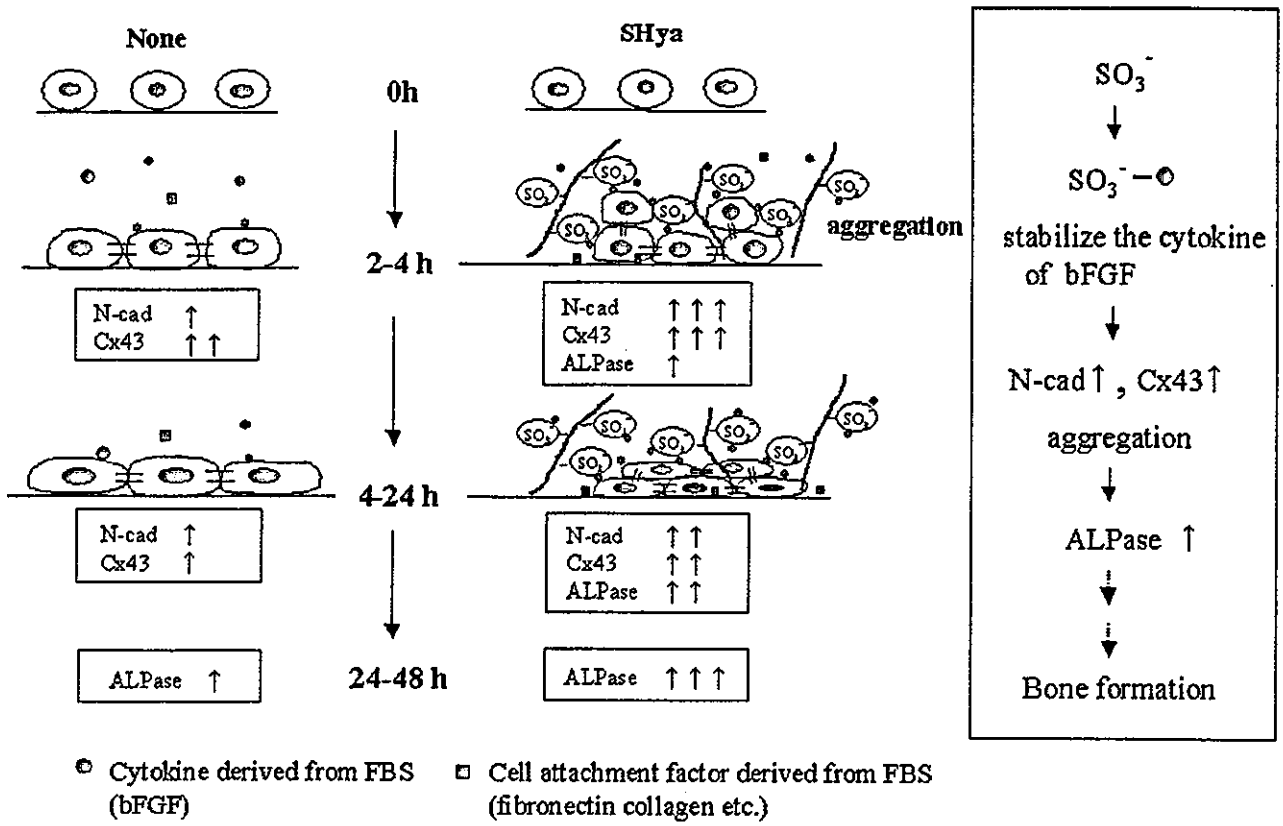

Fig. 10. SHya regulation of rat osteoblast cell differentiation.

ALPase activity was examined using the Azo staining method. The increased ratio and value of rOB cell ALPase activity with addition of SHya was higher than that of the control or Hya addition after 8 h. From the above results, the differentiation of rOB cells is promoted in aggregation, but the degree of proliferation is low as a whole. Therefore, it was shown that SHya controls rOB cell proliferation and differentiation, and that it especially promotes the differentiation. In this experiment, ALPase activity is enhanced while the expression of N-cad and Cx43 of rOB cells rises (Figs. 3, 6, and 7). Therefore, the expression of N-cad and Cx43 of rOB cells forming aggregations rises, and seems to promote the differentiation function, ALPase activity is enhanced with the aggregation formation of rOB cells.

In this experiment, 10% FBS was included in the culture medium. A serum of the usual 5–10% was included for the general culture medium used by the cell cultures of in vitro. The serum contained many components such as hormone, growth factor, cell adhesion molecule, and transportation protein [32]. Therefore, SHya interacted with the serum component, and it seemed to affect the cell. We examined the effect on the rOB cells by adding SHya to the serum-free medium. As the result, aggregations were not formed. However, when SHya coexisted with the serum, rOB cells formed aggregations. The interaction between the cell aggregation and the serum component such as FN, bFGF, and SHya was examined. In the case of FN, there was no effect on the cell aggregation. However, in the case of bFGF, cell aggregations were observed in both conditions with and without 2.1SHya. Therefore, it seems to relate the function of bFGF to the cell aggregation. N-cad expression of osteoblast by bFGF has been reported [22].

From these results, the effects of SHya on rOB cell function were not from the SHya alone; the data indicated that SHya affected rOB cell aggregation, proliferation, and differentiation by interacting with the serum component such as FGF and ECM (Fig. 10).

In conclusion, early expression of N-cad and Cx43 by SHya is the key to forming aggregations and enhancing the ALPase activity in rOB cells.

Acknowledgments

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The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes

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Abstract

Polyelectrolyte complexes (PEC) were prepared from chitosan as the polycation and several synthesized functional anion polysaccharides, and their effects on cell attachment, morphology, proliferation and differentiation were estimated using normal human osteoblasts (NHOst). After a 1-week incubation, PEC made from polysaccharides having carboxyl groups as polyanions showed low viability of NHOst on it although the NHOst on it showed an enhancement in their differentiation level. On the other hand, NHOst on PEC made from sulfated or phosphated polysaccharides showed similar attachment and morphology to those on the collagen-coated dish. When the number of NHOst was estimated after 1 week, the number on the PEC was ranged from 70% to 130% of those on the collagen-coated dish, indicating few effects of these PEC on cell proliferation. In addition, NHOst on PEC films made from sulfated polysaccharides differentiated to a level very similar to that observed on the collagen-coated dish, indicating that these PEC films maintain the normal potential of NHOst to both proliferate and differentiate. Measurement of gap junctional intercellular communication of NHOst on PEC revealed that PEC did not inhibit communication, suggesting that PEC films have few effects on cell homeostasis. Thus, PEC made from the sulfated polysaccharide may be a useful material as a new scaffold for bone regeneration.

Keywords: Polyelectrolyte complex; Normal human osteoblasts; Cell proliferation; Cell differentiation; Gap junctional intercellular communication

1. Introduction

The extracellular matrix (ECM) provides an essential three-dimensional (3D) environment for cells to construct several kinds of tissues. The ECM, consisting of numerous kinds of molecules such as proteins, polysaccharides and proteoglycans regulates the behavior of surrounding cells to form tissues and organs precisely [1,2]. For tissue regeneration trials using in vitro techniques, therefore, it is indispensable to develop a synthetic ECM scaffold that functions similarly to the native ECM. For more than a decade, engineering of new tissues by using selective cell transplantation on polymer scaffolds as an artificial ECM instead of tissue transplantation to other living bodies has been studied [3,4]. Recently, many studies on developing a scaffold for tissue regeneration have been done using ECM proteins such as collagen and gelatin [5–7], biodegradable synthetic polymers [8–10] and polysaccharides [11,12]. Because proteins derived from human tissues have many problems such as antigenicity or potential for infection, a biocompatible synthetic polymer or polysaccharide may be preferable for tissue regeneration.

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2.2. Preparation of PEC and PEC-coated dishes

Polyanions were dissolved individually in distilled water (final concentration = 5×10^{-4} mol of ionic sites/l), and the pH of the solutions was adjusted to 7.4 by adding aqueous HCl or NaOH. Chitosan was dissolved in aqueous 0.5% acetic acid solution and the pH adjusted to 6.0. The ratio of the solutions of polyanions and polycation was adjusted in each combination to neutralize the charge balance of PEC. This mixed solution (1 ml/35 mm tissue culture dish) was allowed to stand overnight at room temperature. After removing the supernatant solution, the dish was dried and annealed at 65 °C in an oven. Then, the dishes were washed with distilled water and oven-dried again to form the PEC-coated dish. This dish was sterilized for 3 min in a microwave oven. Water contact angles of PEC films were measured with the sessile drop method [23], and their zeta potentials were measured by Otsuka Electronics Co., Ltd. (Osaka, Japan).

2.3. Cell culture

NHOst were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco, Grand Island, NY) containing 20% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo Japan). The cells were maintained in incubators under standard conditions (37 °C, 5%-CO_2–95%-air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 mM beta-glycerophosphate. NHOst cells (1×10^5 cells/dish/2.5 ml medium) were cultured on PEC-coated dishes to evaluate the effects of their interaction with PEC. In each experiment, the medium was changed three times before GJIC of the cells was measured and their differentiation level was evaluated after a 1-week incubation.

2.4. Estimation of differentiation level of NHOst cultured on PEC films

The proliferation of NHOst cells cultured on PEC films was estimated by Tetracolor One assay (Seikagaku Co., Tokyo, Japan), which incorporates an oxidation-reduction indicator based on detection of metabolic activity. After a 1-week incubation, 50 μl of Tetracolor One solution was added to each test dish, followed by a further 2 h incubation. The absorbance of the supernatant at 450 nm was estimated by μQuant spectrophotometer (Bio-tek Instruments, Inc., Winooski, VT). Estimation of alkaline phosphatase (ALP) activity was performed according to an original procedure by Ohyama et al. [24]. After estimating the proliferation of the NHOst cells cultured on PEC films, the cells were washed by phosphate-buffered saline (PBS(−)), followed by addition of 1 ml of 0.1 M glycine buffer (pH 10.5) containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 8 mM *p*-nitrophenylphosphate sodium salt. After incubating the cells at room temperature for 7 min, the absorbance of the glycine buffer was detected at 405 nm using μQuant to evaluate the ALP activity of the test cells. The amounts of calcium deposited by the cell during a 1-week incubation were evaluated as follows: after fixing the cells in PBS(−) containing 3% formaldehyde and washing the cells with PBS(−), 0.5 ml of 0.1 M HCl was added to each well. The amounts of calcium dissolved in HCl were estimated using a calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to manufacturer's instruction.

2.5. Measurements of GJIC activity

NHOst cultured on PEC films were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these films on the GJIC. FRAP analysis was carried out according to the procedure of Wade et al. [25] with some modifications [21]. Briefly, NHOst were plated on PEC-coated dishes and incubated for 1 or 7 days. The cells were incubated for 5 min at room temperature in PBS(−) containing Ca^{2+} and Mg^{2+} (PBS(+)) and a fluorescent dye, 5,6-carboxyfluorescein diacetate. After washing off excess extracellular dye with PBS(+), the cells in PBS(+) contacting at least two other cells were subjected to FRAP analysis under a Ultima-Z confocal microscope (Meridian Instruments, Okemos, MI) with a 10 × objective lens at room temperature. The cells were photobleached with a 488 nm beam, and recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total of 4 min. The data obtained from more than seven independent cells were expressed as the average ratio of the fluorescence recovery rate to the rate obtained from NHOst cultured on a collagen-coated dish.

2.6. Statistic analysis

All data were expressed as mean values ± standard deviation of the obtained data. The Fisher–Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

3. Results and discussion

When NHOst were cultured on five kinds of PEC films, their morphology and attachment to the film differed with the composition of the PEC. Fig. 2 shows the morphologies of the NHOst adhering to PEC films.

Table 1
Zeta potentials of various PEC prepared on a culture dish

	Culture	S-PEC	CM-PEC	P-PEC	SHA-PEC	HA-PEC
Zeta potential (mV)	−58.7	−28.0	34.5	24.9	−5.7	29.5

Table 2
The cell number and differentiation of NHOst cultured on various PEC films after 1 week

Samples	The cell number (percent against control)	$\frac{\text{ALP activity}}{\text{The cell number}}$ (ratio)	$\frac{\text{Ca amount}}{\text{The cell number}}$ (μg/ratio)
Collagen-coated dish	100.0±17.0	1.00±0.15	3.4±0.5
S-PEC	82.2±6.1	0.98±0.11	10.7±3.6
CM-PEC	6.0±2.6*	0.05±0.08*	27.4±3.0*
P-PEC	130.4±6.3	0.02±0.01*	2.5±0.8
SHA-PEC	71.4±22.1	1.35±0.48	2.1±1.0
HA-PEC	8.1±3.0*	0.52±0.31	38.3±12.3*
Chitosan	79.5±25.0	0.93±0.13	2.7±2.0

*$p<0.01$ against collagen-coated dish.

with a carboxyl group, such as HA-PEC and CM-PEC, showed positive zeta potentials. In addition, P-PEC showed a positive potential less than that of HA-PEC. These data indicate that attachment of NHOst on surfaces with positive zeta potentials is reduced, suggesting the zeta potential of a PEC film partially controls cell attachment and morphology. Although all PEC were prepared by mixing anionic and cationic polysaccharides to neutralize their charge, zeta potential of each PEC film was ranged from −30 to 35 mV as shown in the table. This might indicate that not all anionic and cationic chemical groups were interacted to make PEC and their main chain composition and type of chemical groups may influence their side chain mobility, resulting in different surface zeta potential of each PEC. Details of surface properties of PEC films and their relationship to cell attachment will be reported in the near future.

After 1-week of incubation on various PEC films, the differentiation level of NHOst was estimated by measuring proliferation, alkaline phosphatase (ALP) activity and the amounts of calcium deposited. Table 2 shows the proliferation and ALP activity of NHOst cultured on various PEC films as well as the amounts of calcium deposited on the PEC. The proliferation of NHOst on the PEC is expressed as a percentage of proliferation of NHOst on a normal culture dish. The ALP activity was also calculated as a percentage of the control and normalized using the results of proliferation. In addition, the amount of calcium detected was normalized using the proliferation results as well. After a 1-week incubation, many dark spots, presumably calcium deposits, were observed on the collagen-coated dish and other PEC films (Fig. 2). When NHOst were cultured on CM-PEC or HA-PEC, it was observed that the NHOst aggregates were covered by the calcium deposits. It was reported that a surface with carboxyl group could induce calcium deposition after its incubation in SBF. However, when the PEC were incubated in the medium without NHOst, no calcium deposition was detected. In addition, zeta potential estimation suggests less carboxyl groups are appeared on a surface of the PEC. These indicate that calcium deposition occurred only on aggregated NHOst but not on surfaces lacking NHOst. Therefore, normalization is necessary to estimate the capacity of PEC films to induce NHOst differentiation, although the raw values of deposited calcium or ALP activity are low. In fact, CM-PEC or HA-PEC films show a capacity to induce NHOst differentiation comparable to the collagen-coated dish and other PEC films, judging from the normalized values of deposited calcium shown in the table, even though the ratio of NHOst number on them was only 6–8% of that on a collagen-coated dish. Their ALP activities were, however, much lower than those on the collagen-coated dish. Incubation of the PEC films without NHOst for 1 week resulted in no calcium deposition, irrespective of their composition, suggesting that the PEC films themselves had no effect on calcium deposition. Thus, enhancement of calcium deposition on the PEC films may be ascribed to enhancement of NHOst functions related to their differentiation even though their ALP activity was suppressed. The reason for this inconsistency observed between calcium deposition and ALP activity must be investigated further.

When sulfated polysaccharides were used to prepare PEC films, proliferation of NHOst on the PEC films was 70–80% of that on a collagen-coated dish, and ALP activity was very similar to that on the collagen-coated dish. This suggests that sulfated polysaccharide PEC

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The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes

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Abstract

Polyelectrolyte complexes (PEC) were prepared from chitosan as the polycation and several synthesized functional anion polysaccharides, and their effects on cell attachment, morphology, proliferation and differentiation were estimated using normal human osteoblasts (NHOst). After a 1-week incubation, PEC made from polysaccharides having carboxyl groups as polyanions showed low viability of NHOst on it although the NHOst on it showed an enhancement in their differentiation level. On the other hand, NHOst on PEC made from sulfated or phosphated polysaccharides showed similar attachment and morphology to those on the collagen-coated dish. When the number of NHOst was estimated after 1 week, the number on the PEC was ranged from 70% to 130% of those on the collagen-coated dish, indicating few effects of these PEC on cell proliferation. In addition, NHOst on PEC films made from sulfated polysaccharides differentiated to a level very similar to that observed on the collagen-coated dish, indicating that these PEC films maintain the normal potential of NHOst to both proliferate and differentiate. Measurement of gap junctional intercellular communication of NHOst on PEC revealed that PEC did not inhibit communication, suggesting that PEC films have few effects on cell homeostasis. Thus, PEC made from the sulfated polysaccharide may be a useful material as a new scaffold for bone regeneration.

Keywords: Polyelectrolyte complex; Normal human osteoblasts; Cell proliferation; Cell differentiation; Gap junctional intercellular communication

1. Introduction

The extracellular matrix (ECM) provides an essential three-dimensional (3D) environment for cells to construct several kinds of tissues. The ECM, consisting of numerous kinds of molecules such as proteins, polysaccharides and proteoglycans regulates the behavior of surrounding cells to form tissues and organs precisely [1,2]. For tissue regeneration trials using in vitro techniques, therefore, it is indispensable to develop a synthetic ECM scaffold that functions similarly to the native ECM. For more than a decade, engineering of new tissues by using selective cell transplantation on polymer scaffolds as an artificial ECM instead of tissue transplantation to other living bodies has been studied [3,4]. Recently, many studies on developing a scaffold for tissue regeneration have been done using ECM proteins such as collagen and gelatin [5–7], biodegradable synthetic polymers [8–10] and polysaccharides [11,12]. Because proteins derived from human tissues have many problems such as antigenicity or potential for infection, a biocompatible synthetic polymer or polysaccharide may be preferable for tissue regeneration.

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2.2. Preparation of PEC and PEC-coated dishes

Polyanions were dissolved individually in distilled water (final concentration = 5×10^{-4} mol of ionic sites/l), and the pH of the solutions was adjusted to 7.4 by adding aqueous HCl or NaOH. Chitosan was dissolved in aqueous 0.5% acetic acid solution and the pH adjusted to 6.0. The ratio of the solutions of polyanions and polycation was adjusted in each combination to neutralize the charge balance of PEC. This mixed solution (1 ml/35 mm tissue culture dish) was allowed to stand overnight at room temperature. After removing the supernatant solution, the dish was dried and annealed at 65 °C in an oven. Then, the dishes were washed with distilled water and oven-dried again to form the PEC-coated dish. This dish was sterilized for 3 min in a microwave oven. Water contact angles of PEC films were measured with the sessile drop method [23], and their zeta potentials were measured by Otsuka Electronics Co., Ltd. (Osaka, Japan).

2.3. Cell culture

NHOst were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco, Grand Island, NY) containing 20% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo Japan). The cells were maintained in incubators under standard conditions (37 °C, 5%-CO_2–95%-air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 mM beta-glycerophosphate. NHOst cells (1×10^5 cells/dish/2.5 ml medium) were cultured on PEC-coated dishes to evaluate the effects of their interaction with PEC. In each experiment, the medium was changed three times before GJIC of the cells was measured and their differentiation level was evaluated after a 1-week incubation.

2.4. Estimation of differentiation level of NHOst cultured on PEC films

The proliferation of NHOst cells cultured on PEC films was estimated by Tetracolor One assay (Seikagaku Co., Tokyo, Japan), which incorporates an oxidation-reduction indicator based on detection of metabolic activity. After a 1-week incubation, 50 μl of Tetracolor One solution was added to each test dish, followed by a further 2 h incubation. The absorbance of the supernatant at 450 nm was estimated by μQuant spectrophotometer (Bio-tek Instruments, Inc., Winooski, VT). Estimation of alkaline phosphatase (ALP) activity was performed according to an original procedure by Ohyama et al. [24]. After estimating the proliferation of the NHOst cells cultured on PEC films, the cells were washed by phosphate-buffered saline (PBS(−)), followed by addition of 1 ml of 0.1 M glycine buffer (pH 10.5) containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 8 mM *p*-nitrophenylphosphate sodium salt. After incubating the cells at room temperature for 7 min, the absorbance of the glycine buffer was detected at 405 nm using μQuant to evaluate the ALP activity of the test cells. The amounts of calcium deposited by the cell during a 1-week incubation were evaluated as follows: after fixing the cells in PBS(−) containing 3% formaldehyde and washing the cells with PBS(−), 0.5 ml of 0.1 M HCl was added to each well. The amounts of calcium dissolved in HCl were estimated using a calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to manufacturer's instruction.

2.5. Measurements of GJIC activity

NHOst cultured on PEC films were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these films on the GJIC. FRAP analysis was carried out according to the procedure of Wade et al. [25] with some modifications [21]. Briefly, NHOst were plated on PEC-coated dishes and incubated for 1 or 7 days. The cells were incubated for 5 min at room temperature in PBS(−) containing Ca^{2+} and Mg^{2+} (PBS(+)) and a fluorescent dye, 5,6-carboxyfluorescein diacetate. After washing off excess extracellular dye with PBS(+), the cells in PBS(+) contacting at least two other cells were subjected to FRAP analysis under a Ultima-Z confocal microscope (Meridian Instruments, Okemos, MI) with a 10 × objective lens at room temperature. The cells were photobleached with a 488 nm beam, and recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total of 4 min. The data obtained from more than seven independent cells were expressed as the average ratio of the fluorescence recovery rate to the rate obtained from NHOst cultured on a collagen-coated dish.

2.6. Statistic analysis

All data were expressed as mean values ± standard deviation of the obtained data. The Fisher–Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

3. Results and discussion

When NHOst were cultured on five kinds of PEC films, their morphology and attachment to the film differed with the composition of the PEC. Fig. 2 shows the morphologies of the NHOst adhering to PEC films.

Table 1
Zeta potentials of various PEC prepared on a culture dish

	Culture	S-PEC	CM-PEC	P-PEC	SHA-PEC	HA-PEC
Zeta potential (mV)	−58.7	−28.0	34.5	24.9	−5.7	29.5

Table 2
The cell number and differentiation of NHOst cultured on various PEC films after 1 week

Samples	The cell number (percent against control)	$\frac{\text{ALP activity}}{\text{The cell number}}$ (ratio)	$\frac{\text{Ca amount}}{\text{The cell number}}$ (μg/ratio)
Collagen-coated dish	100.0±17.0	1.00±0.15	3.4±0.5
S-PEC	82.2±6.1	0.98±0.11	10.7±3.6
CM-PEC	6.0±2.6*	0.05±0.08*	27.4±3.0*
P-PEC	130.4±6.3	0.02±0.01*	2.5±0.8
SHA-PEC	71.4±22.1	1.35±0.48	2.1±1.0
HA-PEC	8.1±3.0*	0.52±0.31	38.3±12.3*
Chitosan	79.5±25.0	0.93±0.13	2.7±2.0

*$p<0.01$ against collagen-coated dish.

with a carboxyl group, such as HA-PEC and CM-PEC, showed positive zeta potentials. In addition, P-PEC showed a positive potential less than that of HA-PEC. These data indicate that attachment of NHOst on surfaces with positive zeta potentials is reduced, suggesting the zeta potential of a PEC film partially controls cell attachment and morphology. Although all PEC were prepared by mixing anionic and cationic polysaccharides to neutralize their charge, zeta potential of each PEC film was ranged from −30 to 35 mV as shown in the table. This might indicate that not all anionic and cationic chemical groups were interacted to make PEC and their main chain composition and type of chemical groups may influence their side chain mobility, resulting in different surface zeta potential of each PEC. Details of surface properties of PEC films and their relationship to cell attachment will be reported in the near future.

After 1-week of incubation on various PEC films, the differentiation level of NHOst was estimated by measuring proliferation, alkaline phosphatase (ALP) activity and the amounts of calcium deposited. Table 2 shows the proliferation and ALP activity of NHOst cultured on various PEC films as well as the amounts of calcium deposited on the PEC. The proliferation of NHOst on the PEC is expressed as a percentage of proliferation of NHOst on a normal culture dish. The ALP activity was also calculated as a percentage of the control and normalized using the results of proliferation. In addition, the amount of calcium detected was normalized using the proliferation results as well. After a 1-week incubation, many dark spots, presumably calcium deposits, were observed on the collagen-coated dish and other PEC films (Fig. 2). When NHOst were cultured on CM-PEC or HA-PEC, it was observed that the NHOst aggregates were covered by the calcium deposits. It was reported that a surface with carboxyl group could induce calcium deposition after its incubation in SBF. However, when the PEC were incubated in the medium without NHOst, no calcium deposition was detected. In addition, zeta potential estimation suggests less carboxyl groups are appeared on a surface of the PEC. These indicate that calcium deposition occurred only on aggregated NHOst but not on surfaces lacking NHOst. Therefore, normalization is necessary to estimate the capacity of PEC films to induce NHOst differentiation, although the raw values of deposited calcium or ALP activity are low. In fact, CM-PEC or HA-PEC films show a capacity to induce NHOst differentiation comparable to the collagen-coated dish and other PEC films, judging from the normalized values of deposited calcium shown in the table, even though the ratio of NHOst number on them was only 6–8% of that on a collagen-coated dish. Their ALP activities were, however, much lower than those on the collagen-coated dish. Incubation of the PEC films without NHOst for 1 week resulted in no calcium deposition, irrespective of their composition, suggesting that the PEC films themselves had no effect on calcium deposition. Thus, enhancement of calcium deposition on the PEC films may be ascribed to enhancement of NHOst functions related to their differentiation even though their ALP activity was suppressed. The reason for this inconsistency observed between calcium deposition and ALP activity must be investigated further.

When sulfated polysaccharides were used to prepare PEC films, proliferation of NHOst on the PEC films was 70–80% of that on a collagen-coated dish, and ALP activity was very similar to that on the collagen-coated dish. This suggests that sulfated polysaccharide PEC

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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

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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

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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-

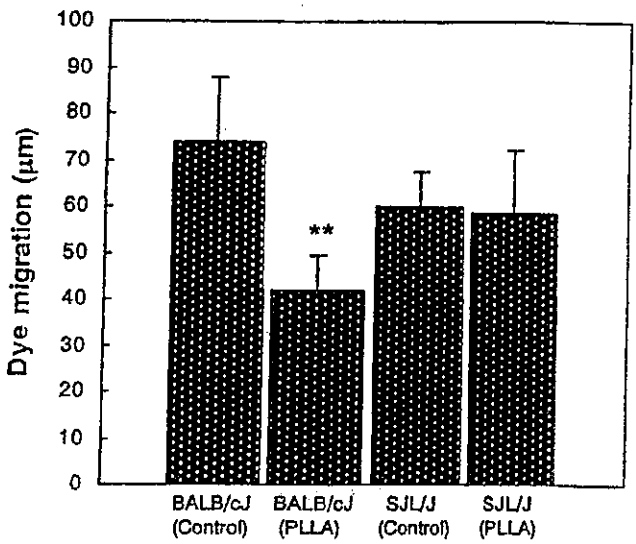


Figure 1. Statistical analysis of the SLDT assay. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. GJIC was significantly inhibited in PLLA-implanted BALB/cJ mice cells compared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

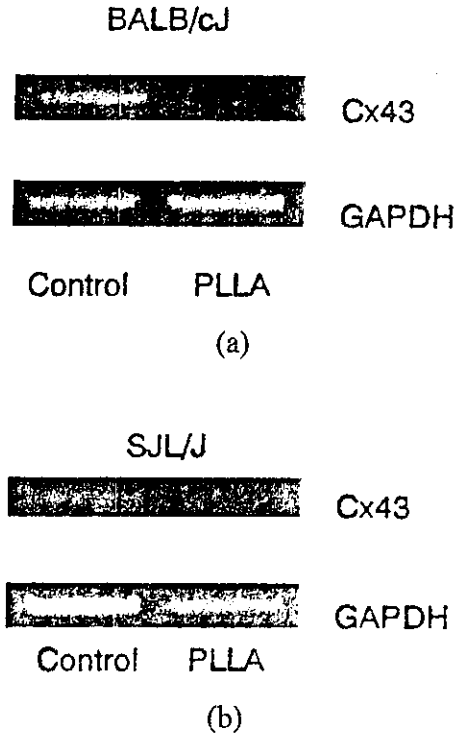


Figure 2. mRNA expression of Cx43 by RT-PCR analysis. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. SYBR Green I stained PCR products after agarose gel electrophoresis showed 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

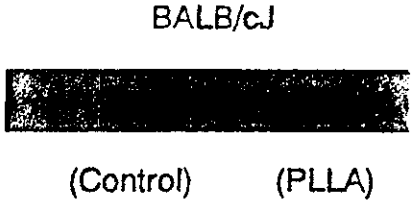


Figure 3. Protein expression of Cx43 by Western blot analysis. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. Total level of protein expression such P_0, P_1, and P_2 levels were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

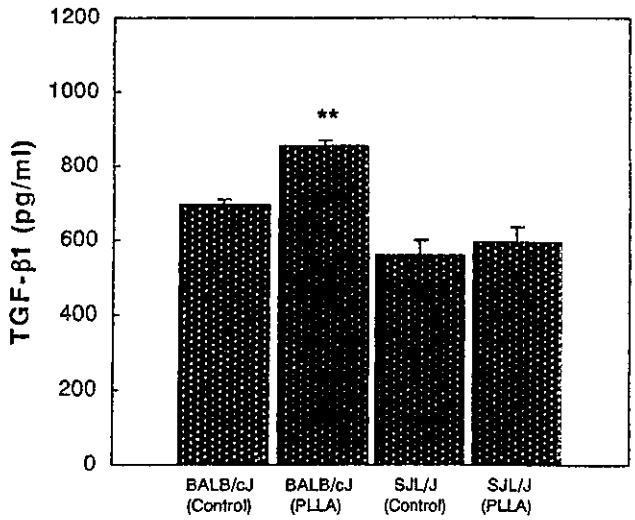
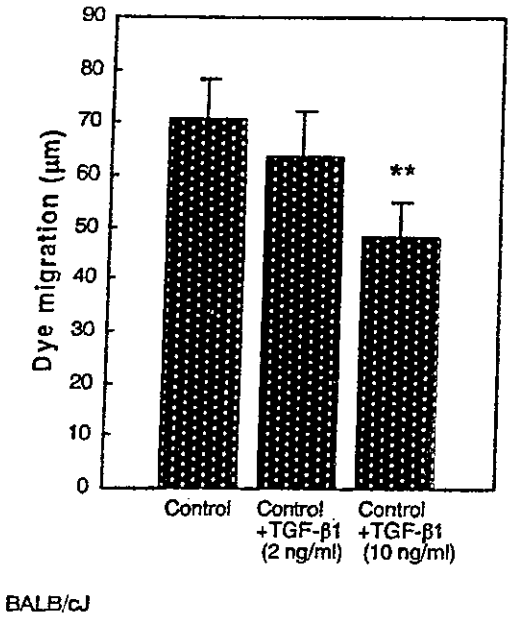


Figure 4. Statistical analysis of TGF-β1 cytokine assay by ELISA. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. Secretion of the TGF-β1 level was significantly increased in PLLA-implanted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

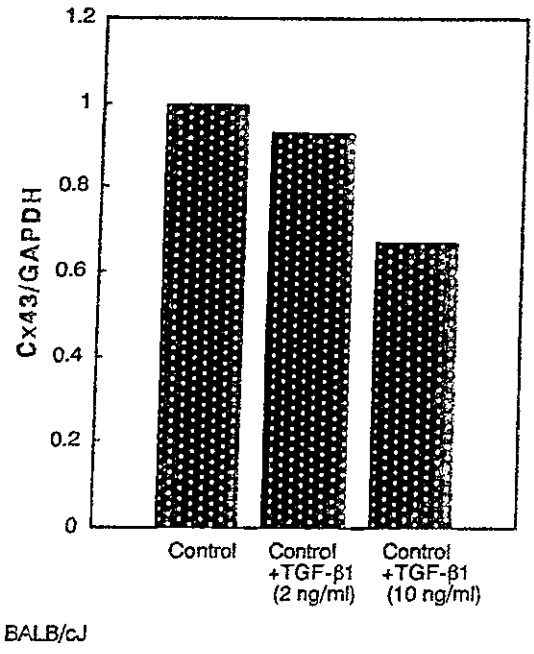
modification in the Cx43 protein was shown to be involved in impaired GJIC and could be associated with tumorigenesis. Therefore, it is suggested that the inhibitory effect of PLLA on GJIC might be caused by the alteration in the Cx43 protein, causing enhancement of tumorigenesis. Moreover, Moorby and 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.

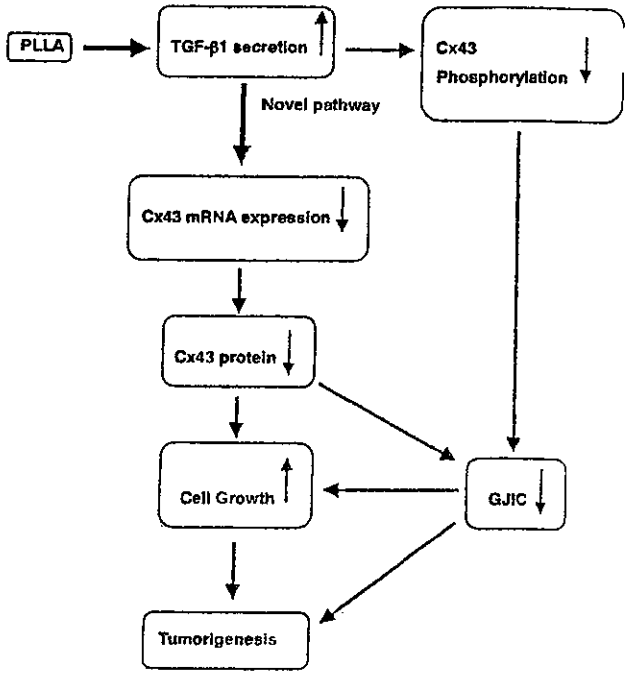


Figure 6. Schematic representation of the pathway of tumorigenesis induced by PLLA in BALB/cJ mice.

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).

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細胞組織医療機器等の製品化のためのガイドライン，環境整備について

医療機器・細胞組織医療機器関連の薬事法の改正が昨年度からスタートしている。その内容は，(1) 多様性に富んだ医療機器のリスクに応じた新クラス分類とその承認制度の見直し，(2) 細胞組織医療機器が含まれる生物由来製品の感染リスクに応じた安全対策の充実，(3) 市販後安全対策の抜本的見直しが急ピッチで進められている。また，第三者認証制度の導入において必要な規格・基準の整備も行われている。本稿では，細胞組織医療機器や新たな制度である生物由来製品の薬事法の改正内容について記載した。

土屋利江

1. 細胞組織医療機器等の薬事法改正について

平成12年12月26日付けでヒトまたは動物由来成分を原料として製造される医薬品等の品質及び安全性確保についての医薬安全局長通知（医薬発第1314号）において，別添1「細胞組織利用医薬品等の取扱い及び使用に関する基本的考え方」と別添2「ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」の2つの文書が示された。

別添1の基本的考え方は，ヒトや動物の細胞・組織から構成される医薬品および医療機器（平成17年度から医療用具は，医療機器に名称変更される予定）について，品質および安全性の確保ならびに細胞・組織の取扱いに関する科学的および倫理的妥当性を確保するための方策をまとめている。

別添2の指針は，「基本的考え方」に基づき，ヒトの細胞・組織に培養処理等の加工を施して製造される医薬品および医療機器について，品質および安全性の確保のために必要な基本的技術要件を定めており，治験前に厚生労働省に提出する品質および安全性の確認申請時に，必要な添付資料の内容を示している。

平成13年3月28日付けで「薬事法施行規則の一部を改正する省令等の施工について（細胞組織医薬品及び細胞組織医療用具に関する取扱いについて）」（医薬発第266号）省令および告示が公布され，平成13年4月1日より施行されている。

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Reform of Biological Products Regulation and Guidelines for Manufacturing Tissue Engineered Medical Products

ヒトまたは動物の細胞または組織より構成された医療機器および医薬品に関して科学技術の進歩に伴う感染症への対策が急務となり，ドナースクリーニング，感染因子の不活化など，ドナーに由来する感染症への対策，培養などの処理により細胞または組織が有害な性質のものとならないことの確認など，品質および安全性を確保するために特別の対策が必要とされ，改正された。

2. 細胞組織医療機器等の適用範囲

ヒトまたは動物の細胞または組織より構成される医療機器および医薬品であり，動物由来の組織を利用して承認を取得している生体弁や心のう膜も含んでいる。

3. 改正概要

承認申請書の記載方法の変更（詳細は，最新の薬事法で確認のこと）や，GMP*関係省令についても一部改正された。その概要は，細胞組織医療機器などを製造または輸入するにあたり，細胞もしくは組織由来または製造工程中の感染症などの伝播による危険性を排除し，不適切な製造や取扱いによる品質および安全性上の問題の発生を防止するために，製造管理と品質管理に必要な要求事項を定めた。

具体的には，

(1) 細胞組織医療機器等の製造所の構造設備の基準への適合，原料の受入れ，加工処理，製品の保管等を行う区域について，他区域からの区分，必要な構造及び設備の要求。

* GMP: 製造許可の要件として製造所の構造設備から製造工程全般にわたる製造管理及び品質管理について製造業者が遵守すべき基準

(2) 加工処理の「加工」とは疾病の治療や組織の修復又は再建を目的として，細胞または組織の人為的増殖，細胞または組織の活性化を目的とした薬剤処理，生物学的特性改変，遺伝子工学的改変，非細胞または非組織成分とのハイブリッド化，カプセル化等を施すこと。

(3) 細胞組織医療機器等は，生物学的製剤等と同様に原料として使用する人，動物，植物または微生物から得られたものに係る事項や使用動物の規格に関する事項について，製品標準書を作成。

(4) 細胞組織医療機器等の製造，保管及び出納ならびに衛生管理に関する記録については，遅発性感染症の危険性を否定し得ないことから，安全性の確保上必要な情報を得るために，少なくとも所定の期間記録を保存することとなっている。即ち，特定生物由来製品の場合，医療機関での患者使用記録の保管期間は20年間とし，製造業者等での提供者・製造記録の保管期間は30年間と規定。生物由来製品では，製造業者等での提供者・製造記録の保管が求められ，人血液成分以外の成分に関する記録は，10年間，人血液成分を含む場合の人血液成分に関する記録は，30年間保管となっている。(生物由来製品の特性に応じて保管期間は異なる。最新版の薬務公報に目を通し，現時点での正確な情報を入手し，確認すること。)

(5) 細胞又は組織の取り違えや細菌，真菌，ウイルス等の伝播の危険性を避けるために，製造工程において複数のドナーからの細胞又は，組織を同一室内で同時期に取扱ったり，交叉汚染を引き起こすような保管方法をとらないこと。更に，ドナー又はドナー動物ごとに細胞や組織，中間製品及び製品を管理する必要がある。

(6) 原料となる細胞又は組織について，後述した内容において，適格なものであることを確認し，その結果に関する記録を作成すること。

(i) 当該細胞又は組織を採取した施設

(ii) 当該細胞又は組織を採取した年月日

(iii) 当該細胞又は組織が人に係るものである場合には，ドナースクリーニング（ドナーについて，問診，検査等による診断を行い，細胞組織医薬品の原料となる細胞又は組織を提供する適格性を有するかどうかを判定することをいう）のためのドナーの問診，検査等による診断の状況。

(iv) 当該細胞又は組織が動物に係るものである場合には，ドナー動物の受入の状況並びにドナースクリーニング（ドナー動物について，試験検査及び飼育管理を行い，細胞組織医薬品の原料となる細胞又は組織を提供する適格性を有するかどうかを判定することをいう）のためのドナー動物の試験検査及び飼育管理の状況

(v) 当該細胞又は組織を採取する作業の経過（採取する作業経過に関する記録と採取作業において微生物等に汚染されていない旨が確認できるものであること）。

(vi) (i)から(v)までに掲げるもののほか，細胞組織医療機器等の品質の確保に関し必要な事項（製造に使用する試薬に関する試験検査結果を指す）。

(7) 「施設」は組織を採取した医療施設もしくは動物の細胞又は組織を採取した施設を指す。

(8) 「適格性を有する」とは，「細胞組織医薬品及び細胞組織医療用具に関する基準」の

以下のいずれにも該当し，原料となる条件を満たしているもの。

細胞組織医療機器について，薬事第42条の規定に基づき，品質及び安全性確保の観点から，原料又は材料となる細胞又は組織に関する基準を定めている（平成13年3月厚生労働省告示第101号関係)。この基準を満たさない細胞又は組織は，品質及び安全性についての情報が十分でないことから，製造業者は，これら細胞又は組織を原料又は材料として医薬品又は医療機器として製造すべきでない。

「基準の概要」

医薬品又は医療機器の原料又は材料となる細胞又は組織については，

必要な衛生管理と人員を持つ施設で採取されていること。
ドナースクリーニングが適切に行われていることが確認できること。
採取作業が適切に行われていることが確認できること。
必要な記録を確認できること。

が必要である。

ドナースクリーニングの項目等具体的な内容については，個別の製品ごとに異なることから，具体的事項については承認申請書に記録する。

4. 生物由来製品に関する制度の概要

(1) 生物由来製品に関する制度の創設について

人又は動物の細胞，組織等に由来する原材料を用いて製造される生物由来製品は，その特性として，原材料の汚染に由来する感染リスク等について，注意を払う必要がある。生物由来というこの共通特性に着目し，原材料採取・製造から市販後に至る，一貫した安全性確保体制を導入し，製品の安全性を図るために創設された。

(2) 生物由来製品に関する制度の主な内容

(a) 生物由来製品及び特定生物由来製品の指定

製品の感染症リスクを考慮した科学的評価に基づき，指定を行い，生物由来製品は約700製品，特定生物由来製品は約280製品について指定し，公表した。

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,