SMOOTH MUSCLE CELL CULTURE IN MICROCHANNEL TOWARD CONSTRUCTION OF MULTILAYERED VASCULAR TISSUE IN MICRO-SCALE

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ABSTRACT

We here report a new culturing method to produce micro vascular-mimetic tissue consisting of smooth muscle cells (SMCs) and endothelial cells (ECs) toward the practical use of tissue engineering. To achieve the stable coculture, the requirements for SMC culture in microchannels are clarified by basic experiments. Then, a new microchip that satisfies those requirements was developed by bonding glass substrate and porous polycarbonate membranes. Finally, the stable coculture of SMC and ECs in confined micro-space was demonstrated for the first time. Those knowledge and new system would contribute not only to the vascular tissue engineering but also to the cellular engineering in micro-space.

KEYWORDS

Vascular tissue, Smooth muscle cell, Curvature

INTRODUCTION

Recent progress in tissue engineering has led to clinical successes for several functional tissues [1]. However, a major limitation remains a lack of methods to produce thick tissues due to the restrictions of passive diffusion of biomolecules. There is a need for micro-scale vascular tissues to construct large organs *in vitro*. We have previously reported the basic culture and recovery of ECs utilizing a separable microchip [2] and been investigating SMC culture condition [3]. Next, multilayered tissue consisting of SMCs and ECs should be constructed (Fig. 1). However, it is difficult to culture SMCs in microchannel because they are intrinsically stretch cells and there are still no reports. To achieve the concept, stable culture of SMCs in microchannel is necessary. In this present paper, the obstructive factors to SMC growth in closed microchannel were firstly identified by 3 basic experiments. Then, a new microchip that satisfied those requirements is designed and the stable coculture of SMCs and ECs in closed microchannels was demonstrated for the first time.

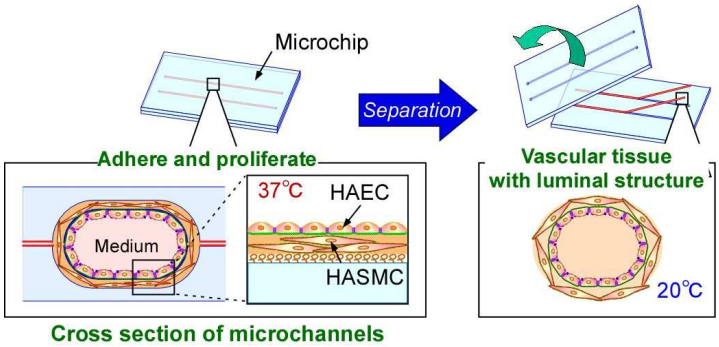

Figure 1: Total concept of this study. Multilayered SMCs and ECs are cultured in a microchannel. After cell culture, they are harvested separating the microchip.

IDENTIFICATION OF OBSTRUCTIVE FACTORS ON SMC GROWTH

Vascular SMCs are easily cultured on conventional cell culture dish, but difficult to be cultured to confluent state in closed glass microchannels. They are a kind of muscle cells in vascular wall and stretch along the long axis of spindle shape. So, larger metabolic requirement and growth inhibition by surrounding geometry are supposed to be severe problem in micro-scale culture compared to other cells. Cell culture procedure in the microchip is divided to adhering term and increasing term. In adhering term, suspended cells are introduced into the microchannel and allowed to adhere on the surface stopping the perfusion for 2 hours. After adhering term, medium is constantly perfused and cells increase to confluent state. In this report, According to this idea noted above, O_2 consumption, nutrients, and surrounding surface curvature were considered in each term.

Firstly, O_2 consumption in adhering term was tested. Glass-bottom glass microchip and glass-bottom PDMS microchip were prepared and the microchannels were coated by collagen. Then, human arterial SMCs (HASMCs) were loaded and the medium flow was completely stopped (Fig. 2a). After 2 hours, the survival rate was evaluated as the proportion of adhering cells to whole cells in microchannels. Fig 2b shows the significant difference of survival rate between two microchannels. While almost 100% of SMCs adhered and survived on the bottom of the PDMS microchannel, survival rate in the glass microchannel was about 65%. In this experiment, the difference between two conditions is only the O_2 permeability of microchannel walls. So this difference in cell adhesion is caused by O_2 supply. And this result showed HASMCs in glass microchannel were exposed to serious O_2 deficiency even in first 2 hours. On the other hand, almost 100% of survival rate in PDMS microchannel indicates that the amount of other nutrients would be efficient in this term.

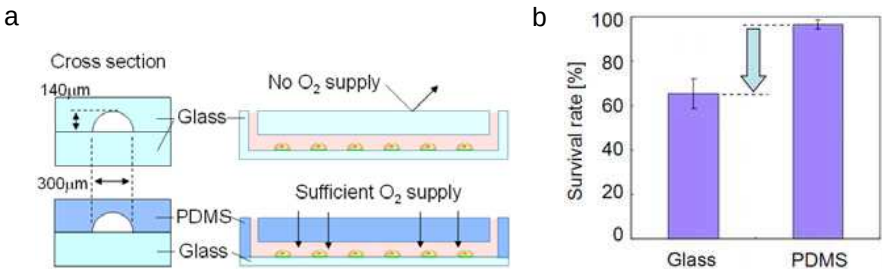

Figure 2: Investigation of O2 effect. (a) Schematic of experiment. (b) Survival rate of SMCs after 2hours from introduction.

Secondly, the medium supply in increasing term was tested in pseudo experiment. In the case of cell culuture in microchip, changing flow rate results in variable shear stress acting on adhering cells. To exclude the shear stress effect, this experiment was done in conventional cell culture dish. HASMCs were seeded at 5×10^4 cells/cm^2 and cultured with exchanging 4 mL of medium in different frequency (A: every 8 hours, B: every 16 hours, C: 24 hours, D: no exchange). Fig 3 shows the growth curves of HASMCs in each dish and no significant difference in inclination that means the increasing speed was observed. From this result, at least 4mL/day (C) of medium supply is enough to grow HASMCs. This amount of flow rate is roughly equivalent to 0.15 μL/min in microchannel whose diameter is 300μm and far less than that of normal cell culture (several μL/min). So it is confirmed that the conventional condition could have supplied enough nutrients to SMCs in microchannel, contrary to expectation. Although the amount of medium perfusion rate to supply enough nutrients could be roughly estimated, we could not discuss the O_2 supply, because these dishes are exposed to atmosphere throughout the experiment. So, O_2 deficiency in this term should be improved together with that in adhering term in some way.

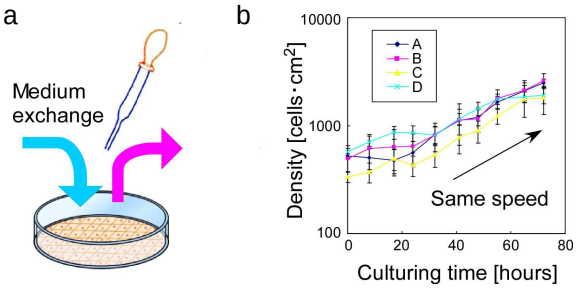

Figure 3: Investigation of medium supply (a) Schematic of experiment. (b) Growth curves of HASMCs in each dish.

At last, surface curvature of the substrate was tested. The microchannel in glass microchips has curving surface because of the fabrication by wet etching process. To check the effect of curvature on cellular growth, HASMCs were cultured on two kinds of microgrooves that had semicircular and rectangular cross-sections (Fig 3a). Although the seeding densities were same on all surfaces (Fig 3b), significant difference was showed in surface coverage between curving and flat surface after 120hours (Fig 3c). The same tendency was observed also on the PDMS substrates (data is not shown). Those results indicate that curvature does not affect on the adhesion of HASMCs but matters to the proliferation in increasing term. Some papers have reported the strange migration and unique metabolism of living cells on micro fabricated surface [4, 5]. However, the mechanism of suppression is still unclear and the detail should be investigated by further research.

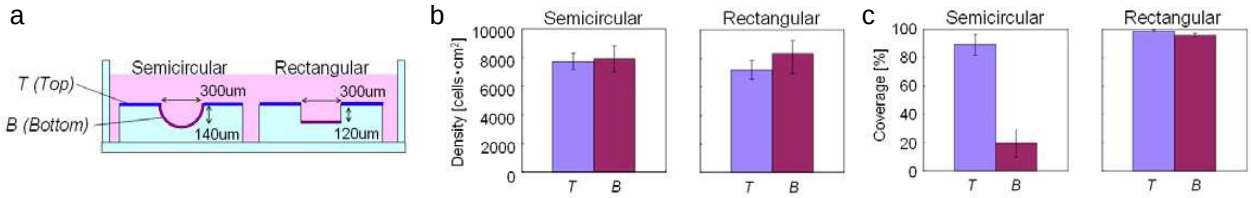

Figure 4: Investigation of curvature effect. (a) Schematic of substrates. (b) Adhering density of SMCs after 2hours from seeding. (c) Coverage of SMCs after 120 hours.

Throughout these experiments, we concluded that (a) O_2 deficiency in both terms (b) curving surface in increasing term are the obstructive factors on the growth of HASMCs in closed microchannels.

SMC CULTURE IN A NEW MICROCHIP

To achieve the stable culture of HASMCs, a new microchip was developed. To exclude O_2 deficiency and growth suppression by curvature, new microchip was prepared sandwiching glass substrate with penetrating microchannel (width: 400μm, thickness: 700μm) by two porous polycarbonate membranes applying low temperature bonding technique (Fig. 4). Briefly, a glass substrate was cleaned by piranha solution. In parallel, two sheets of porous PC membrane were prepared and modified with Aminopropyltriethoxysilane (APTES). Then, both sides of the glass substrate were contacted to APTES-modified PC membranes and bonded by heating at 110℃ for 12 hours with applying 100N of pressure. This microchannel has all flat surfaces and efficient O_2 is supplied from the outside. Then, it was connected to syringe pump and dipped into the medium.

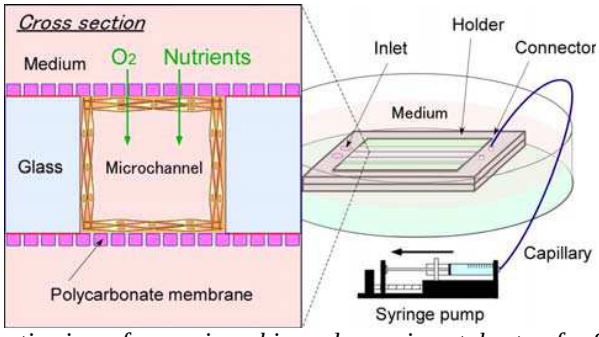

Figure 5: Schematic view of new microchip and experimental setup for SMC culture.

After coating the collagen onto the microchannel, HASMCs were loaded at 7×10^6cells/mL and the perfusion is stopped for 2hours. After cell adhesion, the medium was perfused at 2μL/min. After 48hours, HASMCs reached to confluent state (Fig). Next, human arterial endothelial cells (HAECs) at 5×10^6cells/mL were introduced into the microchannel and the perfusion was stopped for 2hours. After adhesion of HAECs, the medium was perfused again at 2μL/min. After 50 hours from the introduction of HAECs, they adhered onto the confluent HASMCs stiffly. Comparing to the previous result, it is obvious that the SMCs could be cultured successfully. This is the first demonstration of coculture of SMCs and ECs in microchannel and would provide significant guides for vascular tissue engineering and indicates that the coculture of SMC and EC in the microchannel needs a new design of a separable microchip.

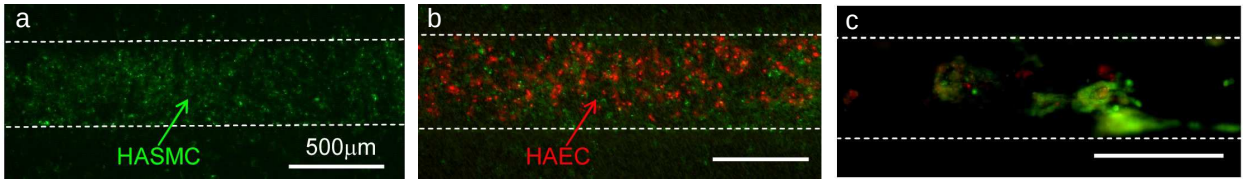

Figure 6: Cultured cells in the microchip (a) Confluent SMCs (green) after 50hours from seeding (b) Cocultured SMCs and ECs (red) after 50hours from EC seeding. (c) Previous result in the normal separable microchip: SMCs underwent necrosis after 15hours from EC seeding.

CONCLUSION

In this report, we clarified the obstructive factors for SMC culture in the closed microchannels. O_2 deficiency in the adhering term and growth suppression by curving surface in increasing term were the critical problems for SMC proliferation. Then, a new microchip that solved those problems was developed and the successful coculture of SMCs and ECs were demonstrated for the first time in the microchannel. Those knowledge and technique would contribute to the cellular engineering in micro-space including micro vascular tissue engineering.

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REFERENCES

[1] J. Yang, M. Yamato, T. Shimizu, H. Sekine, K. Ohashi, M. Kanzaki, T. Ohki, K. Nishida and T. Okano, *Reconstruction of functional tissues with cell sheet engineering*, Biomaterials, pp. 5033-5043, (2007).
[2] T. Yamashita, Y. Tanaka, N. Idota, K. Sato, K. Mawatari and T. Kitamori, *Cultivation and recovery of vascular endothelial cells in microchannels of a separable micro-chemical chip*, Biomaterials, pp. 2459-2465, (2011).
[3] T. Yamashita, Y. Tanaka, Y. Sugii, K. Mawatari and T. Kitamori, *Construction of vascular-mimetic tissue in a separable microchip*, Proc. Micro Total Analysis Systems 2010, Groningen, The Netherlands, pp. 1316-1318, (2010).
[4] JY. Park, DH. Lee, EJ. Lee, SH. Lee, *Study of cellular behaviors on concave and convex microstructures fabricated from elastic PDMS membranes*, Lab Chip, pp. 2043-2049, (2009)
[5] M. Ochsner, M. Textor, V. Vogel and ML. Smith, *Dimensionality controls cytoskeleton assembly and metabolism of fibroblast cells in response to rigidity and shape*. PloS One, 5, pp. e9445, (2010).

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