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Amino acid sequence preferences to control cell-specific organization of endothelial cells, smooth muscle cells, and fibroblasts

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Effective surface modification with biocompatible molecules is known to be effective in reducing the life-threatening risks related to artificial cardiovascular implants. In recent strategies in regenerative medicine, the enhancement and support of natural repair systems at the site of injury by designed biocompatible molecules have succeeded in rapid and effective injury repair. Therefore, such a strategy could also be effective for rapid endothelialization of cardiovascular implants to lower the risk of thrombosis and stenosis. To achieve this enhancement of the natural repair system, a biomimetic molecule that mimics proper cellular organization at the implant location is required. In spite of the fact that many reported peptides have cell-attracting properties on material surfaces, there have been few peptides that could control cell-specific adhesion. For the advanced cardiovascular implants, peptides that can mimic the natural mechanism that controls cell-specific organization have been strongly anticipated. To obtain such peptides, we hypothesized the cellular bias toward certain varieties of amino acids and examined the cell preference (in terms of adhesion, proliferation, and protein attraction) of varieties and of repeat length on SPOT peptide arrays. To investigate the role of specific peptides in controlling the organization of various cardiovascular-related cells, we compared endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblasts (FBs). A clear, cell-specific preference was found for amino acids (longer than 5-mer) using three types of cells, and the combinational effect of the physicochemical properties of the residues was analyzed to interpret the mechanism. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: cell specific; cell adhesion; cell proliferation; amino acid index; peptide array

Introduction

The life-threatening risks that occur after implantation of medical devices and products are mostly due to the disruption of the biological environment of the location. With medical devices and products for cardiovascular treatments, such disruption-induced side effects could directly end patients' lives. The most common risk with cardiovascular implants is stenosis caused by thrombosis and neointimal hyperplasia [1]. Thrombosis is caused by the atypical attraction of serum proteins, platelet, and circulating blood cells to damaged or absent endothelial surfaces [2]. Neointimal hyperplasia, which is characterized by excessive smooth muscle cell (SMC) growth is also a critical risk caused by damage to the endothelial layer together with expansion pressure in the cases of stent implants [3,4]. Side effects such as these, which occur with cardiovascular treatments, are commonly contradictory. It is known that when restenosis is effectively suppressed by the elution of cell growth inhibition reagents, for e.g. by the use of drug eluting stents, proper endothelialization is inhibited [5]. Therefore, to overcome such defects, the ideal surface coating of a cardiovascular implant should not only inhibit overgrowth of SMCs but also enhance the growth of the endothelial cells (ECs) for successful endothelialization [4].

One of the most promising strategies in regenerative medicine is to lower the risks of cardiovascular implants by modifying the device surface with biological molecules (such as proteins [6–9], glycosaminoglycan [10], chemokines [11], and protein-derived peptides [12–15]) to mimic the natural biological atmosphere for rapid and prolonged repair by the native cellular system [16]. Fibrin, collagen, fibronectin (FN), and elastin are frequently chosen biological molecules for medical device coating because of the antithrombosis effects of these molecules. Because endothelialization is the most critical event involved in both thrombosis and restenosis, biological molecules with the ability to enhance endothelialization have attracted attention. CD34 antibodies have been utilized to coat stent surfaces to capture endothelial progenitor cells for rapid and effective

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endothelialization [17]. However, most large biological molecules, such as the anti-CD34 antibody, are obtained from nonhuman hosts; therefore, the risk of unexpected infection is a concern that has led to medical restrictions. Considering this risk, artificially synthesized peptides derived from human proteins could serve as ideal molecules because of their biocompatibility and safety assurance.

Many short peptides have been shown to enhance cellular adhesion by surface conjugation. RGDS (Arg-Gly-Asp-Ser) is the most commonly studied short peptide from extracellular matrix (ECM) and binds to integrins on cells to form strong cellular adhesions [18]. Besides RGDS, short peptides, such as LDV (Leu-Asp-Val) [19], YIGSR (Try-Ile-Gly-Ser-Arg) [20], and PHSRN (Pro-His-Ser-Arg-Asn) [21], have been reported to enhance cellular attraction to the material surface. These peptides are ideal model peptides and reveal that even short peptides can serve as cell adhesion molecules.

In the natural biological repair system, cells specifically localize to their correct location to form a well-organized cellular system; therefore, there are few chances to explore the abovementioned integrin ligands on the complex ECM surface. As a result, we hypothesized that there might be a cellular preference for more broad candidate molecules with similar physicochemical properties, such as a bias toward certain varieties of amino acid or peptide, on ECM surfaces that could explain cell-specific adhesion and proliferation mechanisms. To investigate our hypothesis, we examined the amino acid preferences that control the cellular organization in cardiovascular tissue. We chose three cell types, ECs, SMCs, and fibroblasts (FBs), that typically have roles in cardiovascular tissues and compared the relative preferences of these cells for specific amino acids and repeated sequences. For the cell-peptide interaction assay, we introduced a peptide array-based interaction assay of solid-bound peptides and anchorage-dependent cells (PIASPAC) method [22–24], an application of a SPOT peptide array technique [25]. By combinatorial examination of the peptide array, we could compare the cellular preferences in adhesion and proliferation. The accumulation of serum-derived proteins was also examined to determine its effect on cell-specific adhesion to peptides. Finally, to propose a design strategy for biomimetic polymers, we analyzed the relationship between cell specificity and the physicochemical properties of amino acids by using amino acid indices and multivariant analysis. To our knowledge, this is the first detailed analysis comparing the amino acid preferences of cardiovascular-related cells.

Materials and Methods

Cells

Normal human umbilical vein ECs (Kurabo Industries Ltd., Osaka, Japan) were maintained in HuMedia-EG2 (Kurabo Industries Ltd.) and designated as ECs. SMCs (Cell Applications, Inc., San Diego, CA, USA) were maintained in smooth muscle growth medium (Cell Applications Inc.) and designated as SMCs. Normal human dermal FBs (Kurabo Industries Ltd.) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Corporation, Carlsbad, CA, USA) at 37 °C under 5% CO_2 and designated as FBs. Penicillin streptomycin (Life Technologies Corporation) was used as antibiotics in the DMEM. All the cells were used in assays within four to six passages.

Peptide Array Synthesis

A cellulose membrane (grade 542; Whatman, Maidstone, UK) was modified using Fmoc-β-Ala-OH (Watanabe Chemical Industries, Ltd., Hiroshima, Japan) as the N-terminal basal spacer by 1-Methylimidazole, redistilled, +99% (Sigma-Aldrich, St. Louis, MO, USA), and *N*,*N*′-diisopropylcarbodiimide (DIPCI) (Watanabe Chemical Industries). Fmoc-11-aminoundecanoic acid (Watanabe Chemical Industries) was linked as an additional spacer between the candidate peptide and the cellulose by the cocktail of DIPCI and 1-Hydrozybenzotriazole (HOBt, anhydrous) (Watanabe Chemical Industries) (volume ratio 1 : 4), and which was optimized for better interaction with the cells. Fmoc amino acids (0.5 M) (Watanabe Chemical Industries) were also activated by the cocktail of DIPCI and HOBt, and spotted twice with a peptide autospotter (ASP222; Intavis Bioanalytical Instruments AG, Köln, Germany) in accordance with the manufacturer's instructions. Peptides were elongated by conventional Fmoc chemistry using the 20% piperidine (Watanabe Chemical Industries) as the removal agent of side-chain protecting groups. By the repeated numbers of elongation steps, peptide spots were designated as 1-mer (one elongation step), 5-mer (five elongation steps), and 7-mer (seven elongation steps). The final deprotection step of side chains was carried out by the cocktail of m-cresol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), thioanisole (Tokyo Chemical Industry Co., LTD., Tokyo, Japan), 1,2-Ethanedithiol (EDT) (Watanabe Chemical Industries), and trifluoroacetic acid (TFA) (Watanabe Chemical Industries) = 1: 6 : 3 : 40, respectively, for 3 h. The synthesized array membrane was then thoroughly washed three times for 2 h with diethyl ether (Wako Pure Chemical Industries), methanol (Wako Pure Chemical Industries), and Dulbecco's phosphate-buffered saline (PBS; pH 7.2) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Finally, the array was soaked in methanol (Wako Pure Chemical Industries) and dried on a clean bench.

Peptide Array-Based Interaction Assay of Solid-Bound Peptides and Anchorage-Dependent Cells (PIASPAC)

The cell assay on SPOT arrays was carried out according to a previously described method [22] with slight modifications. Briefly, from the synthesized peptide array, each spot corresponding to different peptides was punched out as a disk and embedded in a 96-well plate, and after soaking the punched disks with the appropriate cell culture medium, 1.5×10^4 cells/well were directly seeded on the disks. Cells and peptide disks were incubated for 1 h for cell adhesion assays and for 3 days for cell proliferation assays. After three repeat washes of PBS to remove unattached cells by pipetting, the viable cells were stained with calcein AM (Life Technologies Corporation) for 30 min, and fluorescence intensity was measured on a Fluoroskan Ascent (type 374; Labsystems, Helsinki, Finland) with 485 nm excitation and 538 nm emission. For reproducibility, the data of triplicate spots from two experiments were averaged. To normalize the fluorescence intensities to compare the cellular preferences, each average fluorescence intensity was divided by the average negative control (no peptide, linker only) value, which was set to 1.0, to obtain a relative preference ratio (adhesion or proliferation) for each sequence. The assay scheme is depicted in Figure 1. Peptides that exerted their effects equally in all of the cells were considered to be 'peptides with no cell preference (nonspecific peptides)', and peptides that indicated a biased effect to particular cell were considered to be 'peptides with cell preference (specific peptides to target cells)'. For example, when the number of ECs on a peptide spot disk

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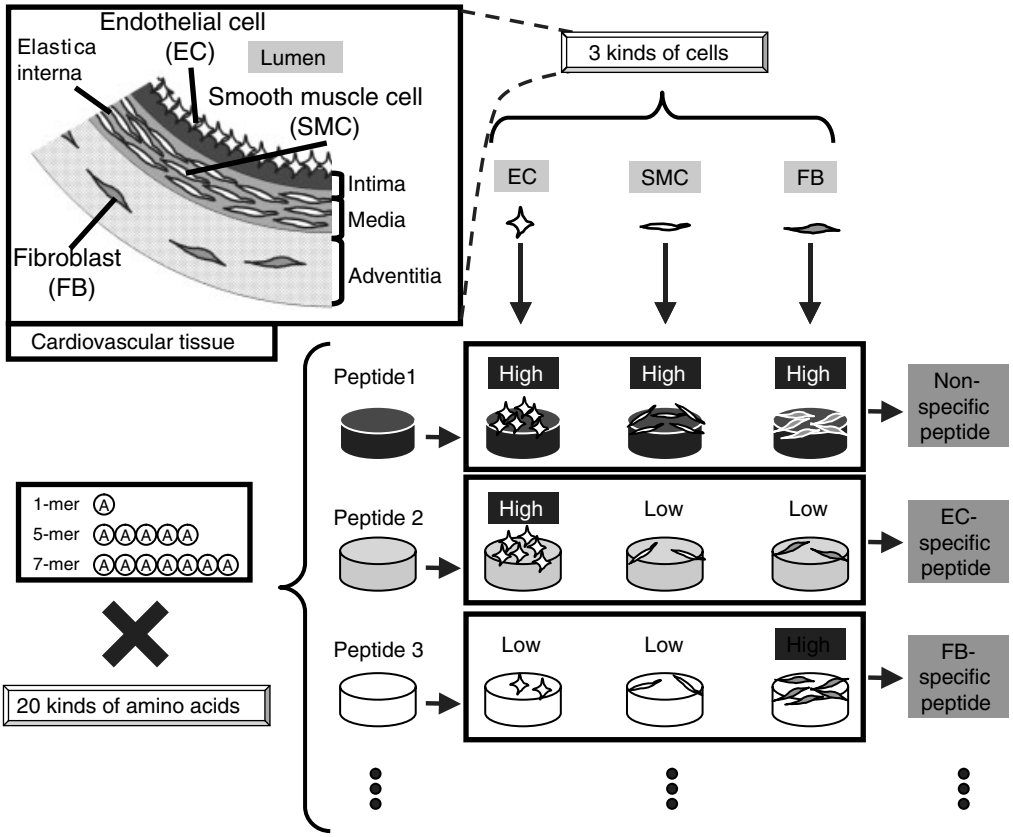

Figure 1. A schematic illustration of this study. Three kinds of cells that comprise cardiovascular tissues were chosen for determining the cell specificity. Twenty kinds of amino acids were chosen to design tandem repeat peptides for investigating the cell adhesion and proliferation. Cells were seeded to each peptide sequence on the peptide array spot and evaluated as the relative cell adhesion or proliferation rate for each cell type.

is larger than any other cell types, it is designated as EC-specific peptide.

Protein Attraction Assay on Peptide Array

The synthesized peptide arrays were washed five times with PBS (pH 7.2), and the membranes were allowed to dry under sterile conditions. Arrays were blocked with 1% bovine serum albumin (BSA) in PBS for 12 h at 4 °C. After blocking, the arrays were incubated for 1 h at 37 °C with DMEM containing 10% fetal bovine serum (Life Technologies Corporation) in order to assay the binding activity of each spot with serum-derived protein such as FN or vitronectin (VN). After continuous washes with PBS, the arrays were hybridized with anti-rabbit human FN IgG (Novotec, Saint Martin La Garenne, France) or anti-rabbit human VN IgG (Chemicon, Tokyo, Japan) diluted to a concentration of 1/500 or 1/1000 with PBS containing 0.25% BSA for 2 h at 37 °C. After several washes with Tris-buffered saline containing 0.05% Tween-20 (T-TBS; pH 7.2), arrays were hybridized with anti-rabbit IgG-conjugated Alexa 488 (Life Technologies Corporation) diluted to a concentration of 2 μg/ml with PBS containing 0.25% BSA for 1 h at 37 °C. After several washes with T-TBS at 37 °C, the fluorescence intensities of spots were scanned with a FLA-7000 (Fujifilm, Tokyo, Japan) with 473 nm excitation and 520 nm emission. The scanned spot image was analyzed with ArrayGauge Ver.2.0 (Fujifilm), and the fluorescence intensity of each spot was calibrated. Each array was designed to contain triplet spots, and two duplicate experiments were averaged as the data. The averaged fluorescence intensity of each sequence was normalized by subtracting the fluorescence intensity of the same sequence without the addition of the first antibody.

Scanning Electron Microscope (SEM) Analysis

Cells were treated according to the cell assay protocol described for the PIASPAC method, and the cells on the peptide disks were fixed with 4% glutaraldehyde (Wako Pure Chemical Industries) for 12 h at 4 °C. After further fixation with osmium tetroxide (PGM Chemicals (Pvt) Ltd., New Germany, USA) for 30 min at room temperature, samples were dried with t-butylalcohol (Wako Pure Chemical Industries) using a VFD-20 drying apparatus (Hitachi Ltd., Tokyo, Japan) and plasma coated with osmium tetroxide using an osmium plasma coater (Nihon Lazor Denshi, Ichinomiya, Japan). The SEM images were obtained using an S-800 electron microscope (Hitachi Ltd.).

Results

Comparing Cell Preference of Amino Acids in Cardiovascular Tissues

We compared three types of normal human cells (ECs, SMCs, and FBs) that contribute to cardiovascular tissues to investigate the cell-specific preference of particular amino acids, which may determine the effect of ECM on specific cells (Figure 1). In evaluating cell adhesion (with 1 h incubation), an amino acid repeat number more than five provided a relative cell-specific preference (Figure 2(A),

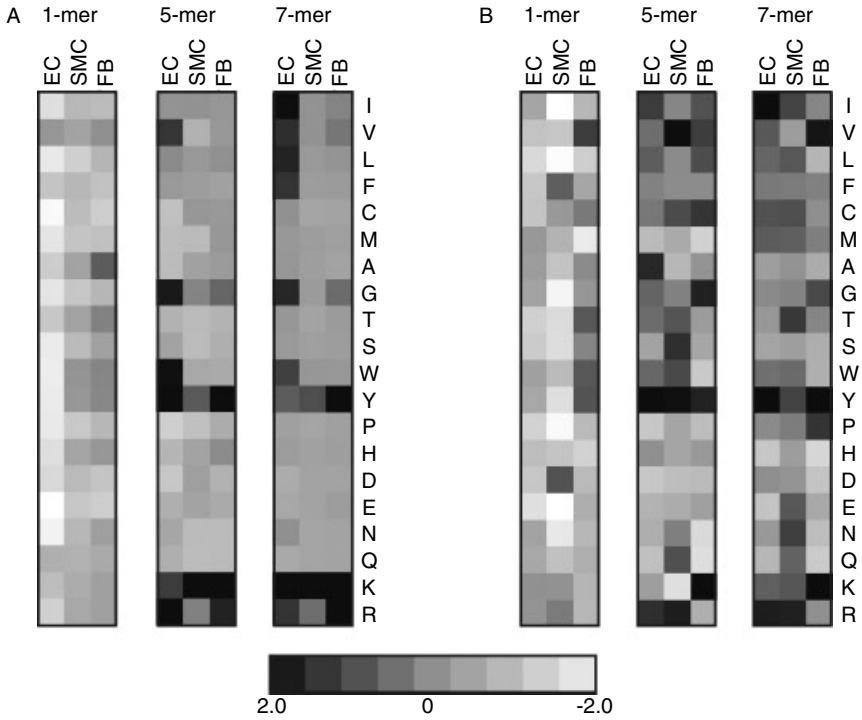

Figure 2. The heat map of cell adhesion and proliferation on simple repeats of 20 kinds of amino acids. The intensity of cell adhesion (A) and proliferation (B) were determined for each cell and each peptide and indicated as gradation of colors. (A) Each cell was seeded on the each peptide array spot and incubated for 1 h. Arrays were then washed three times and stained with calcein AM for fluorescent detection. Signal was measured by a fluorescent plate reader at Ex485/Em538. (B) Seeded cells were incubated for 3 days and stained by calcein AM. Samples were then washed and measured in the same way as for the adhesion assay. The values were normalized in each cell type. Black color indicates high adhesion (+2.0) and white color indicates low adhesion (−2.0).

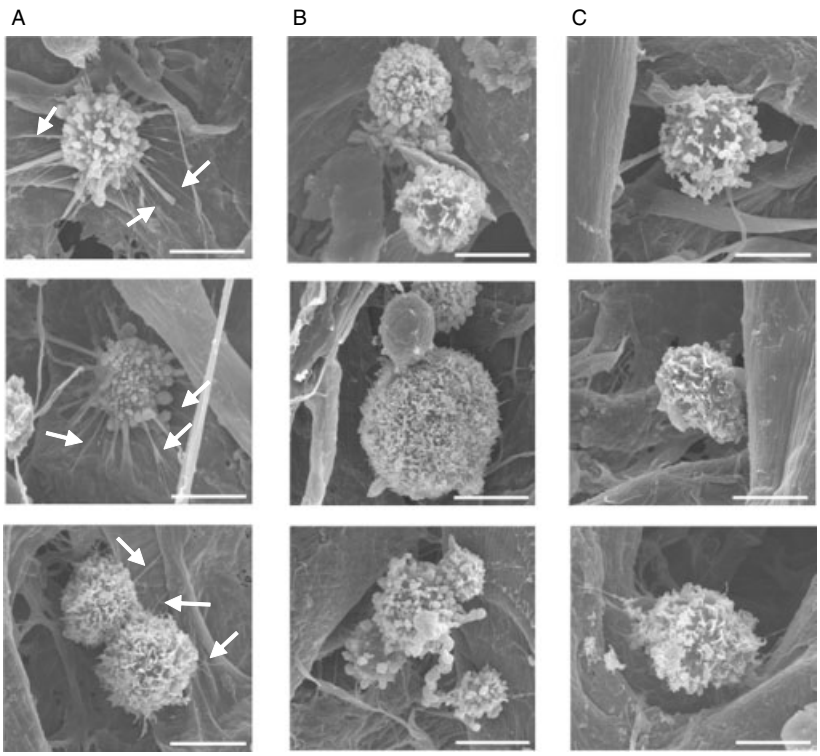

Figure 3. SEM image of cells on EC-specific peptide (hepta-Ile). SEM images (three fields of views) of morphology after 1 h of cell adhesion are shown. (A) ECs, (B) SMCs, and (C) FBs. All images are in the same magnification (scale bar is 10 μm). The large fibrous three-dimensional background is due to the cellulose support of SPOT array. Arrows indicate filopodias or ECM fibers from cells.

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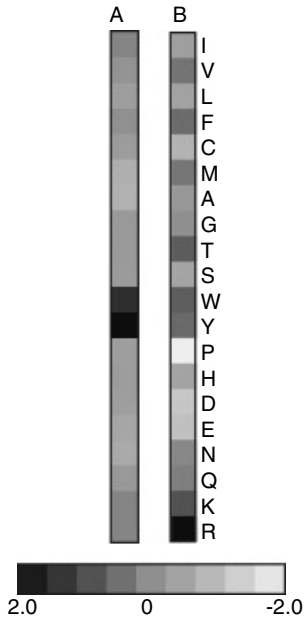

Figure 4. Heat map of ECM molecule binding to 20 kinds of amino acids (7-mer). Interaction with (A) fibronectin and (B) vitronectin. The peptide array was incubated with the medium containing 10% serum for 1 h. Arrays were then hybridized by anti-rabbit human fibronectin IgG or anti-rabbit human vitronectin IgG for 2 h then hybridized with anti-rabbit IgG-conjugated Alexa 488 for 1 h. Arrays were scanned by FLA-7000 (Ex473/Em520). The fluorescent intensity values were firstly subtracted from the array results with ECM protein and second antibody (without first antibody), and normalized for each ECM protein and illustrated as Figure 2.

see for detailed data in Figure S1 and Table S1, Supporting information).

In particular, longer repeats of hydrophobic amino acids (isoleucine, valine, leucine, and phenylalanine) were found to contribute to enhance EC adhesion compared to the other two cell types, especially peptides with seven repeated steps of elongation. Isoleucine, valine, and leucine contributed proportionally to promote adhesion of ECs, indicating a stronger effect with shorter repeats. In contrast, two positively charged amino acids (arginine and lysine), which are conventionally considered to have cell adhesion properties, were found to be too universal to control specific cellular organization. But the positively charged amino acid histidine demonstrated no preference between these three cell types. And two negatively charged amino acids (asparatic acid and glutamic acid), which are considered not to have cell adhesion properties, were found to be nonadhesion properties.

Figure 2(B) indicates the proliferation rates (after a 3-day incubation) on the different amino acids (detailed data in Figure S2 and Table S1, Supporting information). This result also shows that the residues, such as charged residues, previously shown to have cell adhesion properties indicate no preference for cell type, but that isoleucine has a preference for enhancing ECs and valine has a preference for enhancing FBs. In spite of the wide inhibitory preferences of SMCs, such as hydrophobic amino acids, enhancive preference of SMCs was not clear. These results suggest that the amino acid preference for SMCs is largely different than that of ECs and FBs. Throughout the experiment, preference data from peptides synthesized by less than five repeated elongation steps were found to have larger standard error (ECs on three repeated elongation steps: 21.5% of average; SMCs on three repeated elongation steps: 20.2% of average; FBs on three repeated elongation steps: 59.1% of average) (Figures S1 and S2; 3-mer data not shown). However, a similar tendency was also clearly observed in the short proliferation assay (1 day) (data not shown); therefore, such an amino acid preference effect could be firm with longer peptides. In this aspect, we focused the comparison between 1-mer and longer peptides.

SEM Analysis of Cell Morphology on Preferred Amino Acids

To investigate the detailed effect on the cells of particular amino acids, cell morphology was monitored by SEM (Figure 3). The hepta-Ile (array spot with seven repeated steps of elongation with Ile) was chosen as the best EC-specific peptide. On hepta-Ile, relatively high numbers of adherent ECs were observed compared to other cells (Figure 3(A–C)). Filopodias and fibers of ECM from ECs were found on hepta-Ile (arrows indicated) than on both the negative controls (other cell types on the same peptide disk), indicating that ECs prefer the peptide-coated surface for adhesion (Figure 3(A)). This result supports that the biological effect is triggered by cell-specific preference on such preference amino acids.

Involvement of Serum-Derived ECM Proteins in Cellular Adhesion Preferences

Each of the PIASPAC assays described above was carried out in serum- or serum-related supplement-containing medium to mimic the natural cellular *in vivo* conditions. However, in these assays, the cellular preference for amino acids could be explained by a dominant effect of the amino acid itself, of the serum-derived proteins that accumulate on amino acids, or both. Therefore, we examined the accumulation rate of FN and VN, two of the major ECM proteins that affect cell adhesion and proliferation, on amino acid-repeated sequences on the peptide arrays. FN was found to accumulate on tyrosine (Figure 4(A), see for detailed data in Figures S3 and S5(A)), residues that contain aromatic side chains. VN was found to accumulate on lysine and arginine, positively charged residues (Figure 4(B), see for detailed data in Figures S4 and S5(B)). These three amino acids were the universal cell-attracting residues (i.e. no cellular preference) (Figure 2). Although our hybridization scheme cannot deny the probability to detect false-positive signal from the nonspecific accumulation of primary antibody to high density peptides, the found cell-specific amino acids (such as Ile and Val) did not show ECM protein accumulation. Therefore, we concluded that any dominant effect of serum-derived proteins on the cell preference of identified amino acids is unlikely.

Confirmation of Controlling Cell Adhesion and Proliferation by Designed Cell-Specific Peptides

To further confirm the possibility of cell-specific organization controlled by amino acid preferences, we newly designed peptides consisting of selected amino acids that indicated cellular specificities (Figure 5, see for detailed data in Figure S6). To design 30 peptides, 9 amino acids were selected to represent three categories; (category 1) inhibitory peptides without cell specificity (aspartic acid and glutamic acid), (category 2) enhancive peptides without cell specificity (lysine, arginine, and tyrosine), and (category 3) enhancive peptides with EC-specificity (phenylalanine, isoleucine, and leucine). In each category, amino acids were randomly selected to build 7-mer peptides. Interestingly, EC-specificity could be designed by the combination of any type of EC-specific amino acids. With

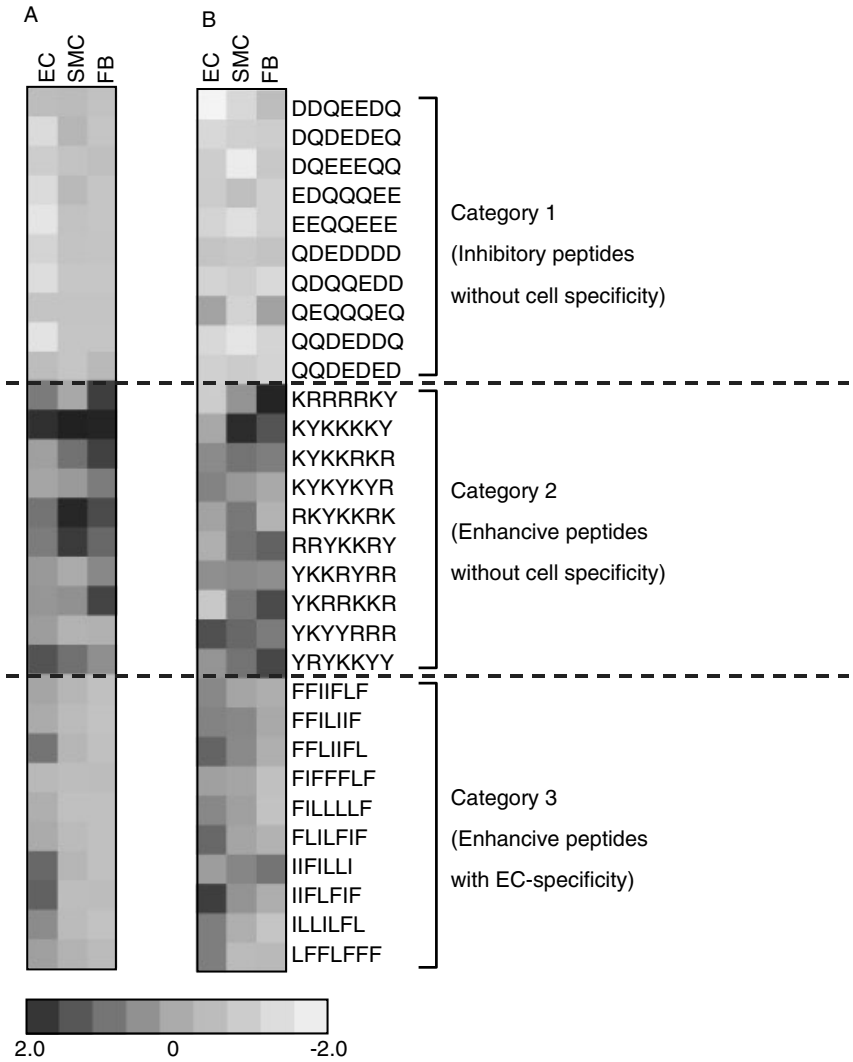

Figure 5. Heat map of cell adhesion and proliferation on random 7-mer sequences consist of selected amino acids. The following nine amino acids were selected to represent three categories: (i) inhibitory peptides without cell specificity (Asp, Glu, and Gln), (ii) enhancive peptides without cell specificity (Lys, Arg, and Tyr), and (iii) enhancive peptides with EC-specificity (Phe, Ile, and Leu). The intensity of cell adhesion and proliferation were determined and illustrated as Figure 2.

nonspecific amino acids, clear change in the cell-specific effect was not observed by combinations. Such results indicate that such simple physicochemical properties of amino acids have the potential to control the cellular organization.

Discussion

In this study, we reported for the first time the cell-specific preferences of particular amino acids in adhesion and proliferation by comparing three types of typical cardiovascular cells. We examined the cell preferences of simple repeats of amino acids to investigate our hypothesis that the ECM functions to control cellular self-organization *in vivo* and that this process would be controlled not only by ligand-specific rare domains (such as RGD) but also by the physicochemical properties of the surface environment provided by the ECM proteins. To assay these cell-specific preferences in peptide interactions in a combinatorial manner, we utilized our PIASPAC method, the application of SPOT array to directly assay cell adhesion and proliferation.

From the cell preference assay for adhesion and proliferation, we found that there are largely two types of cell preference in cardiovascular cells; ECs and FBs prefer repeats of hydrophobic residues, and SMCs have less of a preference for adhesion but prefer repeats of aromatic residues for proliferation (Figure 2). It was also found that the amino acids that contributed to cell-specific adhesion also contributed to proliferation. Because no single amino acid was preferred by particular cell, we concluded that domain-like physicochemical properties are more important for cell preference than the exact residue. Such amino acid preference was also confirmed with an assay of other random sequence peptides, which consisted of EC-specific amino acids (Figure 5). We also confirmed that the nonspecific preference of aromatic side chains amino acids and (tyrosine) positively charged amino acids (lysine and arginine) are probably due to the attracted serum-derived proteins (FN and VN) on these peptides (Figure 4). These observations support our hypothesis that the control of cell-specific organization can be maintained by physicochemical-

based affinities that accept broad candidate molecules that form domain-like property on the surface of ECM, rather than sequence-based affinities, such as ligand-receptor interactions.

We also found several simple amino acid effects that control cellular organization: longer elongation of isoleucine attracted ECs and eliminates SMCs and FBs (Figure 3). The introduction of isoleucine also contributed to provide EC-specific effect on random sequences of peptides (Figure 5). Because these amino acid preferences were assayed using serum-containing medium, peptides containing these amino acids could be promising practical candidates to enhance proper cell organization on medical devices and products. However, it should be noted that the more the elongation step increases, the more the impurity of peptide spot would appear in some amino acids by insufficient synthesis. In this aspect, our 7-mer peptide spots may include fewer percentages of perfect 7-mer repeats with some types of amino acids. Combining the facts that our spots consist of single amino acid (produce no mismatch sequences) and our data has high reproducibility (Figures S1 and S2), we consider that there are accumulative effect of amino acids on determining cell-specific preferences.

If physicochemical-based affinity can control the cell-specific organization, a rule based on amino acid indices should support the design of artificial molecules or polymers for medical device coatings. To extract the physicochemical rule to design cell-specific peptides, we analyzed the total data (20 peptides of repeated amino acids and 30 peptides of randomly selected amino acids) by classification and regression tree (CART) (Table S1). CART analysis automatically calculates the combination of physiochemical variables, as opposed to manually interpreted by a researcher, to obtain the final classification model. In other words, the selected variable from such analysis reflect the exhaustive consideration of all possible combinations of candidate physicochemical properties for the best classification. For the classification, we divided our data in three categories (Table S1) (i) enhancive peptides without cell specificity (ALL_Enh), (ii) inhibitory peptides without cell specificity (ALL_Inh), and (iii) enhancive peptides with EC-specificity (EC_SP). To classify the three categories of peptides, 13 amino acid indices (Table S2) [26–36] from AAindex1 (http://www.genome.ad.jp/dbget-bin/www_bfind?aaindex) [37] were examined by CART for best parameter combination. By this objective analysis, we found that the isoelectric point (threshold $= -4.418$) is the primary property that classifies 'adhesive peptides' and 'nonadhesive peptides'. The result also indicated that the combination of isoelectric point (>-4.418) and the hydropathy ($>+3.241$) was found to be a defining characteristic of EC-preferred structures (Figure S7). Although such analysis is still limited in our achieved data, such interpreted rule would characterize the surface physicochemical property to control the cell-specific organization on medical devices. The concept of using a medical device coating material to enhance proper cell organization could be important for overcoming the contradictory effects of endothelialization and stenosis.

Whether the minimum functional molecule for providing physicochemical-based affinities for cell self-organization is 'domain consists of amino acids' or 'domain consists of peptides' is still not clear. Therefore, further investigation is needed to understand the mechanism of controlling cellular organization for next-generation medical device coating.

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Supporting information

Supporting information may be found in the online version of this article.

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