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# Morphological differentiation of bone marrow stromal cells into neuron-like cells after co-culture with hippocampal slice

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【 学位論文題目 】

Morphological differentiation of bone marrow stromal cells into neuron-like cells after co-culture with hippocampal slice
（海馬スライス共培養下における骨髄間質細胞の神経様細胞への形態的分化）

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## Abstract

Adult green mice marrow stromal cells were co-cultured with hippocampal slices. Differentiation to neuron-like or non neuron-like cells occurred exclusively inside slice boundaries starting at day 3, and then decreased gradually over 35 days. Neuron-like cells tended to form network-like connections around day 14. The use of retinoic acid greatly increased the number of differentiated cells, and the most effective concentration was $10^{-6}$M. NeuN immunohistochemistry was positive in 9.6% ± 1.7% of morphologically differentiated neuron-like cells. Both GFAP and Iba1 immunostaining were negative. We concluded that bone marrow stromal cells can be differentiated into neurons, and direct contact with the host brain tissue is essential for this to occur. Retinoic acid significantly increases the number of differentiated cells, as has been reported with other stem cells.




## Introduction

As bone marrow stromal cells (BMSCs) are a promising source for stem cells for replacement therapy, we thought to study its capacity to be morphologically differentiated to neuron cells in a new in vitro model " organotypic hippocampal slice culture"that is similar to in vivo and easly manipulated and which to our knowledge first time to used as a model to study BMSCs differentiation. We thought also to study the effects of retenoic acid on the process of differentiatiation.

## Material and methods

The hippocampi of postnatal day 10 Sprague-Dawley rats were dissected under aseptic conditions, and 400 $\mu$m slices were cut with a tissue chopper. Five slices were placed on each culture plate using a Millicell-CM 0.4 $\mu$m. The slices were cultured in 6-well culture clusters at 34 °C in 5% $CO_2$ with 1 ml per well of a standard slice culture medium.

Bone marrow was extracted from the femur of adult green mice. BMSCs were separated by their attachment to the plastic culture dish. An aliquot of $4.8 \times 10^5$ cells was applied evenly to each culture plate on the surface of five slices and on the membrane inserts to cover an area of 314 $mm^2$ in total. The area of each hippocampus slice was about 10 $mm^2$, so the estimated number of BMSCs was

15,280 cells per slice. A set of BMSCs cultured alone on the membrane inserts under the same conditions served as a control.

We observed the morphological changes using a confocal microscope and a fluorescent microscope up to 35 days and counted the number of morphologically differentiated cells between days 3 and 14. The counting was done in a blinded manner using an objective 20X lens of fluorescent microscope and dividing the slice surface into 12 visual fields. Four culture plates with 20 hippocampal slices were used for each time point. Neuron-like differentiated cells were polygonal, angular (with pointed corners), or spindle shaped with a size ranging between 10 $\mu$m and 70 $\mu$m, and they had long axon-like processes. Cells having small cell bodies and multiple short processes shorter than the cell body were classified as "non neuron-like cells," which might have included immature neuron-like cells or non-neuronal cells. Round small cells without processes were not counted because they became debris late in culture.

Secondly, we added $10^{-5}$ M of RA dissolved in DMSO at the final concentration of 0.1% to the same number of slices and evaluated the promotion of differentiation by RA during the same time period. Lastly, we tested the effects of different concentrations of RA, ranging from $10^{-4}$ M to $10^{-8}$ M, and counted differentiated cell numbers in 20 slices for each dose at day 5 to reveal dose dependency. A set of slices with untreated medium and another set with medium treated with DMSO 0.1% but without RA served as controls.

Immunohistochemistry was processed with anti-NeuN, GFAP, and Iba1 as 1ry antidodies. Texas red avidin was used as a fluorescent marker after secondary antibodies, and examined with a confocal microscope.

The results were expressed as mean ± SEM per slice. The seven groups used to test the effects of different concentrations of RA were compared by ANOVA followed by Dunnett's multiple comparison test.

## Results

The majority of co-cultured BMSCs maintained their round cell body after plating, but they gradually died and became debris. Neuron-like differentiation occurred mostly within the hippocampal slice boundaries, and was detected from day 3 on. The mean length of the axon-like processes was 56 $\mu$m, which increased to 109 $\mu$m on day 5, 166 $\mu$m on day 7, and 247 $\mu$m on day 9. The length became difficult to measure around day 14 because of network-like formations between differentiated cells. Differentiation of BMSCs was not affected by their location either on white or gray matter. BMSCs outside the hippocampal slice but on the Millicell-CM membrane showed no differentiation. BMSCs were observed only on the surface of

the slice. The axon-like processed penetrated into the tissue but migration of BMSCs has never been observed.

The number of neuron-like cells was 31.4 ± 2.79 on day 3. This number decreased on the following days and became 6.8 ± 0.8 on day 14. RA at $10^{-5}$ M increased the number of the neuron-like cells at each time point. The number was 36.60 ± 3.50 on day 3 and 8.2 ± 0.66 on day 14. The number of non neuron-like cells was 29.8 ± 5.23 on day 3 and 16.4 ± 1.08 on day 14 without RA, while it was 61.4 ± 9.37 on day 3 and 75.8 ± 10.37 on day 14 with RA. RA increased the number of differentiated cells of both neuron-like and non neuron-like cells ($P<0.05$). RA effects showed dose dependency, with the maximum effect on both neuron-like and non neuron-like cell morphology at $10^{-6}$ M ($P<0.01$). The control with 0.1% of DMSO showed a decrease in the numbers of both cell types. Immunohistochemistry using anti-NeuN revealed positive staining in 9.6% ± 1.7% of neuron-like cells with co-localization between green fluorescent protein (GFP) of the BMSCs and red fluorescent color of NeuN examined by Z-scanning under the confocal microscope. The morphologically fully mature cells with long axon-like processes were negative for NeuN, while those less mature with short processes (<100$\mu$) were positive for NeuN. We failed to get positive staining with GFAP and Iba1 for microglia. A few differentiated cells had double green nuclei, suggesting fusion between two differentiated BMSCs cells or differentiation to a sympathetic or ganglia cell.

## Discussion

Our results showed the capacity of BMSCs to differentiate morphologically into neuron-like cells that were positive for NeuN staining in 9.6% ± 1.7% of cells. These results are consistent with previous studies both in vitro and in vivo. Our observation that this differentiation occurred exclusively within the boundaries of hippocampal slices strongly suggests the importance of direct contact between BMSCs and the host brain tissue for occurrence of differentiation.

The possibility of the presence of surface immunoglobulin-binding proteins that may give false-positive results with neuronal markers was not detected during our confocal examination with Z-scanning, which would otherwise show a ring staining pattern.

Our results show that RA has an enhancing dose-dependent effect on the number of differentiated BMSCs. This effect has been reported with embryonic stem cells differentiating into neural lineage. Various methods of induction of differentiation were recently developed to partially differentiate human, rat, or mouse adult BMSCs into neuron-like cells. Those methods include combinations of different growth factors and RA. In our study we followed RA-treated slices for 14 days, during

which there was an initial significant increase followed by a steep decline in the number of the differentiated neuron-like cells, ending at levels close to those of the control. We suggest that the cells that were forced to differentiate by exposure to RA have decreased viability. Furthermore, the recent clinical issue of RA being associated with depression or depressive episodes may in some way be related to its effects on neurons and progenitor cells and therefore warrants further investigation. The estimated number of the co-cultured cells was 15,280 cells per slice; however, the number after culture is much lower due to the death of BMSCs after culture and the fact that there are a limited number of viable stem cells among the total population of BMSCs. GFAP- and Iba1-positive cells were not detected. This also adds to the controversy about the differentiation to astrocytes that has recently been refuted by some authors. Although many of the differentiated cells showed glial morphology, we could not prove it immunohistochemically. This may be due to immaturity of the cells at the time of the immunohistochemical study performed on day 5 after co-cultur. The possibility of contaminating BMSCs with neural stem cells in the nerve fibers innervating the bone during extraction is an unproved hypothesis. Contamination is probably unlikely to occur with the technique of simple flushing of the bone marrow used in this study.

The issue of cell fusion in bone marrow transplantation has been raised recently and seems to be very rare. This study showed that differentiated cells contained a single nucleus similar to those of the surrounding cells except on a rare occasion.

## Conclusion

In conclusion, we have demonstrated the ability of transplanted BMSCs to differentiate to neuron-like cells in an organotypic hippocampal slice culture model. This differentiation required direct contact with the host brain tissue. The differentiation was enhanced in a dose-dependent fashion by RA, with the best concentration occurring at $10^{-6}$ M.

神戸大学大学院医学系研究科（博士課程）

# 論文審査の結果の要旨

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| 論文題目 | **Morphological differentiation of bone marrow stromal cells into neuron-like cells after co-culture with hippocampal slice**<br>（海馬スライス共培養下における骨髄間質細胞の神経様細胞への形態的分化） | | |
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（要旨は1，000字～2，000字程度）

骨髄幹細胞は置かれた環境によって色々な細胞に分化することが知られている。骨髄間質細胞は間葉性幹細胞や前駆細胞を含んでおり、適切な環境で培養すると、筋細胞や肝細胞のみならず脳細胞にまで分化することが報告されている。骨髄間質細胞がin vitroにおいて神経細胞や神経膠細胞に分化するという報告がある一方、骨髄間質細胞を脳内移植に移植した時には神経細胞や神経膠細胞の特徴を示すマーカーの発現が移植細胞には見られなかったとする報告もある。申請者は、脳内移植に近い実験条件として海馬スライス器官培養系を用いて骨髄間質細胞の神経系細胞への分化が起こるかどうかを研究した。海馬スライス器官培養系は長期培養が可能な確立された実験系であり、神経細胞と神経膠細胞の正常なネットワークが保持され、細胞間の情報伝達も正常に行われ、また様々な実験的な操作も行い易いといった特徴がある。生後１０日のSprague-Dawley系ラットの海馬を無菌的に取り出し、４００μのスライスを作製した。Millicell-CM 0.4μmを用いて５スライスずつを１培養皿に入れ１ｍｌの標準培養液で培養した。骨髄間質細胞は成人グリーンマウスより分離し、$4.8 \times 10^5$細胞をそれぞれの培養皿（１スライス当たり15,280細胞になる）に添加した。共培養をはじめて５日目まで形態学的変化を共焦点顕微鏡及び蛍光顕微鏡で観察した。神経細胞様に分化した細胞は、多角形あるいはかく張った形を示す、あるいは紡錘形を示し、長い軸索様の突起を持つ。これらの神経細胞様細胞は共培養開始後３日でその大部分が海馬スライスの周辺に出現し、日数を追って軸索長が伸びていく。軸索突起は海馬スライスの組織中に伸びてゆくが、神経細胞体が組織中に移動することは観察されなかった。これらの所見は、骨髄間質細胞の神経系細胞への分化には、骨髄間質細胞が海馬の神経組織と直接接触することが重要であることを強く示唆する。骨髄間質細胞から分化した神経様細胞の数は、共培養３日目で31.4±2.79であったが、１４日目には6.8±0.8に減少した。しかし、レチノイン酸(RA)を培養液中に添加すると、共培養３日目で36.60±3.50、１４日目には8.20±0.8とより多くの細胞が認められた。申請者は、小さな細胞体を持ち、さらに細胞体よりも小さな多く突起を持つ細胞を非神経細胞（未熟な神経様細胞を含む）と定義して、これら非神経細胞数の変化も

観察した。RA 非存在下で非神経様細胞数は、共培養３日目で 29.8±5.23、１４日目では 16.4±1.08 であったが、RA 存在下では、共培養３日目で 61.4±9.37、１４日目には 75.8±10.37 と明らかにより多くの細胞が認められた。従って RA は、骨髄間質細胞の神経様細胞および非神経様細胞の両方への分化を促進すると考えられた。分化した細胞が神経細胞であるか否かを NeuN 抗体で免疫組織化学的検討を行ったところ、添加した骨髄間質細胞の 9.6±1.7%であった。長い軸索を持つ形態学的に成熟した神経細胞は NeuN 抗体には陰性、軸索がまだ短く未熟な神経細胞は NeuN 抗体に陽性と言われている。一方、ミクログリアに特徴的と言われている GFAP および IBA1 の抗体で染色される細胞は、骨髄間質細胞由来の非神経様細胞に検出出来なかった。形態学的には神経膠細胞様に見えなが、GFAP および IBA1 の抗体には陰性であった理由は明らかではないが、GFAP や IBA1 がまだ発現していない未熟な神経膠細胞様細胞である可能性が考えられた。

以上、本研究は、骨髄間質細胞から神経細胞および神経膠細胞を分化誘導出来るか否かについて、海馬スライス培養系モデルを用いて研究したものであるが、従来知られていなかった、骨髄間質細胞から神経系細胞への分化には骨髄間質細胞と神経組織の直接接触が重要であること、またレチノイン酸が分化誘導を明らかに促進することなど、重要な知見を得たものとして価値ある集積であると認める。よって、本研究者は、博士（医学）の学位を得る資格があると認める。