Effect of Cell-Adhesive-Molecule-Coated Poly(lactide-*co*-glycolide) Film on the Cellular Behaviors of Olfactory Ensheathing Cells and Schwann Cells

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ABSTRACT: The surface properties of polymeric devices that are used to repair damaged nervous tissue are a point to be considered for axon regeneration in the nervous system. In this study, we investigated the interaction of olfactory ensheathing cells (OECs) and Schwann cells (SCs) with poly(lactide-*co*-glycolide) (PLGA) film surfaces coated with various cell-adhesive molecules. We used cell adhesives such as fibronectin, fibrinogen, laminin, vitronectin, poly(D-lysine), and poly(L-lysine) to coat PLGA film surfaces. We cultured 1×10^4 cells/cm^2 (OECs or SCs) on coated or uncoated PLGA film surfaces, and then we examined the cell attachment and proliferation by cell count and scanning electron microscopy observation. In addition, we evaluated relative messenger RNA expression of neuronal cell-adhesion molecules by reverse transcription polymerase chain reaction. Cell count results revealed differences caused by initial cell adhesion related to protein adsorption on the PLGA surface. In addition, neurite outgrowth and the proliferation rate of OECs or SCs revealed differences according to the presence of serum in the medium. As a result, we recognized that the attachment and proliferation of OECs or SCs were affected by specific cell-adhesive molecules. In conclusion, the selection of optimal adhesive molecules is an important consideration for manufacturing nerve guidance and neural cell cultures for tissue-engineered nerve regeneration. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 1243–1251, 2008

Key words: biocompatibility; biological applications of polymers; biomaterials; hydrophilic polymers; proteins

INTRODUCTION

Self-repair or regeneration of the damaged central nervous system, including spinal cord injury (SCI), in adult mammals is known to be limited because injury to the adult mammalian spinal cord results in progressive tissue damage, which causes permanent functional deficits.[1–3] Furthermore, axonal regeneration in the damaged spinal cord is limited to sprouting immediately in the region surrounding the injury.[2,3] Thus, most research into SCI focuses on promoting axonal regrowth and reducing neuronal degeneration.[4–7] Three major approaches to promoting axonal regeneration are (1) cell transplantation, (2) addition of neurotrophic factors, and (3) biomaterial scaffolds. Many studies have recently demonstrated that special cells such as olfactory ensheathing cells (OECs) and Schwann cells (SCs) have the ability to induce remyelination in SCI. These cells, transplanted into the injured spinal cord, improve functional outcome and promote axonal regeneration. In addition, these cells release neurotrophic factors that are known as inducing factors of remyelination, such as brain-derived neurotrophic factor and nerve growth factor, and it is possible to expand a culture to maintain their characteristics.[4–6,8–11]

In reported biomaterials, biodegradable materials have been shown to have more advantages than nonbiodegradable ones.[12] Recently, much research into the development of nerve guidance has considered poly(α-hydroxy acid)s such as polyglycolide, polylactide, and their copolymer [poly(lactide-*co*-glycolide) (PLGA)]. However, the surfaces of these polymers are hydrophobic and tend to unfavorably influence their cell compatibility in the initial stage of attachment. The interaction of cell materials and cell extracellular matrices is an important factor for cell proliferation and differentiation for use in tissue-engineered nerve regeneration.[13–19] The induction and growth of neuritis must occur for the regeneration of nerves on polymeric guidance. For this reason, the properties of polymeric devices that are used to regenerate nervous tissue are a point to be

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considered for providing surfaces favorable to axonal regeneration of injured neurons. For extracellular matrices such as synthetic polymers, the surface characteristics play an important role in the regulation of cell morphology, polarity (orientation), adhesion, spreading, and growth.[20–25]

Protein or polypeptide precoating and subsequent OEC or SC seeding on materials such as tissue-engineered scaffolds for nerve regeneration have attracted intense interest as a means of improving regeneration,[12,16,26–32] but few comparative studies with a wide range of proteins (known as cell-adhesive proteins) or polypeptides (known as cell-adhesive polypeptides) are available.[32] In our previous study, we confirmed the attachment and growth behavior of SCs onto PLGA films coated with several cell-adhesive proteins and polypeptides such as fibronectin (FN), fibrinogen (FG), laminin (LM), vitronectin (VN), poly(D-lysine) (PDL), and poly(L-lysine) (PLL).[33] We recognized that the attachment and proliferation of SCs were promoted by specific adhesive molecules such as FN, FG, and LM from the results of a previous study. In this study, we compared OEC and SC attachment and proliferation behavior on PLGA films coated with adhesive proteins or polypeptides. Because most cells attach poorly to hydrophobic surfaces without prior coating with one of these proteins or polypeptides, the hydrophobic PLGA film surfaces used in this study represent an ideal system with which to explore the effect of variations in the proteins or polypeptides on cell attachment for neural cell cultures. In addition, we confirmed a correlation between the protein or polypeptide type and neural cell-adhesion molecule (N-CAM) expression.

EXPERIMENTAL

Materials

PLGA (Resomer RG756, 90,000 g/mol) was purchased from Boehringer Ingelheim Co. (Ingelheim, Germany). We used cell-adhesive proteins and polypeptides such as FN, FG, LM, VN, PDL, and PLL, which were purchased from Sigma Co. (St. Louis, MO). Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, trypsin–ethylene diamine tetraacetic acid, and other cell culture reagents were purchased from Gibco BRL Co. (Grand Island, NY). In addition, polymerase chain reaction reagents were purchased from Invitrogen Life Technologies, Inc. (Groningen, The Netherlands).

Preparation of protein- or polypeptide-coated PLGA films

PLGA films were fabricated as follows. A 5 wt/vol % PLGA solution was dissolved in dichloromethane, and 6 mL of the PLGA solution was poured onto a glass Petri dish. Then, we performed solution casting and allowed it to dry at room temperature. The average film thickness was 100 $\pm$ 30 μm. We used films that were sterilized by immersion in 70% ethyl alcohol, washed with distilled water (DW), and dried on a clean bench. All the proteins and polypeptides except VN were dissolved and diluted with filtered DW to make 100 μg/mL solutions. For VN, 5 μg/mL solutions were made with filtered DW. The protein or polypeptide solutions (2 mL) were placed on PLGA films. In the case of an untreated PLGA film (control UN), it was incubated in the presence of DW. After 1 h of incubation at 37°C, the films were gently rinsed with 3 mL of DW twice to remove unadsorbed or weakly adsorbed proteins or polypeptides and dried at room temperature. The proteins or polypeptides were coated on the films by this process, which was repeated three times. The films surfaces were analyzed by electron spectroscopy for chemical analysis (ESCA; Escalab MK II, V.G. Scientific Co., East Grinstead, UK). The ESCA apparatus was equipped with an Al Kα radiation source with 1487 eV and 300 W at the anode. The nitrogen 1s peaks from the survey scan spectra were used for the analysis of the proteins or peptides adsorbed on the surfaces.

Neuronal/glial cell culture on the surfaces

OECs were isolated from the olfactory bulb of a female rat according to the modified Nash method.[34] Primary-cultured SCs were obtained after the predegeneration of rat sciatic nerves as described by Morrissey et al. in a previous study.[35] The OECs and SCs, routinely cultured in tissue culture polystyrene flasks (Falcon, San Jose, CA) at 37°C under a 5% CO_2 atmosphere, were harvested after a treatment with 0.25% trypsin (Gibco BRL). The protein- or polypeptide-adsorbed films were equilibrated with phosphate-buffered saline (PBS) for 30 min. Noncoated PLGA films were also used as controls. After the removal of the PBS solution from the films, the cells ($1 \times 10^4/cm^2$) were seeded on the film surfaces. The culture medium was DMEM without serum. DMEM with 10% FBS was also used to examine the effect of serum on the cell adhesion and proliferation behavior.

Observation of cell adhesion and proliferation

The number of attached cells on the film surface was measured 1 and 3 days after the culture. The cell-attached surfaces were rinsed with PBS and treated with trypsin. The cell density on the surfaces was estimated by the counting of the number of detached cells with a hemacytometer. Adhered cells on films were fixed with 2.5% glutaraldehyde (Sigma Chemi-

TABLE I
Sequences of Primers

Messenger RNA	Sequence	Annealing temperature (°C)	Fragment length (bp)
GAPDH	F: 5′-TGAACGGGAAGCTCACTGG-3′ R: 5′-TCCACCACCCTGTTGCTGTA-3′	60	307
N-CAM (140 kD)	F: 5′-GTCTGTCACCCTGGTGTGTG-3′ R: 5′-GTGGACGTTCTCCAGTGAT-3′	55	351

cal Co.). After dehydration by an ethanol/water series (50, 60, 70, 80, 90, and 100%) for 10 min each, we observed the morphology of cells for 1 day after cell seeding with microphotographs taken with a scanning electron microscope (S-2250N, Hitachi Co., Tokyo, Japan). Using reverse transcription polymerase chain reaction, we analyzed N-CAM expression of cells on coated PLGA surfaces in an *in vitro* culture. N-CAM is a homophilic binding glycoprotein expressed on the surface of neurons, glia, and skeletal muscle. N-CAM has been implicated as having a role in cell–cell adhesion and neurite outgrowth.[36,37] Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instructions and quantified with an ultraviolet–visible spectrophotometer (SmartSpec Plus, Bio-Rad Laboratories, Inc., Hercules, CA) at 260 and 280 nm. Table I presents the primer sequences, cycling conditions, and predicted size. Products were analyzed by agarose gel electrophoresis and visualized with SYBR Green I (Cambrex Bio Science Rockland, Inc., Maine) staining under a UV illuminator. The relative expression of N-CAM was normalized by a housekeeping gene [glyceraldehyde 3-phosphate dehydrogenase (GAPDH)].

Statistical analysis

Data are presented as the mean ± standard deviation of quadruplicate cultures. Statistical analysis was performed with a Student *t* test (independent difference). Results were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Characterization of the protein- or polypeptide-coated surfaces

The protein- or polypeptide-coated surfaces were analyzed with ESCA. The nitrogen 1s signal from the survey scan spectra was used as an indicator of surface protein or polypeptide adsorption because no nitrogen content of the pure protein or polypeptide powders was detected on the film surfaces. The nitrogen content of the pure protein and polypeptide powders was in the range of 11–15%, depending on the types of proteins and polypeptides,[38] as determined by ESCA (Table II). They were mainly derived from peptide bonds (for proteins) and pendant groups (for polylysine). The nitrogen content of film surfaces adsorbed with the proteins was much higher than that of the surfaces adsorbed with polylysine, and this indicated the larger amount of protein adsorption on those surfaces. The small amount of protein adsorption in VN was probably caused by the application of a solution with a low protein concentration (5 μg/mL) in comparison with the other protein solutions (100 μg/mL). We compared the nitrogen signals of the protein- and polypeptide-coated film surfaces with those of the pure powder. The relative adsorbed amounts of proteins and polypeptides in Figure 1 were determined as follows:[38]

$$\text{Relative adsorbed amount} = (\text{Nitrogen \% from adsorbed protein or polypeptide}) / (\text{Nitrogen \% from pure protein or polypeptide powder})$$

TABLE II
ESCA Results for Protein- and Polypeptide-Adsorbed PLGA Surfaces

Adsorbed protein or polypeptide	atom %[a]			N (%) of pure powder	Concentration (μg/mL)
	C	O	N		
Uncoated PLGA (UN)	56.56	43.44	0	—	—
FN	60.49	23.84	15.67	12.5	100
FG	59.96	24.08	15.96	11.6	100
LM	60.11	25.96	13.93	15.1	100
VN	56.58	42.11	1.31	—[b]	5
PDL	56.29	43.23	0.48	14.0	100
PLL	57	42.16	0.83	13.4	100

[a] Data from ref. 25.
[b] Not determined.

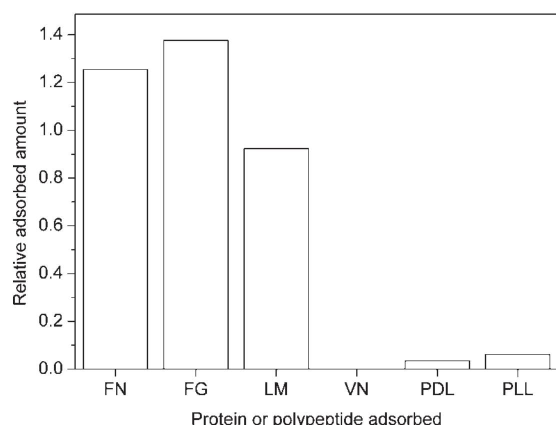

Figure 1 Relative adsorbed amounts of proteins and polypeptides on PLGA film surfaces.

As shown in Figure 1, the proteins were largely adsorbed on the PLGA film surface, probably because of hydrophobic interactions of the protein molecules with the hydrophobic PLGA surface. The relative adsorbed amounts of proteins (0.34–1.38) seemed to be nearly the value of the monolayer coverage. Therefore, we could assume that the proteins used in this study were coated on the PLGA film surfaces with almost monolayer coverage.[14,38]

OEC attachment and proliferation on coated surfaces

As shown in Figure 2(a), the OECs cultured in a serum-free medium adhered much more to the specific protein- or polypeptide-coated film surfaces, such as FN and FG, than the UN. The number of attached OECs on the coated film surfaces at 1 day after seeding was as follows: FN ≒ FG ≫ UN ≒ LM ≒ VN ≒ PDL ≒ PLL. The proliferation ratio of OECs dramatically increased at LM and VN in comparison with the others for 3 days. On the basis of this result, cell attachment was especially affected by FN and FG among the adhesives; on the other hand, cell proliferation was closely related to LM and VN among the adhesives. We could observe similar results in SC experiments. However, OEC attachment increased in FN and FG in comparison with SC attachment. In addition, we observed a higher proliferation ratio of OECs versus SCs. We presumed that these results were caused by the culture of OECs being relatively favorable versus that of SCs under the serum-free condition.

When OECs were cultured in a 10% serum containing medium, the cell-adhesion behavior on the surfaces was different from that under the serum-free medium condition. As shown in Figure 2(b), cell adhesion on the UN in the serum-containing medium increased greatly in comparison that under the serum-free medium. We observed differences in the initial cell adhesion on different surfaces. OEC adhesion in the 10% serum containing medium (at 1 day after culturing on films) on the PLGA film surfaces coated with proteins or polypeptides was FN ≒ FG > PLL ≫ control (UN) ≒ LM ≒ VN ≒ PDL, and OEC proliferation in the 10% serum containing medium was dramatically increased on most surfaces, except the UN, at 3 days after seeding. We observed little difference in the initial cell attachment caused by the content of serum in the medium. These results were caused by the presence of serum (FBS) in the medium. FBS contains many adhesives such as FN, LM, and VN as well as nutritive proteins.[39] It seems that these components of FBS affected not

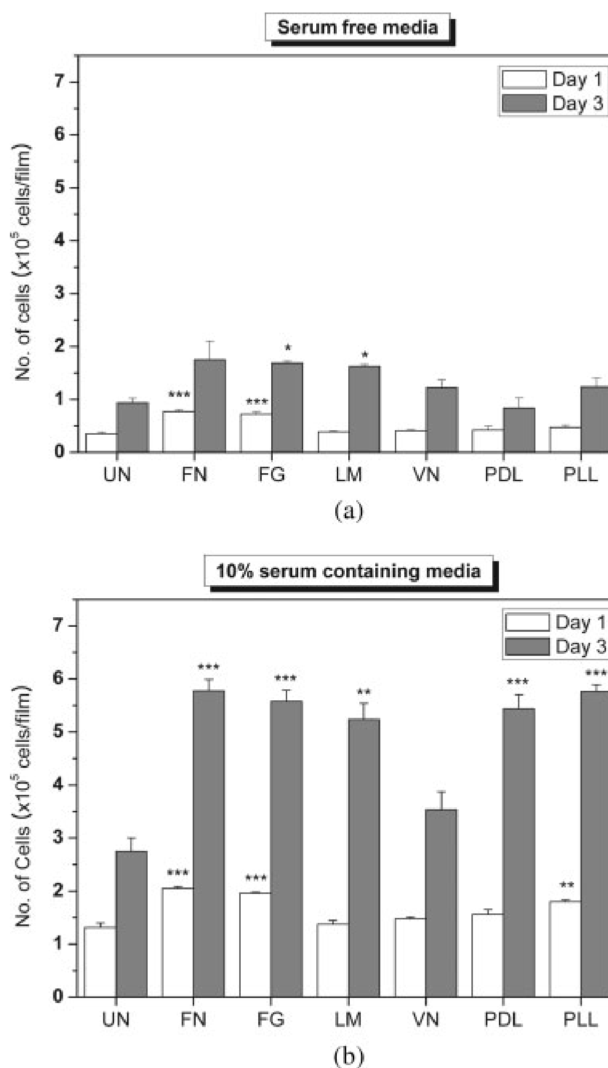

Figure 2 Number of OECs adhering to PLGA film surfaces coated with proteins or polypeptides: (a) serum-free media and (b) 10% FBS containing medium. Asterisks denote significant differences in comparison with UN for each day (*$P < 0.05$, **$P < 0.02$, ***$P < 0.01$).

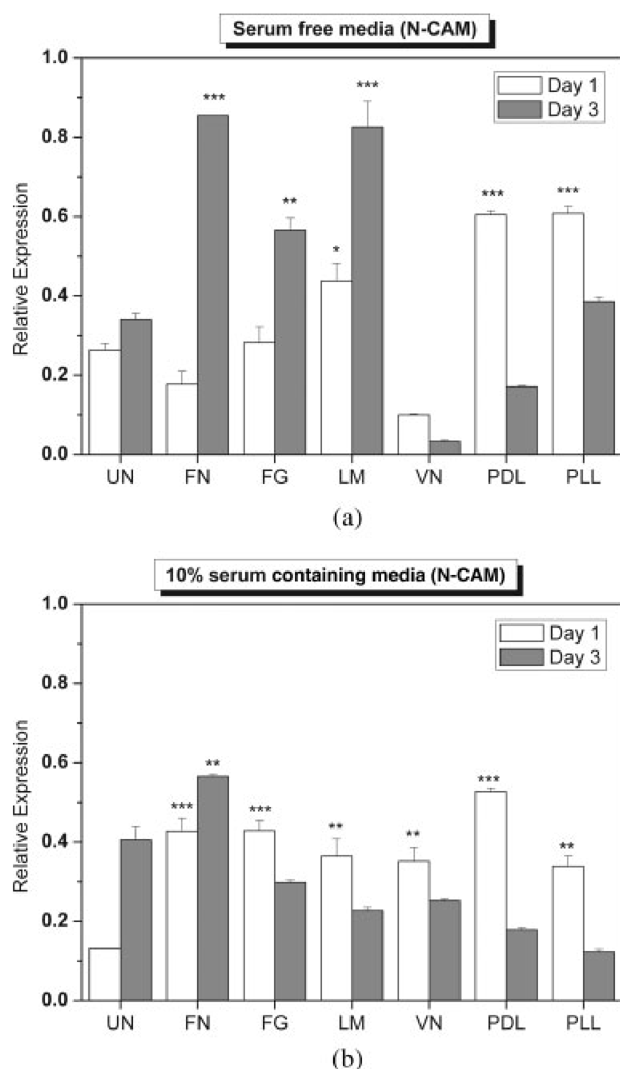

Figure 3 Relative expression of N-CAM in OECs cultured on PLGA film surfaces coated with proteins or polypeptides: (a) serum-free media and (b) 10% FBS containing medium. Asterisks denote significant differences in comparison with UN for each day (* $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$).

only cell proliferation but also cell attachment. However, cell proliferation had an influence on the content of serum in the medium (data not shown).

N-CAM expression of OECs

N-CAM is an important molecule with respect to neuronal/glial cell adhesion.[36,37] We confirmed the difference in the cell activity according to the types of adhesives by a comparison of N-CAM expression. Figure 3 shows the relative expression of N-CAM in OECs cultured on coated surfaces. In the serum-free medium, N-CAM at PDL and PLL was highly expressed in comparison with the others at 1 day. FN, FG, and LM were significantly different in comparison with the others at 3 days. However, N-CAM expression in the 10% serum containing medium was different from that under the serum-free medium condition. In the 10% serum medium, we confirmed similar expression intensity of N-CAM to most surfaces, except the UN and VN, at 1 day. On the other hand, only FN and LM had remarkable expression intensity in comparison with the UN at 3 days. Moreover, the expression intensity of OECs decreased in the 10% serum containing medium in comparison with the SC experiment. It seems that decreased intensity of N-CAM was related to the lower proliferation ratio for the culture of OECs in the 10% serum medium.

OEC morphologies on coated surfaces

The morphology of attached OECs did not differ according to the types of proteins or polypeptides, as determined by scanning electron microscopy (SEM; Fig. 4). Filopodia and lamellipodia, related to cell spreading and activity, were observed to lead to good formation of OECs attached onto film surfaces. Most attached OECs appeared in a neurite-like cell process on the film surface. For SCs, we observed similar results. However, we observed no difference in OECs according to the presence of serum, unlike SCs. These results meant that the attachment, proliferation, and morphology of OECs were less affected by serum in comparison with SCs. In addition, OECs were not influenced with respect to a change in the morphology by coated or uncoated surfaces.

SC attachment and proliferation on coated surfaces

As shown in Figure 5(a), the SCs cultured in the serum-free medium were attached much more to the specific protein- or polypeptide-coated film surfaces such as FN and FG than the control (UN). The number of attached SCs on the coated film surfaces at 1 day after seeding was FG $>$ FN $\gg$ UN $\fallingdotseq$ VN $>$ LM $\fallingdotseq$ PDL $\fallingdotseq$ PLL, and the proliferation ratio of SCs dramatically increased at LM and VN in comparison with the others at 3 days. We concluded that FG and FN were closely associated with the attachment of SCs. In addition, LM and VN had an influence on the proliferation of SCs cultured in a serum-free medium.

As SCs were cultured in the 10% serum containing medium, the cell attachment and proliferation on the surfaces were different from those under the serum-free medium condition. As shown in Figure 5(b), the cell adhesion on the UN in the serum-containing medium increased greatly in comparison with that in the serum-free medium. We could not observe a difference in the initial cell adhesion among the different surfaces. The SC attachment on

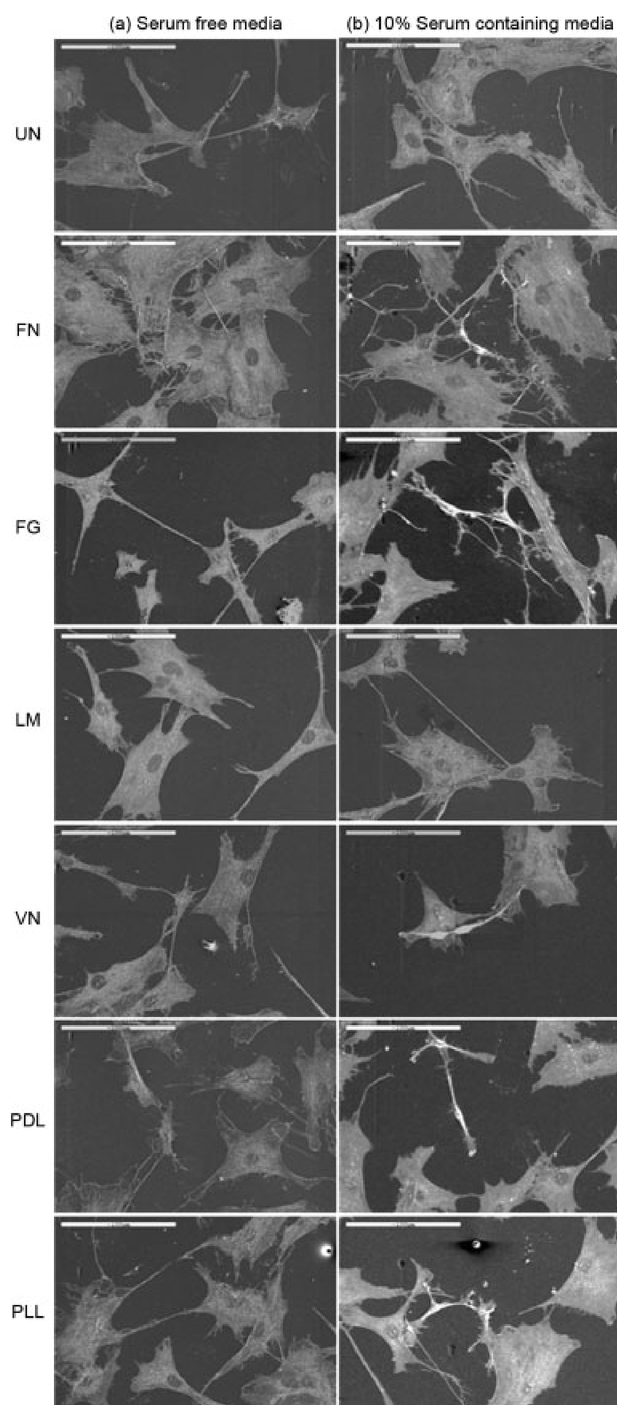

Figure 4 SEM microphotographs of OECs revealing the morphology of the attachment. OECs were cultured in media with or without serum on PLGA film surfaces coated with proteins or polypeptides for 1 day (scale bar = 100 μm).

the PLGA film surfaces coated with proteins or polypeptides in the 10% serum containing medium (at 1 day after culturing on films) was VN > LM ≒ FG ≒ FN ≒ PLL > UN > PDL, and SC proliferation in the 10% serum containing medium dramatically increased at FG and VN in comparison with the others at 3 days after culturing on films. In addition, we observed little difference in the initial cell attachment caused by the content of serum in the medium. However, cell proliferation had an effect on the content of serum in the medium (data not shown). In these results, we recognized that SC attachment and proliferation were affected by specific adhesives such as FN, FG, and LM as well as the presence of serum in the medium, similarly to OECs.

N-CAM expression of SCs

Figure 6 shows the relative expression of N-CAM in SCs cultured on coated surfaces. In the serum-free

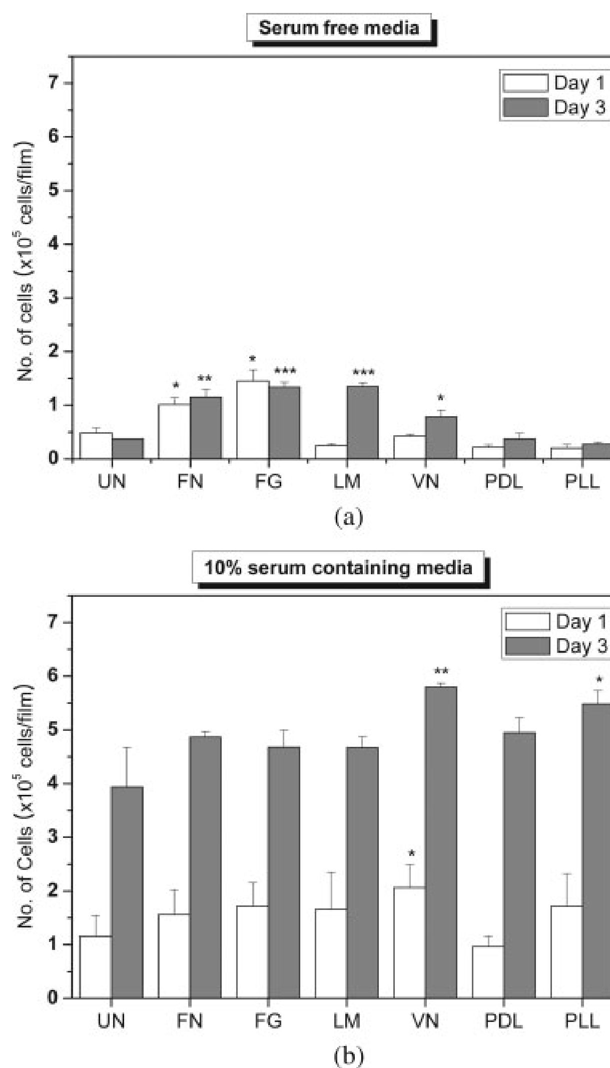

Figure 5 Number of SCs adhering to PLGA film surfaces coated with proteins or polypeptides: (a) serum-free media and (b) 10% FBS containing medium. Asterisks denote significant differences in comparison with UN for each day (*$P < 0.05$, **$P < 0.02$, ***$P < 0.01$).

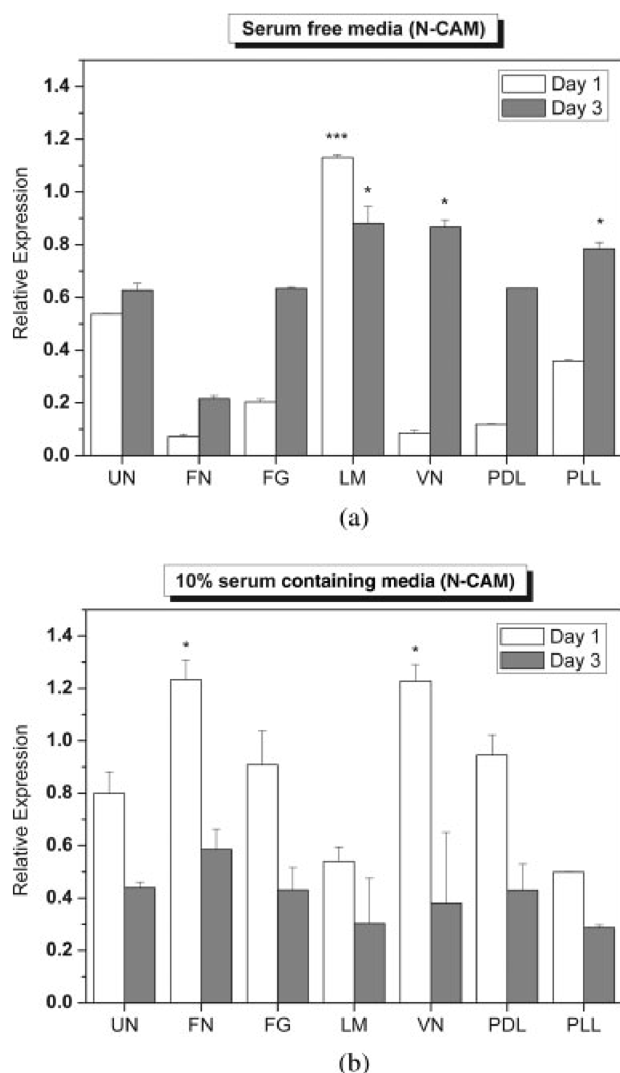

Figure 6 Relative expression of N-CAM in SCs cultured on PLGA film surfaces coated with proteins or polypeptides: (a) serum-free media and (b) 10% FBS containing medium. Asterisks denote significant differences in comparison with UN for each day (*$P < 0.05$, **$P < 0.02$, ***$P < 0.01$).

medium, N-CAM highly expressed to LM in comparison with the others at 1 day. LM, VN, and PLL were significantly different in comparison with the others at 3 days. However, N-CAM expression in the 10% serum containing medium was different from that under the serum-free medium condition. We found relatively high expression of N-CAM messenger RNA at FN and VN in comparison with the others at 1 day and no difference for the surfaces at 3 days. On the basis of this result, we concluded that specific adhesives such as FN, LM, and VN induced relatively high N-CAM secretion into attached cells. In addition, these results appeared to be related to the results for cell attachment and proliferation. Therefore, we recognized that N-CAM expression had an effect on the proliferation and morphology of attached cells.

SC morphologies on coated surfaces

The morphology of attached SCs did not differ according to the types of proteins and polypeptides,

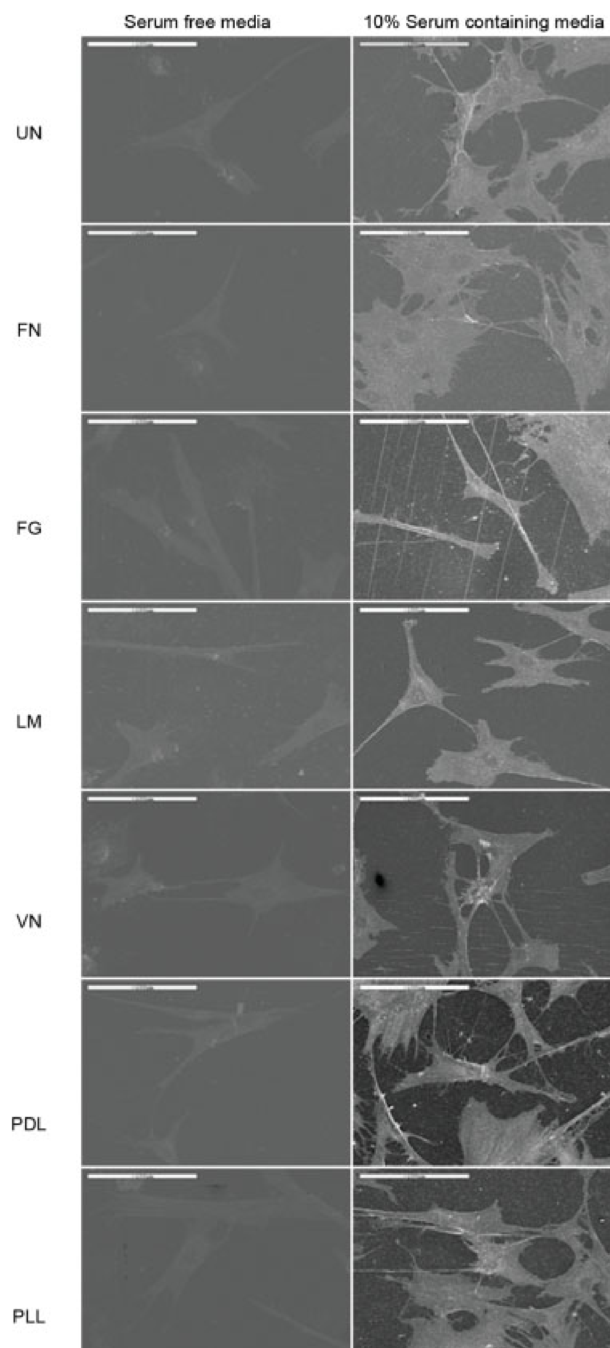

Figure 7 SEM microphotographs of SCs revealing the morphology of the attachment. SCs were cultured in media with or without serum on PLGA film surfaces coated with proteins or polypeptides for 1 day (scale bar = 100 μm).

as determined by SEM (Fig. 7). Filopodia and lamellipodia, related to cell spreading and activity, were observed to lead to good formation of SCs attached onto film surfaces. Most attached SCs appeared in a neurite-like cell process on the film surface. It seems that the type of adhesive was not an important factor with respect to cell spreading and activity. However, we observed differences in the SC morphology according to the presence of serum. SCs in the serum-free medium had a relatively flattened morphology different from that of normal SCs. This result was caused by the fact that the presence of serum in the medium was closely related to cell activation. On the basis of this result, the presence of serum in the medium was closely connected with SC morphology as well as proliferation.

OECs and SCs are known as neural cells having similar properties. However, we confirmed that these neural cells, having similar properties, differ in attachment and proliferation according to factors such as adhesive molecules and the presence of serum. These results suggest that a suitable surface considering the cell types or culture condition improves cell attachment and proliferation. We confirmed that FN, FG, and LM were suitable adhesive molecules with respect to coating efficiency and improvement of the attachment and growth of OECs and SCs on the basis of these results.

CONCLUSIONS

In this research, we experimented with the interaction of neural cells (OECs and SCs) and PLGA, which has attracted attention recently as nerve guidance. In summary, the results of this research are as follows:

- The adsorbed amounts of proteins and polypeptides revealed differences according to types under identical coating conditions.
- FN and FG importantly affected the initial attachment of OECs and SCs.
- Cell proliferation was influenced by adhesive proteins and polypeptides such as PDL and PLL.
- In the case of SCs, serum, in comparison with the type of material surface, had a greater influence on proliferation and morphology.
- The proliferation and morphology of OECs were affected by serum less than SCs.
- N-CAM expression was closely related to cell attachment and proliferation.

The selection of an optimal adhesive molecule is an important consideration for manufacturing nerve guidance and neural cell cultures for tissue-engineered nerve regeneration. In conclusion, we expect this research to provide a guideline to decisions about adhesive molecules for modified cell compatibility of PLGA.

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