The Use of Elastin Mimetic Peptides and Cell Attachment Motifs to Improve Angiogenesis

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ABSTRACT

The composition of the extracellular matrix (ECM) is an important determinant of the extent of cell growth and function in all tissues. Various components in the extracellular matrix are involved in regulating cell attachment, proliferation and proper functioning. These are crucial processes in the process of angiogenesis, where endothelial cells interact with the extracellular matrix of blood vessels to function effectively. In addition to these functions, the two main components in the ECM of blood vessels, collagen and elastin, also provide structural support. This paper first studies the ability of elastin mimetic peptides to promote these processes. Then the effect of addition of a cell attachment peptide sequence, such as RDGS, in improving these processes is examined. Confocal microscopy is used to observe cell morphology and proliferation on surfaces coated with the different peptides. These results demonstrate the improvement in cell adhesion and proliferation in the presence of the RGDS sequence. Finally, the possibility of creating better scaffolds by incorporating both collagen and elastin is considered.

INTRODUCTION

Angiogenesis is the formation of new capillaries from pre-existing blood vessels. It is a complex process involving a coordinated sequence of humoral and cellular interactions (Laschke, 2006). This process is vital for vascular remodeling and maturation (Robinet, 2004). It would be beneficial to be able to stimulate the formation of blood vessels in-vitro. This can also allow rapid vascularization of artificially engineered tissues and thus ensure its long term survival and function.

This first requires an understanding of the structural composition of blood vessels. The walls of blood vessels can be divided into three layers: the intima, composed of a layer of endothelial cells, the media, containing smooth muscle fibres and elastic connective tissue, and the adventia, composed of elastic and collagenous connective tissue. The composition and the sizes of each layer vary for the different types of blood vessels.

In the blood vessels, collagen provides strength and support. Elastin, made by linking tropoelastin molecules, also serves a crucial function. It provides elasticity to the walls and allows elastic recoil after the passage of blood through the vessel at high pressure. Collagen and elastin make up the connective tissue of the blood vessels, which serves as a scaffold for the endothelial cells and imparts mechanical properties to the blood vessels that allow them to withstand the stresses experienced during blood flow.

In addition to its structural function, collagen also influences cell adhesion, cell proliferation, cell-cell and cell-ECM communication, and differentiation (Khew, 2007), indicating its
usefulness as a scaffold for tissue engineering purposes. Use of animal-derived collagen has several complications associated with it such as host tissue rejection, disease transmission, low purity, and poor reproducibility (Khew, 2007). Thus it has been attempted to mimic the structure and function of collagen by synthesizing collagen mimetic peptides. These peptides contain the repeating Gly-xxx-yyy sequences found in triple helical collagen as well as GFOGER, which has been identified as the major integrin-receptor binding locus within type I collagen. Integrins recognize and bind to this sequence, enabling cell adhesion and proliferation (Khew, 2007). Previous studies have successfully used such synthetic peptides to achieve substantial cell adhesion and proliferation of hepatocytes and fibroblasts (Khew, 2007).

Other studies have also shown that elastin derived peptides can influence cell migration and proliferation in blood vessels (Robinet, 2004). These properties have been attributed to the VGVAPG hexapeptide sequence, commonly found in tropoelastin. However these peptides lack a cell attachment motif. In this regard, an elastic mimetic peptide with such a motif (such as RGDS) can lead to improved cell adhesion and proliferation. These peptides could be incorporated into a scaffold along with collagen. Such an environment closely mimicking the in-vivo conditions can promote effective angiogenesis.

**MATERIALS AND METHODS**

**Peptide synthesis**

All peptides shown in Table 1 were synthesized on an automated Multipep peptide synthesizer. Fluorenylmethoxycarbonyl (Fmoc) – protected amino acids were used. The required amounts of amino acids and PyPob (the activator), as indicated by the software, were weighed. All amino acids, except Phe and Pro, were dissolved in dimethylformamide (DMF). The activator, PyPob was also dissolved in DMF. Fmoc-Phe and Fmoc-Pro were dissolved in 1-methyl-2-pyrrolidone (NMP).

Piperidine (30% v/v), and N-methylmorpholine (NMM) (45% v/v), used as the base, were both prepared in DMF and poured into their designated containers. The required amount of pure 1-methyl-2-pyrrolidone (NMP), as indicated by the software was also poured into its container. The first peptide was assembled on Fmoc-Ser-Wang resin, the second on Fmoc-Gly-Wang resin, and the third on Fmoc-Pro-Wang resin. The resins were placed in syringes and washed twice with DMF. Peptide synthesis was carried out by a stepwise double coupling method, with each coupling reaction allowed to proceed for 30 minutes at room temperature.

**Table 1. Three different peptides synthesized.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tr>
<td>Elastin Peptide 1 (EP1)</td>
<td>GGGGVGVAPGVGVAPGVGVAPGRGDS</td>
</tr>
<tr>
<td>Elastin Peptide 2 (EP2)</td>
<td>GGGGVGVAPGVGVAPGVGVAPG</td>
</tr>
<tr>
<td>Short Peptide 1 (SP1)</td>
<td>GRGDSP</td>
</tr>
</tbody>
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The Fmoc protection group was removed with 30% (v/v) piperidine in DMF for 15 minutes twice. The resin was then washed in DMF 4 times. Cycles consisting of deprotection, washing, double couplings, and washing were repeated till the desired sequence was obtained. After the
peptide synthesis was completed, the peptides were washed twice with DMF and placed in a vacuum dry oven for a day.

After the peptides are dried, the resin group was cleaved. First, a cocktail solution, composed of 95% trifluoroacetic acid (TFA), 2.5% deionised water and 2.5% triisopropylsilane, was prepared. 3 ml of the cocktail solution was added to each dried peptide and placed in a rotating mixer for 3 hours. Cold tert-butyl methyl ether was prepared in a tube and placed in a -20°C fridge.

After 3 hours, the dissolved peptide was filtered into cold ether using a syringe. The resin remained in the syringe. This solution was centrifuged at 4°C and 7500 rpm for 30 minutes. The ether was poured off, and the tube placed in a pressurized oven for 3 days. The peptides were freeze-dried after they were removed from the oven. The peptides were dissolved completely in 40mL of ultrapure water each and stored at -20°C for 30 minutes. The peptides were then dried by placing them in the lyophiliser machine.

The peptides were purified by HPLC. An Agilent Zorbax 300SB-C18 reverse phase column (5 µm particle size, 300Å pore size, 25 x 1.0 cm) was used. Two buffer solutions were used: buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile). Method file was selected from the software, the flow rate was set to 4 ml/min and the injection volume to 50µl. A linear gradient of 10% of B to 45% of B in 30 minutes was used. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy performed on a Bruker AutoFlex II MALDI-TOF mass spectroscope, along with analytical reverse phase HPLC confirmed the purity of the peptides to be greater than 95%.

**Cell culture**

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell growth medium (ECGM) supplemented with 0.4% (w/v) ECGS/H, 2% (v/v) FCS, 0.1 ng/ml EGF, 1ng/ml basic fibroblast growth factor (bFGF), 1 µg/ml hydrocortisone, 50 ng/ml amphotericin B and 50 µg/ml gentamicin.

**Confocal Microscopy**

Glass cover slips were placed in Nunclon Delta TC Microwell plates. They were then coated with 100µL of 50µg/mL solution of EP1, EP2, mixture of EP2 and SP1, and collagen at 4°C overnight. The blank was not treated with any peptide. This was followed by blocking with 100µL of 1% heat-denatured bovine serum albumin overnight and washing with PBS. Then 100µL of HUVEC cell suspension in serum-free ECGM was added and incubated for 2 days.

Following incubation, the media was aspirated completely. The cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature. The cover slips were then washed once with PBS, twice with a solution of PBS + 100mM NH₄Cl and finally once with PBS again. Then the samples were treated with 0.2% saponin and left standing for 15 mins. Blocking buffer was then added and allowed to incubate for 1 hour. Finally, each cover slip was washed four times with PBS. Confocal microscopy was then carried out to observe cell morphology and proliferation.

**RESULTS AND DISCUSSION**
MALDI-TOF mass spectroscopy

Figure 1. MALDI-TOF mass spectroscopy results for (A) Elastin peptide (EP) 1, (B) Elastin peptide (EP) 2, and (C) Short peptide (SP) 1.

As shown in the figure, distinct peaks were observed for all three peptides using MALDI-TOF mass spectroscopy. This indicates that the purity of all peptides is greater than 95%. Subsequently, the peptides were coated on glass cover slips and seeded with cells.

Confocal microscopy

The following page shows the images of samples obtained using confocal microscopy. Important indicators of the effectiveness of a scaffold are cell adhesion and proliferation. As such, confocal microscopy was performed to observe these factors. A cell morphology in which the cells are elongated due to the arrangement of the cytoskeleton demonstrates good cell adhesion. Further, a high cell density (also illustrated by the large number of nuclei), suggests significant cell proliferation.

As shown in the figure, elastin peptide containing the cell adhesion motif (EP1) supports extensive cell attachment and proliferation as compared to the negative control (blank). The morphology and cell density observed in Fig. 2b and 2c illustrate this positive effect of EP1 on the endothelial cell growth. When coated with the elastin peptide lacking the cell adhesion motif (EP2), however, cell attachment is poorer. This is illustrated by the near-spherical or non-elongated morphology of some cells (Fig 2d, 2e). Cell density also seems lower than that for EP1, suggesting poorer cell proliferation and spreading. When coated with a mixture of EP2 and SP1, cell adhesion and proliferation are comparable to that of EP1. A possible explanation could be that the short peptide promotes cell adhesion, while the elastin mimetic peptide subsequently allows significant cell proliferation. To better compare the two samples, namely EP1 and the mixture of EP2 and SP1, more quantitative assays such as cell adhesion assay, cell proliferation assay or total DNA quantification should be carried out.

The last sample shows cells seeded onto a surface coated with collagen protein alone. Being a vital component of the extracellular matrix, collagen would be expected to encourage considerable cell growth. The appearance of cells and the high cell density seen in Fig. 2g is in agreement with the expected outcome. This also suggests the importance of collagen in the ECM of blood vessels. Cell attachment is the initial process that occurs when cells are seeded onto a surface. It is thus vital for the success of a scaffold. In keeping with this explanation, the results demonstrate the importance of incorporating the cell attachment motif to engineer an effective scaffold.
Figure 2. Confocal microscopy images for different samples: (A) Blank (no peptides), (B) Elastin peptide (EP) 1 at 20x, (C) Elastin peptide (EP) 1 at 40x, (D) Elastin peptide 2 at 20x, (E)
Elastin peptide 2 at 40x, (F) Elastin peptide 2 and Short peptide 1 at 40x, and (G) Collagen at 20x. The nuclei are stained blue.

SCOPE FOR FURTHER INVESTIGATION

This paper examines the effect of an elastic mimetic peptide and a cell attachment motif on endothelial cell adhesion and proliferation. The results show that the cell attachment motif significantly improves both factors.

To create a more effective scaffold, both collagen and elastin can be incorporated as both play crucial roles in the functioning of blood vessels. This can be accomplished by coating the surface with both collagen mimetic peptides (containing the typical Gly-xxx-yyy and GFOGER sequences) and elastin mimetic peptides (with the RDGS sequence). Another option would be to produce a cryogel incorporating collagen in its structure. Elastin mimetic peptides including the cell attachment motif can be further immobilized within this porous 3D structure. The pores within the structure could allow better cell penetration. The three dimensional structure of the scaffold, along with the functional effects of collagen and elastin in encouraging cell growth, could substantially improve the process of angiogenesis and provide an opportunity to engineer blood vessels in-vitro.

REFERENCES


