ACS Chemical Neuroscience

Research Article

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Engineering Three-Dimensional Collagen-IKVAV Matrix to Mimic Neural Microenvironment

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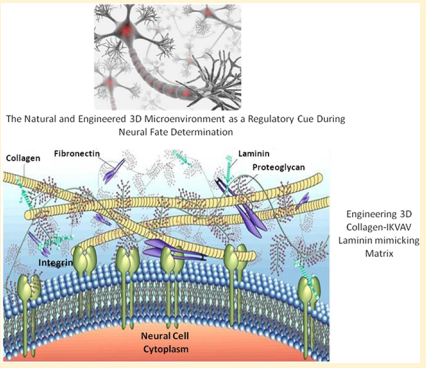
ABSTRACT: Engineering the cellular microenvironment has great potential to create a platform technology toward engineering of tissue and organs. This study aims to engineer a neural microenvironment through fabrication of three-dimensional (3D) engineered collagen matrixes mimicking in-vivo-like conditions. Collagen was chemically modified with a pentapeptide epitope consisting of isoleucine-lysine-valine-alanine-valine (IKVAV) to mimic laminin structure supports of the neural extracellular matrix (ECM). Three-dimensional collagen matrixes with and without IKVAV peptide modification were fabricated by freeze-drying technology and chemical cross-linking with glutaraldehyde. Structural information of 3D collagen matrixes indicated interconnected pores structure with an average pore size of 180 μm. Our results indicated that culture of dorsal root ganglion (DRG) cells in 3D collagen matrix was greatly influenced by 3D culture method and significantly enhanced with engineered collagen matrix conjugated with IKVAV peptide. It may be concluded that an appropriate 3D culture of neurons enables DRG to positively improve the cellular fate toward further acceleration in tissue regeneration.



KEYWORDS: *Tissue engineering, 3D matrix, peptide, collagen, IKVAV*

Engineering the cellular microenvironment is very important to fabricate three-dimensional (3D) models toward better understanding of cell−tissue interactions and regenerative medicine technology.[1−5] Biodegradable materials are at the core of fabrication of 3D engineered tissues together with cell culture technology. Three-dimensional in vitro technology aims to create a platform filed that are suitable for cell−cell interactions as they do in vivo. We are not able to mimic these interactions in common tissue culture dishes or 2D in vitro culture systems. Therefore, such an engineered design would be necessary to address the above challenges. Natural extracellular matrix (ECM) plays important roles in creating microenvironment for cell−cell as well as cell−tissue interactions. Therefore, 3D in vitro models should have similar structure as ECM does. These similarities are in terms of biological, chemical, and physical composition. Several 3D structures have been already developed for cell culture in scaffolds since they provide larger surface area for cell attachment and proliferation than 2D tissue culture dishes.[6−10]

This study aims is to establish a simple 3D in vitro platform technology to analyze the proliferation capability of dorsal root ganglion (DRG) cells under in-vivo-like conditions. We characterized and studied cellular behavior of DRG cells by testing their potential proliferation by culturing them in an in-vivo-like condition. The data described in the current study suggests that this approach may be widely applicable to many stem cell populations. In the present technology, the molecular design of such an in-vivo-like condition was undertaken to design a 3D collagen matrix incorporated the pentapeptide epitope isoleucine-lysine-valine-alanine-valine (IKVAV), which

Received: March 28, 2013
Accepted: May 24, 2013
Published: May 24, 2013

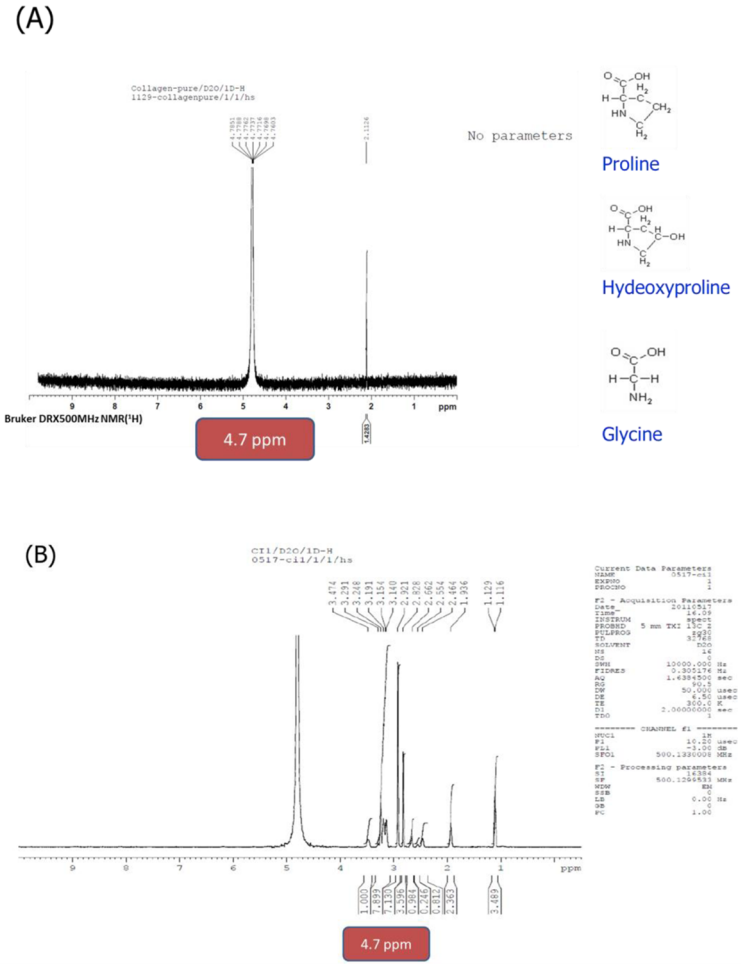


Figure 1. ^{1}H NMR (500 MHz) spectrum analysis of collagen (A) and collagen-IKVAV peptide (B).

is found in laminin and is known to promote neurite sprouting and to direct neurite growth.[11,12] As the control for bioactivity, we fabricated a 3D collagen matrix without further modification. We show that 3D collagen matrix modified with IKVAV peptide can be used as platform technology to enhance proliferation of DRG cells.

RESULTS AND DISCUSSION

Functionalization of Collagen with IKVAV Peptide. Collagen-IKVAV peptide based conjugate was prepared by peptide bound formation between activated carboxyl groups of collagen and primary amino groups of IKVAV peptide. Collagen was initially activated with CDI, and the obtained activated derivative was allowed to react under basic conditions with primary amino groups of IKVAV peptide. The content of substituted IKVAV moieties was determined from nitrogen content (%N) and found to be nearly 50% (50 IKVAV moieties in each 100 repeating units) as shown in ^{1}H NMR analysis in Figure 1. We applied 2,4,6-trinitrobezenesulfonic acid (TNBS) method to determine the percentage of amino groups introduced into collagen. It was found that the percentage of amino groups introduced into collagen was nearly 47.8 mol/mol carboxyl groups of collagen based on the calibration curve prepared by using β-alanine.

Morphology of Collagen-IKVAV Peptide Matrix and Cytotoxicity Assay. Figure 2A shows a light microscopy photograph of collagen-IKVAV peptide matrix, and Figure 2B

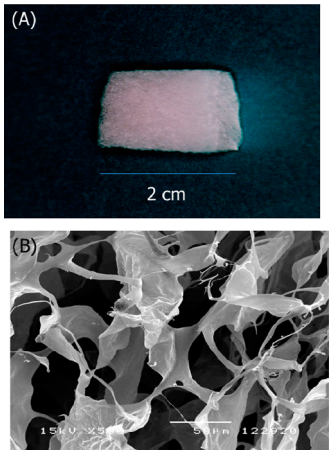


Figure 2. Light microscopy photographs (A) and cross-sectional SEM photographs of collagen-IKVAV peptide matrix (B).

shows a cross-sectional SEM photograph of collagen-IKVAV peptide matrix. Structural information of 3D collagen matrixes indicated interconnected pore structure with an average pore size of 180 μm. Figure 3 shows the appearance, stability, and maintaining ability of 3D collagen matrix with and without IKVAV peptide conjugation after cross-linking with 0.4 wt % glutaraldehyde solution in 0.2 vol % acetic acid for 24 h at 4 °C. Irrespective of the peptide introduction, 3D collagen matrix was

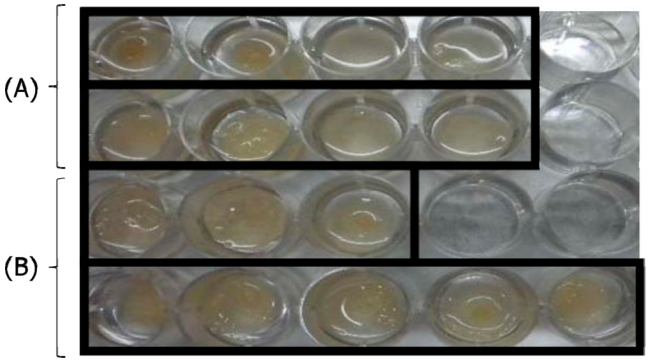


Figure 3. Light microscopic photographs of a collagen-IKVAV matrix (A) and unmodified collagen matrix (B) after cross-linking with 0.4 wt % glutaraldehyde solution in 0.2 vol % acetic acid for 24 h at 4 °C.

stable and the original shape of 3D matrix was maintained for a long time in aqueous as well as in biological solutions.

To observe the cytotoxic effects of the modified and unmodified 3D collagen matrix with and without IKVAV on L929 mouse fibroblast cell lines in vitro, the MTT test was performed. The results obtained with the modified 3D collagen matrix with IKVAV peptide indicate that no inherent toxicity can be attributed to the modifications. Our results clearly indicated that the cell viability for the unmodified and modified 3D collagen matrixes are 98.4% and 98.6%, respectively, after 5 days, which means that the cytotoxicity of the 3D collagen matrix on the L929 cells is not significant (data are not shown).

Cell Attachment and Proliferation in 3D Collagen Matrix with or without IKVAV Peptide Conjugation. Our observation confirmed significant difference in the cell number between 3D collagen matrixes with and without modification with IKVAV, VVIAK, RGD, and RGD-IKVAV peptides with that of 2D tissue culture dishes (Figure 4). As expected, chemical modification of 3D collagen with commonly used cell-adhesive RGD peptide significantly increased cell adhesion and the level of enhancement was almost as the same as modification with IKVAV peptide. Interestingly, chemical modification of collagen with both RGD and IKVAV peptides significantly enhanced cell adhesion among all other 3D collagen matrixes. Modification of 3D collagen with VVIAK as a scrambled peptide of IKVAV did not show any enhancement on cell adhesion and the level of cell attachment was almost as the same as 3D unmodified collagen matrix.

The proliferation profiles of cells cultured in the 3D collagen matrixes modified with IKVAV peptide and other peptides showed that the cell number tended to increase significantly higher with incubation time compared with those cultured in the 3D collagen matrixes without modification with IKVAV, VVIAK, RGD, and RGD-IKVAV peptides. Figure 5 shows the proliferation profiles of cells cultured in 2D static tissue culture dishes, 3D collagen matrix, and 3D collagen matrixes modified with IKVAV, VVIAK, RGD, and RGD-IKVAV peptides. Irrespective of the cell culture systems, the cell number tended to increase with incubation time. Among the three different peptide modifications, the initial cell proliferation was the highest for 3D collagen matrix modified with IKVAV as well as in combination with RGD-IKVAV peptides. Modification of 3D collagen with VVIAK as a scrambled peptide of IKVAV did not enhance cell proliferation, and the level of cell proliferation was almost as the same as that of the 3D unmodified collagen matrix. In contrast, combination of two peptides, RGD as commonly used cell-adhesive peptide and IKVAV as neural cell receptor, significantly enhanced cell proliferation. These data suggest that collagen-IKVAV binds to only IKVAV receptor(s) while the collagen-RGD interacts with integrin receptor(s) and collagen-RGD-IKVAV interacts with both integrins and the IKVAV receptor(s).

Cell seeding and proliferation are important factors in 3D systems as the initial process for development of tissue and the maintenance of the differentiation phenotype.[16] Collagen is one of the main proteins in the ECM and has been widely applied in clinical medicine, cosmetics, and foods.[17,18] Three-dimensional in vitro systems can provide bigger surface area compared with tissue culture dishes.[19] Several research studies reported that the proliferation of cells cultured in tissue culture dishes was stopped after they reached full confluence, and cell proliferation in 3D culture systems was continued.[20−24] Also, 3D culture systems significantly influenced differentiation of cells through enhancing cells' aggregation that would affect cell function.[25−27] It seems that 3D in vitro systems can mimic in vivo microenvironment conditions in an appropriate way; therefore, cellular behavior would more closely resemble that in the in vivo situation.[28]

Effect of 3D Collagen Matrix-IKVAV on DRG Cellular Fate. To evaluate further cellular fate phenomena, we used combinational technology of 3D collagen matrix modified with or without IKVAV and other different peptides, and neuronal induction medium (with growth factor) or standard medium (without growth factor). Acidic fibroblast growth factor (aFGF)

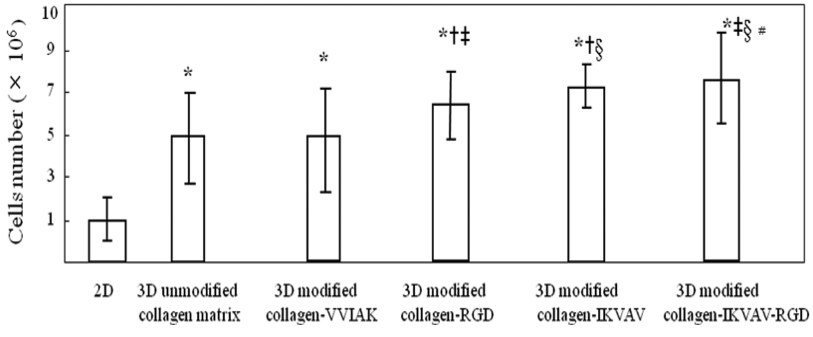


Figure 4. Cell attachment for the 3D collagen matrix with and without VVIAK, IKVAV, RGD, and RGD-IKVAV modification. *, $p < 0.05$; significant relative to 2D culture method. †, $p < 0.05$; significant relative to 3D unmodified collagen matrix. ‡, $p < 0.05$; significant relative to 3D modified collagen-VVIAK matrix. §, $p < 0.05$; significant relative to 3D modified collagen-RGD matrix. #, $p < 0.05$; significant relative to 3D modified collagen-IKVAV matrix.

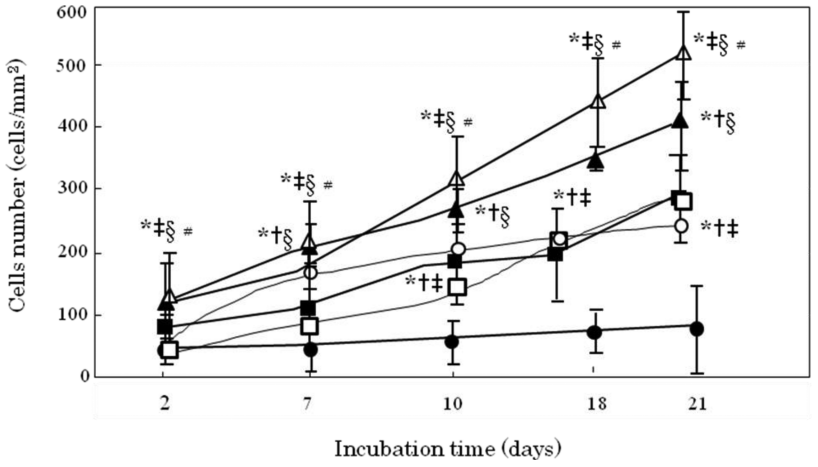


Figure 5. Proliferative profile of cells on 2D culture (●), 3D collagen matrix without modification (■), 3D collagen matrix with VVIAK modification (□), 3D collagen matrix with RGD modification (○), 3D collagen matrix with IKVAV modification (▲), and 3D collagen matrix with RGD-IKVAV modification (△). *, $p < 0.05$; significant relative to 2D culture method. †, $p < 0.05$; significant relative to 3D unmodified collagen matrix. ‡, $p < 0.05$; significant relative to 3D modified collagen-VVIAK matrix. §, $p < 0.05$; significant relative to 3D modified collagen-RGD matrix. #, $p < 0.05$; significant relative to 3D modified collagen-IKVAV matrix.

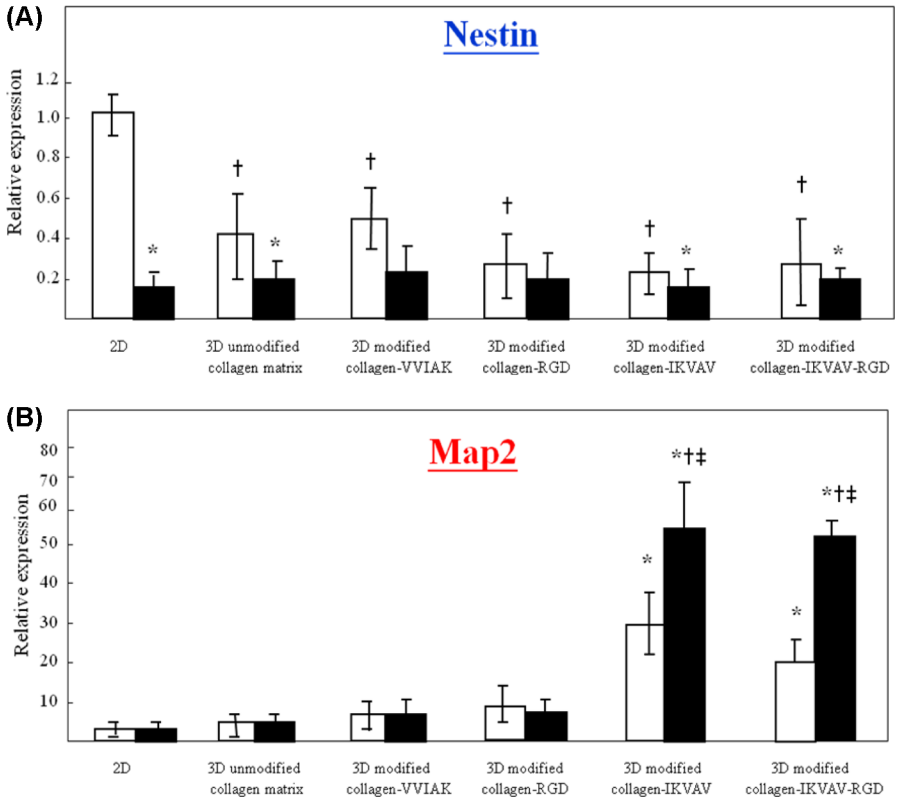


Figure 6. (A) Real-time PCR study of gene expression of Nestin related to DRG cells grown in nerve differentiation (■) and standard medium (□) on 2D and 3D collagen matrix with and without VVIAK, IKVAV, RGD, and RGD-IKVAV modification. *, $p < 0.05$; significant relative to level of gene expression compared to DRG cells cultured in standard medium. †, $p < 0.05$; significant relative to level of gene expression compared to DRG cells cultured in 2D culture method in standard medium. (B) Real-time PCR study of gene expression of Map-2 related to DRG cells grown in nerve differentiation (■) and standard medium (□) on 2D and 3D collagen matrix with and without VVIAK, IKVAV, RGD, and RGD-IKVAV modification. *, $p < 0.05$; significant relative to level of gene expression compared to DRG cells cultured in standard medium. †, $p < 0.05$; significant relative to level of gene expression compared to DRG cells cultured in 2D culture method in standard medium. ‡, $p < 0.05$; significant relative to level of gene expression of DRG cells cultured in nerve differentiation comapred with level of gene expression of DRG cells cultured in standard medium.

was incorporated into each matrix prior to the cell culture. Studies of gene expression by real-time PCR were performed to evaluate the differentiation of cells grown on 3D collagen matrixes with or without modification with IKVAV, VVIAK, RGD, and RGD-IKVAV peptides. Two important key nerve cell related markers, namely, Nestin, an intermediate filament protein expressed by neural cells and Map-2, a marker of mature neurons, were studied. There was weak expression of

neuronal marker in DRG cells cultured in standard medium in 2D culture compared with that in 3D culture as shown in Figure 6. Nestin exhibited a decreased expression in DRG cells grown in neuronal induction medium on both 2D and 3D culture compared with that in standard medium (Figure 6A). Also stronger expression of Map-2 was found in cells cultured on 3D collagen matrixes modified with IKVAV and IKVAV-RGD compared to 2D culture (Figure 6B). In addition, cells cultured on 3D collagen matrix in standard medium demonstrated down regulation of expression of both nerve marker genes.

Surface properties of materials such as topography and chemistry seemed to be appropriate for cell adhesion, proliferation, as well as cell differentiation. In this study, differentiation of DRG cells to neuronal cells was carried out on 3D collagen matrix and neuronal marker expression was studied during the course of differentiation. In order to validate of DRG differentiation, the expression of genes was analyzed by real-time (RT) PCR. RT-PCR indicated the expression of neuronal marker before induction. These data indicate that DRG cells naturally express some neural cell markers. Also, DRG grown in nerve induction medium exhibited a higher Map-2 expression when grown on 3D collagen matrix compared with 2D culture. However, up regulation of Map-2, a protein associated with the maturing neuron, was observed on DRG grown on 3D collagen modified with IKVAV and combination of IKVAV and RGD peptides. IKVAV provides better microenvironment for DRG cells differentiation compared with 2D culture. Improved cellular communication in 3D culture grown in 3D collagen matrix may explain the enhancement of cellular differentiation compared with that in 2D culture. Also, it was demonstrated that IKVAV and IKVAV-RGD peptides appeared to enhance expression of Map-2 marker more than 3D collagen matrix modified with VVIAK peptide, as a scrambled peptide of IKVAV and RGD peptide alone. Perhaps a higher cell density results in a higher degree of cell−cell communication and thus a higher rate of differentiation is expected to increase. These results suggest that 3D collagen matrix modified with IKVAV is more suitable for nerve tissue engineering via DRG cell differentiation. Significant down regulation of Nestin expression in DRG cells cultured in nerve induction medium in both 3D and 2D culture indicate the efficacy of these growth factors in driving DRG cells into prodifferentiated state. On the other hand, our results demonstrated that 3D collagen matrix modified with IKVAV and IKVAV-RGD peptides without using growth factor induced differentiation of DRG cells into neuronal cell and neurotrophic factor are important in neural differentiation. Other research studies have shown that mechanical factors such as nanostructured substrates can induce differentiation of stem cell in the absence of any additional induced factor.[30] However, the mechanisms underlying the down regulation by substrate topology has not been elucidated. Our results clearly indicated protein expression of neuronal cells generated from DRG cells cultured on 3D collagen matrixes modified with IKVAV peptide. However, more studies are needed to further understand the functional cell formation. Therefore, optimal 3D culture conditions should be further investigated.

Synthetic peptide containing the IKVAV sequence has been studied, and its application on neural growth fully tested.[12] It has been shown that IKVAV sequence is one of the principal sites in laminin which regulate cellular behavior.[12] We demonstrate that conjugation of IKVAV peptide to collagen significantly enhanced in vitro proliferation of DRG compared with unmodified collagen. Further characterization and optimization of this method may provide an effective strategy to overcome current limitations associated with in vitro 3D cell culturing, while they simultaneously provide information about cell culture which are known to be a potent regulator of cell functions.

We present here a simple 3D culture system which supports neuronal proliferation under more in-vivo-like conditions. The 3D culture system described here will be suitable for testing neuronal capability of numerous types of stem cells for future cell-based therapeutic applications. The present findings will provide attractive combinational strategy of tissue engineering principles and materials science and engineering for development of regenerative medicine technology.

METHODS

An aqueous solution of type I collagen, prepared from porcine tendon by pepsin treatment, in HCl (3 mg/mL, pH 3.0) was kindly supplied by Nitta Gelatin Inc., Osaka, Japan. Dulbecco's modified Eagle's minimal essential medium (DMEM) and fetal bovine serum (FBS) (lot 2104C) and IKVAV peptides with purity = 90% were purchased from Sigma-Aldrich. Other chemicals were purchased from Sigma-Aldrich and used as obtained. A sequence of VVIAK peptide, as a scrambled peptide of IKVAV, was ordered to be synthesized by Sowa Trading Co. Inc., Tokyo, Japan. RGD (arginine-glycine-aspartic acid) was purchased from Sowa Trading Co. Inc., Tokyo, Japan.

Chemical Modification of Collagen. *1.1. Synthesizing Technique to Conjugate IKVAV Peptide to Collagen.* To conjugate IKVAV peptide to collagen, 1 mg of IKVAV peptide was added to 10 mL of 3% collagen solution in phosphate-buffered saline (PBS, pH 7.0−7.6) containing *N*,*N*-dimethylaminopropyl carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) at the final molar ratio of EDC/NHS/COOH of peptide = 5:5:1. The final mixture was stirred at 25 °C for 1 h to conjugate IKVAV peptide to the primary amino groups of collagen and then dialyzed against double distilled water (DDW) for 48 h at 25 °C by use of a dialysis membrane tube (Spectro/Por membrane, Cut-off M_w = 14 000−18 000) to separate residual IKVAV peptide and EDC-degraded product from collagen-IKVAV prepared. The dialyzed solution was freeze-dried to obtain a powdered collagen-IKVAV. The IKVAV peptide introduction was quantitated by the NMR analysis. The amount of IKVAV peptide conjugation was also analyzed by trinitrobenzene sulfonate (TNBS) method[29] to calculate the molar percentage of NH_2 conjugate to the carboxyl groups of collagen. *β*-Alanine was used to make a calibration curve.

The same procedure was applied to conjugate VVIAK peptide, as a scrambled peptide of IKVAV to collagen to obtain collagen-VVIAK.

1.2. Synthesizing Technique to Conjugate RGD Peptide to Collagen. RGD and EDC were added into 100 mL of double-distilled water (DDW) containing 5 mg of collagen. The mixing molar ratio was fixed at RGD/COOH of collagen 50/3. The final solution was stirred for 18 h at 37 °C. To separate nonreacted nonreacted RGD and EDC from collagen-RGD, we used a dialysis membrane tube (lot No. 041952, cutoff M_w = 12 000−14 000, Nakalai Tesque, Kyoto, Japan) and followed dialyzing against DDW for 48 h at 25 °C. The percentage of NH_2 conjugated into collagen was 37.8 mol/mol COOH of collagen.

1.3. Synthesizing Technique to Conjugate RGD and IKVAV Peptides to Collagen. RGD and EDC were added into 100 mL of DDW containing 5 mg of collagen. The mixing molar ratio was fixed at RGD/COOH of collagen 50:3. Immediately after preparation the above mixture, 1 mg of IKVAV peptide and 10 mL of *N*-hydroxysuccinimide were added to final mixture at the final molar ratio of EDC/NHS/COOH of peptide = 5:5:1. The final solution was stirred for 18 h at 37 °C. To separate nonreacted RGD, IKVAV, and EDC from IKVAV-collagen-RGD we used a dialysis membrane tube (lot no. 041952, cutoff M_w = 12 000−14 000, Nakalai Tesque, Kyoto, Japan) and followed dialyzing against DDW for 48 h at 25 °C. The

percentage of NH_2 conjugated into collagen was 47.6 mol/mol COOH of collagen.

Fabrication of 3D Collagen Matrix. We applied freeze-drying technology to fabricate 3D collagen matrixes. Collagen solution (0.75 mL) with or without modification with IKVAV, VVIAK, RGD, and RGD-IKVIV peptides conjugation was poured into a polystyrene mold (Costar, 24 well; Corning Life Sciences, New York) and immediately was transferred into a freezer (−20 °C). We let the above solutions to be kept inside the freezer for at least 24 h, and thereafter, they were transferred into a freeze-drying machine and the freeze-drying process was applied for at least 48 h to obtain a white dried sponge. We used 0.4 wt % glutaraldehyde solution to chemically cross-link collagen with or without peptides modification. Following DDW washing, the collagen sponges were freeze-dried and then sterilized by 70% ethanol.

Characterization of 3D Collagen-IKVAV Matrix. The structure of the lyophilized collagen-IKVAV scaffold was examined by scanning electron microscopy (SEM) using a razor blade mounted on stubs with colloidal graphite and sputter coated with gold to an approximate thickness of 200 Å. The surface and the bulk of collagen sponges were examined with a JEOL-5300 scanning electron microscope using an accelerating voltage of 100 kV.

In Vitro Cell Culture. Dorsal root ganglion (DRG) cells were isolated from neonatal Sprague−Dawley (SD) rats and treated with mixture of papain, protease, and deoxyribonuclease (0.1%:0.1%:0.03%). Dissociated cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) contacting fetal bovine serum (FBS). The choice of cell was considered by the potential advantages of using DRG cells since primary sensory neurons with cell bodies in DRG cells provide great candidate to study mechanisms regulating axonal regeneration. It has been shown that DRG cells were cultured well on a surface covered with laminin as an ECM protein.[31]

Cell Seeding into 3D Collagen Matrix. Dissociated DRG cells were seeded into 3D collagen matrix with or without IKVAV, VVIAK, RGD, and RGD-IKVIV peptide conjugation similar to our previous publications.[13,14] After cell seeding, we used PBS (−) to remove nonadherent cells. We followed a simple static culture method by placing cell-seeded collagen matrixes into each well of 12-well tissue culture dishes by incubation of dishes at 37 °C in a 95% air/5% CO_2 atmosphere. To compare 3D culture with 2D culture method, we coated the surface of tissue culture dish with the solution of collagen-IKVAV and allowed them to dry overnight in a fume hood. Before cell culture, all coated plates were washed three times with DDW to remove any material not adsorbed to the surface of plates. We studied neural differentiation of DRG cells under the influence of neurotrophic factors, acidic fibroblast growth factor (aFGF), since aFGF is reported to affect the induction, mitosis, cell−cell interactions, and promote stable neurite outgrowth and neuronal differentiation. Acidic fibroblast growth factor (aFGF) was incorporated into each matrix prior to the cell culture by using a 100 μL pipet to entrap the growth factor into collagen matrix.

Real-Time Polymerase Chain Reaction Analysis of Gene Expression. Real-time PCR analyses were conducted for a set of three selected genes as shown in Table 1. Total RNA was extracted from cells cultured on each 3D collagen matrix with or without modification with IKVAV, VVIAK, RGD, and RGD-IKVIV peptides in standard medium as a control and nerve induction medium using QIAzol lysis Reagent Kit (QIAgene) according to the manufacturer's instructions. First-strand cDNA was synthesized from the total RNA using a RT kit (Qiagen). A total sample volume of 25 mL, which comprised 12 μL of SYBR Green Mastermix (Takara), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 2 μL of cDNA, and 9 μL of DDW, was used for the real-time PCR experiments. PCR reaction was carried out using Corrbet Science Rotor-Gene 3000 instrument (Applied Biosystems, Foster City, CA) with the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 10 s, 60 °C for 45 s followed by 50 °C for 1 s, 90 °C for 5 s. Amplification was monitored and analyzed by measuring the binding of the fluorescent dye SYBR Green I to double stranded DNA. Melting curve analysis was done to ensure the absence of primer dimers and nonspecific PCR product amplification. Hypoxanthine phosphoribosyl transferase (HPRT) was used as the housekeeping gene for the normalization of the gene expression data. Therefore, the comparison Ct (ΔΔCt) method was used for gene expression analyses.

Table 1. Real-Time PCR Primer Sequences of Selected Genes

gene	primer sequence
HPRT	R: 5′-TCA GTC CTG TCC ATA ATT AGT CC-3′
	F: 5′-CCT GGC GTC GTG ATT AGT G-3′
Nestin	R: 5′-CAG CAG AGT CCT GTA TGT AGC-3′
	F: 5′-TGG AAC AGA GAT TGG AAG GC-3′
Map-2	R: 5′-GGT TAT TCC ATC AGT GAC TTT GT-3′
	F:5′-ACC AAC TCA TCT CTC CTG TG-3′

DNA Assay. To analyze the number of cells adhered and attached to 3D collagen matrixes, fluorometric quantification of cell DNA was applied.[15] All 3D collagen matrixes with or without modification were immersed in a lysis buffer containing 0.5 mg/mL proteinase K, 0.2 mg/mL sodium dodecyl sulfate, and 30 mM saline-sodium citrate at 55 °C for 12 h. A volume of 100 μL of each cell lysate was mixed with 400 μL of sodium dodecyl sulfate, and the final solution was added to 500 μL of fluorescent solution (30 mM sodium dodecyl sulfate and 1 μg/mL Hoechst 33258 dye). The number of cells attached to each 3D collagen matrix was counted via the fluorescent intensity at Ex 355 nm/Em 460 nm.

Cytotoxicity of 3D Collagen Matrix. Cytotoxicity effects of unmodified and modified 3D collagen matrixes with or without modification were investigated using L929 mouse fibroblast cells cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin.

All the 3D collagen matrixes with and without modification were interacted with the cell culture medium and incubated for 5 days at 37 °C. L929 mouse fibroblast cells were plated in 96-well culture plates at density of 1×10^3 cells/well including 3D collagen matrixes that were prewetted with culture medium. After 24 h, medium of each 96-well was discharged and replaced with 100 μL of fresh medium and 13 μL of MTT solution (5 μg/mL, diluted with RPMI 1640 without phenol red). At the end of 4 h of MTT reaction, media were replaced with 100 μL of isopropanol/HCl. An ELISA plate reader was applied to clarify the percentage of viability at 570 nm and compared with control group of cells cultured without any using of 3D collagen matrixes.

Statistical Analysis. All the data were statistically analyzed to express the mean ± the standard deviation (SD) of the mean. Student's t test was performed, and $p < 0.05$ was accepted to be statistically significant.

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Author Contributions

H.H. conceived the experiment and carried it out. Y.H. provided and fabricated collagen sponge with related data analysis. C.-H.L. designed and carried out the data analysis. Y.-R.C. designed and carried out the data analysis. D.-S.Y. co-wrote the paper. P.-D.H. co-wrote the paper. K.-L.O. co-wrote the paper.

Funding

This study was supported by the Research Grant of NSC 99-2314-B-011-001-MY3.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was performed through National Science Council (NSC) of Taiwan.

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