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学 位 論 文 題 名

A Study on the Effect of Hydrogel Scaffolds on the Behavior of Mouse Embryonic Stem Cells

（ハイドロゲルの物性によるマウス胚性幹細胞の挙動に関する研究）

学位論文内容の要旨

Controlling the differentiation of mouse embryonic stem cells (mESCs) into various cells, usually initiated by embryoid bodies (EBs) formation, is challenging in regenerative medicine. It has been reported that synthetic hydrogel scaffolds with bioactivity have been utilized to direct differentiation of ES cells combining with other environmental and genetic influences. Differentiation of Embryoid bodies (EBs) into particular cell lineages has been extensively studied. There is an increasing interest in the effect of soft hydrogel scaffolds on the behavior of EBs, such as the initial adhesion, dynamic morphology change, and differentiation. In this study, without adding any other bioactive factors in the culture medium, dynamic behaviors of mouse EBs loaded on the surfaces of hydrogels with different surface charge and chemical structures are investigated. The results have significant implications in that the properties of scaffolds can affect the differentiation process of EBs.

Four kinds of synthetic hydrogel scaffolds with different chemical structures were synthesized by radical polymerization: two negatively charged hydrogels with sulfonic acid moieties, poly(sodium p-styrene sulfonate) (PNaSS) and poly (2-acrylamido-2-methyl-propane sulfonic acid sodium salt) (PNaAMPS), one positively charged poly (N,N'-Dimethylaminopropylacrylamide, methylchloride quarternary) (PDMAPAA-Q) gels, and one neutral hydrogels, poly (acrylamide) (PAAm) hydrogels. We also cultured ES cells and EBs on positively charged hydrogels with various cross-linker densities and charge densities. EBs and ES cells were loaded on these hydrogels and a gelatin-coated polystyrene (PS) and incubated in chemically defined ES culture medium without adding of Leukemia inhibitory factor (LIF), an inhibitor against differentiation. In order to evaluate the dynamic behavior of EBs, phase-contrast microscopic observations, microscopy data analysis and semi-quantitative RT-PCR were performed.

In **chapter 2**, EBs adhered quickly to negatively charged PNaSS hydrogels, which facilitates EBs spreading, migration, and differentiation into three germ layers with high efficiency of cardiomyocytes differentiation, similar to that on gelatin coated polystyrene (PS) culture plate. While on neutral PAAm hydrogels, EBs maintained the initial spherical morphology with high expression of pluripotency related markers in the short culture periods, and then showed the

significantly greater levels of selected endoderm markers after long time culture. EBs cultured on negatively charged PNaAMPS gels demonstrated the analogous behaviors with that of neutral PAAm gels at early differentiation phase (Day 4+1). Then their adhesion, spreading and differwasation were quite similar to that on negatively charged PNaSS gels. The correlation between surface properties of hydrogels and EBs differentiation was discussed.

In **chapter 3**, the cell morphology, gene expression, and surface properties of mES cells cultured on positively charged PDMAPAA-Q hydrogels with various levels of elasticity ranged in 8 kPa ~ 600 kPa were measured. We found that ES cells can survive on these positively charged hydrogels. ES cells can adhere and proliferate despite the change of elastic modulus of the substrates, which reached confluence on these hydrogels while retaining the similar expression of undifferentiation related markers. However, the cell-clustered morphologies were well observed on stiffer positive PDMAPAA-Q hydrogels than that on softer gels.

In **chapter 4**, in order to clarify the effects of positive charge and elastic modulus of substrates on EBs dynamic behaviors, EBs were also loaded on positively charged PDMAPAA-Q gels with various cross-linker densities. As a result, EBs can develop verified morphologies and demonstrate different gene expression profiles when the elastic modulus of the gels changed. It is obvious that the interaction between EBs and PDMAPAA-Q gels were higher and EBs polarity and migration were distinct with elastic modulus. As visualized by actin sress fiber, substrate stiffness influences adhesion structures and dynamics, cytoskeleton assembly and cell spreading.

In **chapter 5**, dynamic behaviors of EBs on copolymer gels of P(DMAPAA-Q-co-DMAAm) with various charge densities were investigated. The results showed that the charge density can adjust protein adsorption and then controlled the cell-adhesion to the surface of gels. On low-charge-density hydrogels (F=0.2), EBs adhered to the surface of scaffolds and then scattered partly in monolayer growth and partly in multilayer growth. On intermediate-charge-density hydrogels (F=0.4, 0.6), cells of EBs firstly scattered and expanded on the gel surface, then migrated collectively in specific directions instead of a random way. However, on high-charge-density hydrogels (F=0.8, 1.0), EBs did not adhere tightly to the surface of scaffolds, so they grew in the suspension-like state, maintaining spherical shape. These results indicated that intermediate and low-charge-density hydrogels may provide some guidance signals for enhancing cells movement.

In conclusion, neutral hydrogel culture systems were suitable for the study of cell-cell interaction. Negatively charged gels can promote ES cells proliferation and differentiation. Furthermore, positively charged PDMAPAA-Q gels can be used for the study of EBs morphogenesis. It is expected that these synthetic hydrogels with different properties can be used as excellent scaffolds in fundamental medical research and tissue engineering in the future.

学位論文審査の要旨

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ES細胞はそれ自身では自己復製や分化の方向が決定されておらず、環境入力によりはじめて変化の方向が決定される。ハイドロゲルは3次元網目の中に多量の水を含んでいるので、生体組織と類似するソフトでウェットな物質である。そのため、ハイドロゲルを細胞培養基盤として使い、再生医療への応用を目指す研究が最近盛んに行われている。特に、化学架橋のハイドロゲルは化学種、電荷、弾性率、含水率など様々な物性が系統的に調整することが可能であるため、ゲル基盤の物性が細胞の増殖・分化への影響を検討する基礎研究、更には、諸ニーズに合わせた細胞培養基盤を開発する応用研究において、高い可能性を秘めている。従来の研究で用いたゲルは細胞に対する接着性が低く、ゲル表面に細胞接着タンパクの処理が必要であった。本学位申請者が所属する研究室では、アニオン性高分子ゲルを用いて、表面処理せずに内皮細胞を培養することに成功している。しかし、ハイドロゲルの物性によるマウス胚性幹細胞の挙動の制御などはまだ手つかず状態である。

本学位論文では、中性，負電荷および正電荷を持つハイドロゲル上におけるマウス胚性幹細胞の挙動について評価した。その際に、ゲルの弾性率、膨潤度、電荷密度などの物性を系統的に変化させた。マウス胚性幹細胞（ES）と胚体（EB）を用いて実験を行った結果、以下の結論が得られた。1）マウスES細胞は、未分化性を維持するLIFがない状態でも、中性

なゲル上では、未分化性が維持できることを初めて観察された。2）EB はマイナス電荷を持つゲル上で接着し、心筋細胞への分化効率が高いことが分かった。3）正電荷を持つゲル上で、初めてマウス ES 細胞が生存できることが分かった。4）正電荷を持つゲル上において、ゲルの弾性率の増加に伴って培養した EB の形態が多様性を示すことが明らかになった。これは二次元基盤上で EB が形態形成する初めての例である。

本研究はさらに、ハイドロゲルの電荷や弾性率によって細胞の挙動が大きく異なる原因について、細胞と基盤との相互作用の観点から検討した。中性なゲル上で細胞を培養すると、基盤と細胞の相互作用が弱く、ゲル上での細胞運動が促進され、細胞と細胞の相互作用が強くなる。そのため、二次元基盤上で三次元な細胞構造が形成され、ES 細胞の未分化性維持できる。それに対し、マイナス電荷を持つゲル上で細胞を培養すると、マイナス電荷を持つゲル基盤と細胞の相互作用が強いため、EB は基盤に粘着し、心筋細胞の自発分化が促進されると考えられる。このようなゲル環境は接着依存性細胞の生体内環境と近いため、心筋細胞にとって最適な培養基盤となったと考えられる。また、正電荷を持つゲルは EB の形態形成に強く影響されたと考えられる。これらの知見は胚胎発育について研究を進める上で有意義である。

本研究で得られた成果は合成ハイドロゲルの医療基礎研究や再生医療に応用することが期待される。

よって著者は、北海道大学博士（理学）の学位を授与される資格あるものと認める。