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論文の内容の要旨

Tissue engineering has been emerged as a promising alternative approach to regenerate functional tissues and organs. In general, three factors are necessary for tissue engineering. There are cells, scaffolds and growth factors. Porous scaffolds serve as a platform to support cell adhesion, to control cell distribution, to promote cell proliferation and to guide the tissue formation. Ideally, the scaffolds should provide the same topographical cues and biological cues as those of the *in vivo* microenvironments that surround cells. These cues that provide guidance are especially important for the formation of tissues and organs, for example, blood vessels and nerves, because of their network structures.

Guided formation of blood vessel networks and neuronal networks has been investigated recently using various micropatterned materials and substrata. Both topographical cues and biological cues have been used in micropatterned forms to guide blood vessel network formation in a controlled manner. Porous poly-caprolactone scaffolds with micropatterned grooves have been prepared using a mechanical heat press with a prepared polydimethylsiloxane micropattern mold. The micropatterned grooves led to the guided alignment of vascular smooth muscle cells. It has been discovered that biological molecules have an effect on stimulating the regeneration of tissues such as blood vessel networks and nerves. The biological cues include various biological molecules such as vascular endothelial growth factor (VEGF), nerve growth factor (NGF), fibronectin and arginine-glycine-aspartic acid-serine peptide (RGDS). Micropatterning of the biological molecules in a specific structure has been studied, to manipulate the formation of blood vessel networks and nerves in a controlled manner. By photolithographic and microcontact printing methods, a substantial number of studies have been performed to investigate cell adhesion, proliferation and differentiation on two-dimensional substrates that are micropatterned with various biological molecules. However, cell behavior on two-dimensional substrates cannot explain the real cell behavior in our body because the cells in our body are surrounded by a three-dimensional microenvironment. Spatial micropatterning of biological molecules in three-dimensional porous scaffolds is strongly desirable for the guidance of capillary network and neuronal network formation.

In this study, we at first introduced the micropatterned structures of VEGF in a mesh scaffold of poly (D,L-lactic-co-glycolic acid) (PLGA) to study the guidance effect of micropatterned VEGF. And then we developed a novel method to micropattern biological molecules in porous collagen scaffolds. The micropatterns can tailored by designing a program. Any single biological molecules and their combination can be micropatterned in collagen porous scaffolds by this method. VEGF- and NGF-micropatterned collagen sponges were used to study their guidance effects on network formation of capillary and neuritis.

(1). Guided angiogenesis induced by mesh-structured scaffolds

To confirm whether it is possible to guide the formation of a micropatterned capillary network, we used a mesh-type scaffold to carry VEGF in a micropatterned form. The micropatterned scaffold with VEGF was subcutaneously implanted in nude mice to determine the effect on the micropatterned angiogenesis process.

Four types of scaffolds were prepared: a PLGA mesh disc, a PLGA mesh embedded with VEGF-containing collagen hydrogel (VEGF-embedded mesh), a PLGA mesh coated with VEGF-containing collagen hydrogel (VEGF-coated mesh) and a PLGA mesh coated with collagen hydrogel (collagen-coated mesh). The PLGA mesh discs of a diameter of 10 mm were sterilized by immersing in a 70% ethanol/water solution for 30 minutes and then washed with sterile MilliQ water. These sterile PLGA mesh discs were used as a control. The other types of mesh scaffolds were prepared from the PLGA mesh discs under sterile conditions. The VEGF-embedded mesh was prepared by introducing VEGF-containing collagen hydrogel into the openings of the PLGA mesh discs. VEGF solution (5 μL, 1 μg/μL,) was added to cold 2X PBS (495 μL) and homogenously mixed. A 2 % collagen solution (500 μL) was added to the 500 μL VEGF/PBS solution, while on ice and homogeneously mixed. PLGA mesh discs were immersed in the collagen/VEGF/PBS solution while on ice for 5 minutes and then removed from the collagen/VEGF/PBS solution and placed in an incubator at 37°C for 2 hours to achieve gelation. Following gelation, the VEGF-embedded PLGA meshes were prepared. The VEGF-coated mesh was prepared by coating VEGF-containing collagen hydrogel onto the PLGA multifilaments within the mesh but not within the openings of the mesh. The PLGA mesh discs were immersed in the collagen/VEGF/PBS solution while on ice for 5 minutes. The mesh discs were removed from the collagen/VEGF/PBS solution, and the collagen/VEGF/PBS solution that was present in the openings of the mesh was removed by an air sprayer. Subsequently, the mesh discs that were coated with the mixture solution were placed in an incubator at 37°C for 2 hours to achieve gelation. Following gelation, the VEGF-coated PLGA meshes were prepared. The collagen-coated mesh was prepared by coating collagen hydrogel onto the PLGA multifilaments within the mesh. The same procedure was used here as was used for the preparation of the VEGF-coated PLGA mesh, without VEGF. Observations of the optical microscope and SEM showed that hydrogel had formed in the whole of the mesh and that hydrogel had formed on the PLGA multifilaments of the VEGF-coated mesh and the collagen-coated mesh. The presence of VEGF in the scaffolds was confirmed after freeze-drying and corsslinking. The incorporated VEGF in scaffolds was stained by immunological staining with anti-VEGF antibody. Positive staining was observed in the whole regions of the VEGF-embedded mesh and on the PLGA multifilaments for the VEGF-coated mesh. However, the collagen-coated mesh and the PLGA mesh were not positively stained with anti-VEGF antibody. After implantation, all scaffolds were compared in terms of their effectiveness in inducing the formation of a micropatterned blood vessel network. Micropatterned blood vessel networks were induced along the microstructure of PLGA mesh in all scaffolds after 2 w and 6 w. The micropatterned networks of blood vessel were vaguely generated 2 w after implantation but became distinctly evident 6 w after implantation. The formation of a micropatterned blood vessel network in all of the scaffolds suggests that the PLGA mesh may play an important role in inducing the formation of a micropatterned blood vessel network. It has been reported that biodegradable polymer such as PLGA, can recruit inflammatory cells and endothelial cells after

implantation, resulting in the migration of endothelial cells from existing blood vessel and formation of blood vessels in the peripheral areas surrounding the polymer. The results of immunohistological staining of endothelial cell and microphage demonstrated that the generated capillaries and microphages were formed in the peripheral areas of PLGA multifilaments as like previously reported researches. Independent of the effect on the PLGA mesh, incorporated VEGF in hydrogel elicited the promoted blood vessel generation, and higher blood vessel density than scaffold without VEGF. This research demonstrated the possibility of a micropatterned blood vessel network formation by combining VEGF with structured scaffolds.

(2). Preparation of collagen porous scaffolds with micropatterned structures of biological molecules

A novel method was developed to fabricate micropatterned structures of biological molecules in three-dimensional collagen porous scaffolds by using a dispensing machine. This method allows for precise control of micropattern structures and combination of a few types of biological biomolecules. And it provides a porous structure for easy cell seeding. The method includes three steps. At first, micropatterns of frozen lines of mixture solution of collagen and biological molecules were prepared by a dispensing machine. Collagen aqueous solution (475 μL, 1.0 wt%) was mixed with VEGF, NGF and fibronectin (FN) to make the mixture solutions. The mixture aqueous solution was added in a dispensing machine and ejected through the nozzle on a perfluoroalkoxy (PFA) film-wrapped copper plate that was cooled by a circulation cooler set to -20 °C. The prepared ice micropatterns of was further frozen in a freezer (-30 °C) for 1 h, and kept in a -5 °C low temperature chamber for 1h for temperature balance. Subsequently, the ice micropatternes were covered with collagen aqueous solution (1.0 wt%) that was pre-cooled at -1 °C. The surface of the applied collagen solution was spread to form a flat surface. The set of ice micropatterns covered with collagen solution was were kept at -5 °C to freeze the aqueous collagen solution. The frozen set of ice micropattern template and collagen was freeze-dried for 2 days in a freeze-dryer under a vacuum of 20 Pa. Finally, the freeze-dried collagen sponges were cross-linked by glutaraldehyde vapor that was saturated by 25 % aqueous glutaraldehyde solution. The prepared collagen sponge was washed three times with cold pure water and immersed in a 0.1 M glycine aqueous solution for 12 h to block non-reacted aldehyde groups. Control collagen sponges were prepared by using ice micropattern of collagen aqueous solution without supplement of other biological biomolecules.

The collagen sponges with or without micropatterned biological molecules showed similar gross appearance. Observation with SEM indicated that all the collagen sponges had semilar porous structures. Immunological staining showed that the miropatterned structures of VEGF, NGF and fibronectin were clearly observed in the collagen sponges micropatterned with these biological molecules. VEGF, NGF and fibronectin were immobilized in the collagen sponges in three different micropatterns, square, diamond and circle. The micropatterns can be designed according to requirement. The collagen sponge without these biological molecules was not positively stained. The biological molecules can also be co-immobilized in the collagen sponges with different micropattern structures. Stripe micropatterns of NGF and fibronectin were created in collagen sponge. Any micropattern combination of single or multiple biological molecules can be created by this method.

(3). Guided angiogenesis using VEGF-micropatterned collagen sponge

The VEGF-micropatterned collagen sponge was prepared by the above described method using a stripe micropattern program. The presence of VEGF in collagen sponge was confirmed by immunological staining using anti-VEGF antibody. The VEGF-micropatterned and control collagen sponges were subcutaneously implanted in mice to determine their utility for spatial guidance of angiogenesis. After 6 w, the implants were harvested, and the formation of blood vessels was evaluated. Gross examination showed that more blood vessels were formed in the VEGF-micropatterned collagen sponges than in the control collagen sponges. And the blood vessel network was generated in the VEGF-

micropatterned collagen sponges along the micropattern of the immobilized VEGF. In the control sponges, however, the blood vessel network was formed randomly. The cross-sections of the implants were further stained with an antibody for von Willebrand factor, which is a marker of endothelial cells. In the VEGF-micropatterned collagen sponges, more blood vessels were detected in the VEGF-micropatterned lines than in the regions without the lines and the whole regions of control collagen sponge. From the numbers of positively stained blood vessels, the blood vessel density was calculated in the VEGF-micropatterned regions and in the regions without the VEGF micropattern in collagen sponge. The significantly higher blood vessel density in the VEGF-micropatterned regions than in the regions without VEGF demonstrated the effect of immobilized VEGF. These results demonstrate that the immobilized VEGF promoted blood vessel formation and that the blood vessel network was regenerated according to the micropattern of immobilized VEGF.

(4) Guidance effect of NGF-micropatterned collagen sponge on differentiation of PC12 cells

Collagen sponge with micropatterned NGF stripes was prepared by the above described micropatterning method. The immobilized-NGF micropattern was determined by immunological staining. The NGF-micropatterned and control collagen sponges were used for PC 12 cell culture. PC12 cells are neuron-like cells that differentiate and extend neurites in the presence of NGF. After 7 days of culture in low serum condition, neurites and nuclei were stained. When PC12 cells were cultured in control collagen sponges with low serum conditions with NGF supplementation, PC12 cells expressed neurites outgrowth. However, PC12 cells were cultured in the control collagen sponges without NGF did not express obvious neurites. This result indicated that NGF is required for neurite outgrowth of PC12 cells in collagen sponge. When PC12 cells were cultured in the NGF-micropatterned collagen sponges, many neurites were observed in the collagen/NGF micropattern lines, but very few neurites were observed in the regions between the collagen/NGF micropattern lines. In addition, the percentages of neurite-bearing cells were significantly higher in the soluble NGF supplied and NGF-immobilized collagen sponges than control collagen sponge without soluble NGF supplement. These results indicated that the immobilized NGF in the micropattern kept its bioactivity of inducing neurite outgrowth of PC12 cells and the neurite network of the PC12 cells can be manipulated by controlling the micro pattern of the NGF.

In conclusion, the micropatterned growth factors in three-dimensional porous scaffolds were explored to reveal the availability of guided response *in vitro* and *in vivo* by using two micropatterning methods. In the first method by using PLGA mesh as template for micropatterning, capillary formation was guided along the mesh micropattern and higher capillary density in VEGF-incorporated PLGA meshes were confirmed. The PLGA mesh induced inflammatory response that also contributed to formation of micropatterned capillaries. The second method showed more controllable micropattern structures by using a dispensing machine. Biological molecules with any combination of composition and micropattern structures can be introduced and immobilized in three-dimensional collagen porous scaffolds. VEGF-micropattrend collagen sponges showed guided regeneration of capillary network after implantation. NGF-micropatterned collagen sponges stimulated neurite outgrowth of PC12 cells following the micropattern of the NGF. The micropatterning of growth factors in 3D scaffolds will be useful method to guided tissue regeneration and cell manipulation. The dispersing method will be useful for micropatterning of biological molecules in three-dimensional porous scaffolds in controllable micropattern structures. The micropatterned scaffolds will have a potential application for guided regeneration of tissues and oragns.

審 査 の 結 果 の 要 旨

血管や神経などの生体ネットワークの形成を誘導するために、細胞成長因子をパターン化した多孔質足場材料はきわめて有用であると考えられる。本研究では、血管内皮細胞増殖因子(VEGF)と神経成長因子(NGF)をパターン状に導入した多孔質足場材料を作製し、それを用いて、血管と神経のネットワークの形成を試みた。まず、乳酸とグリコールの共重合体（PLGA）のニットメッシュのネットワークにVEGF導入し、毛細血管の再生を調べた。ヌードマウスの皮下埋植実験より、毛細血管はPLGAニットメッシュのネットワークと本メッシュに導入したVEGFにしたがって、ネットワーク状のパターンを形成した。さらに、マイクロディスペンサーを用いてVEGFとNGFをパターン状に導入したコラーゲン多孔質材料の作製手法を確立した。本方法により、VEGFとNGFの任意のパターンを作製することができた。VEGFパターン化コラーゲン多孔質材料をヌードマウスの背中皮下に埋植し、毛細血管パターンの形成を観察した。NGFパターン化コラーゲン多孔質材料を用いてPC12細胞を培養し、神経突起パターンの形成を観察した。その結果、パターン化したVEGFにより毛細血管が再生され、パターン化したNGFによりPC12細胞の神経突起パターンの形成を誘導することができた。これらの結果により、細胞成長因子をパターン状に固定化した多孔質材料は血管や神経などのネットワークの形成にきわめて有用であることが示された。本研究で得られた成果は再生医療のための材料設計と開発に有用な知見を与え、本研究分野の学術的貢献に資することが期待される。よって、本論文は博士（工学）の学位論文として価値あるものと認める。

平成24年2月10日、数理物質科学研究科学位論文審査委員会において審査委員の全員出席のもと、著者に論文について説明を求め、関連事項につき質疑応答を行った。その結果、審査委員全員によって、合格と判定された。

上記の論文審査ならびに最終試験の結果に基づき、著者は博士（工学）の学位を受けるに十分な資格を有するものと認める。