

Study on the simplifying antibody cocktail technique for isolation of human mesenchymal stromal cells (hMSCs)

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I. INTRODUCTION

Embryonic stem cells(ES) are pluripotent cells derived from blastocysts that can be propagated indefinitely undifferentiated in vitro, can differentiate to all cell lineages in vivo, and can be induced to differentiate to most cell types in vitro.¹⁻⁴⁾ Although ES cells have been isolated from humans^{2,3)}, their use in research as well as therapeutics is encumbered by ethical considerations.⁵⁾ The ability to purify, culture, and manipulate multipotent stem cells from non- embryonic origin would provide investigators with an invaluable cell source to study cell and organ development. In addition, such cells could serve to develop replacement tissues for congenital or degenerative disorders. Stem cells have been identified in most organ tissues, including hematopoietic⁶⁾, neural⁷⁾, gastrointestinal⁸⁾, epidermal⁹⁾, hepatic¹⁰⁾, and mesenchymal stem cells(MSCs).¹¹⁻¹⁴⁾

The bone marrow stroma was originally thought to function mainly as a structural framework for the hematopoietic component of the marrow. Since then, it has become well established that the stroma consists of a heterogeneous population of cells, a subset of which exerts both positive and negative regulatory effects on the proliferation and differentiation of hematopoietic stem cells(HSC) in the marrow through a combination of physical and chemical signals. The stroma also contains other non-hematopoietic cells termed mesenchymal stem cells(MSC), that are capable of both self renewal and differentiation into bone, cartilage, muscle, tendon and fat.

The methods for purification of MSCs are centrifugation, fluorescence activated cell sorter(FACS), magnetic activated cell sorter(MACS) and so on. Only using centrifugation showed low purity percentage. And to use MACS or FACS, we need expensive equipment. Lansdorp PM et al, suggested

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cyclic tetra- molecular complexes of monoclonal antibodies for new type of cross-linking reagent for isolation of specific cells.¹⁵⁾

The purpose of this study is to investigate the simplifying antibody cocktail technique for isolation mesenchymal stromal cell.

II. MATERIALS AND METHODS

1. Bone marrow

BM was obtained from 2 healthy donors who needed iliac bone graft. After iliac bone was obtained, bone marrow was aspirated using 12 gauge bone marrow biopsy needle. The tube with heparin is filled with aspirated BM. Before processing for isolation, the tube was kept in refrigerator. Keeping time was not over 4 hours.

2. RosetteSep(human MSC enrichment cocktail, stem cell technologies)

Lansdorp PM proposed cyclic tetra- molecular complexes of monoclonal antibodies for isolation of specific cell type. The RosetteSep Mesenchymal enrichment cocktail (stem cell technologies) contains bispecific antibody complexes against red blood cells (glycophorin A) and CD3, CD14, CD19, CD38, CD66b.

3. Purification of human mesenchymal stromal cell(hMSC)

50 μ m RosetteSep Mesenchymal Cell Enrichment Cocktail per ml of bone marrow was added and mixed well. The tube was incubated for 20 minutes at room temperature. The sample was diluted with twice volume of buffer(PBS containing 2% FBS and 1mM EDTA) and mixed gently. The diluted sample

was layered on top of Ficoll-Paque. The sample was centrifuged for 25 minutes at 1500rpm, room temperature with brake off. Enriched cell was removed from Ficoll-Paque. We washed enriched cells two times with buffer and resuspend cells in medium for culture

4. Culture of hMSC

We plated the cell in polypropylene dish with expansion medium. Expansion medium consisted of 89% Mesencult medium(stem cell technologies) and 10% Mesencult supplement(stem cell technologies) and 1% antibiotics. We change expansion media every 3-4 days. Once adherent cells were more than 50% confluent, they were detached with 0.25% trypsin-EDTA (Sigma) and replated at a 1:4 dilution under the same culture condition.

5. Osteoblast differentiation

MSCs were cultured in osteogenic media. Osteogenic media consisted of 1% antibiotics, 1% 1M β glycerophosphate, 0.5% 1% ascorbic acid, 0.02% 5x10⁴M Dexamethason, 15% FBS, 82,48% D-MEM. Media was changed every 3 to 4 days. MSCs for osteogenic differentiation were cultured during 14days. We stained the differentiated cells with alizarin red, and measured alkaline phosphatase activity for confirming the osteogenic differentiation.

6. Chondroblast differentiation

To induce chondroblast differentiation, MSCs were trypsinized, and were cultured in 1ml mesencult media(serum free) with 1 μ l TGF- β , in the tip of a 15-ml conical tube to allow aggregation of the cells in micromass suspension culture. Medium was exchanged every 3 days. MSCs for chondrogenic

differentiation were cultured during 14 days. For chondrogenic pellet processing, pellet was fixed in 10% neutral buffered formalin for 15min at room temperature. To dehydrate pellets, the pellets were washed in 70%, 70%, 80%, 90%, 95% for 15 minutes each in 4° C. Then, pellet was washed in xylene twice for 10 minutes each. And we transfer pellet to two changes of 60° C paraffin for 15minutes each. The pellet was embedded in a paraffin block and sectioned. We transfer 5 sections of pellet onto slide glasses and incubate the slides at 60° C for one hour, then cool for 10 minutes.

For Staining, we deparaffinized the pellet sections in xylene (3 x 3 min), 100% ethanol (2 x 3 min), 95% ethanol (2 x 3 min) then 70% ethanol (1 x 3 min) and stained the pellet in Weigert's iron hematoxylin for four minutes, then destain in fresh acid alcohol (1ml concentrated HCl in 100ml 70% ethanol) and rinse in tap water. Then we stain in 0.02% aqueous fast green FCF for three minutes, then wash in 1% acetic acid for 30 seconds. The pellet was stained in 0.1% aqueous Safranin-O for five minutes. We dehydrate in 95% ethanol (2 x 5 min), 100% ethanol (3 x 5 min), then xylene (3 x 5 min) and then mount sections in synthetic resin.

we stained the slide with 0.5% toluidine blue and 1% borax for confirming chondrogenic differentiation.

7. Adipocyte differentiation

To induce adipogenic differentiation, MSCs were cultured in DMEM, 10%FBS, 1% antibiotics to confluent state. After confluent state, MSCs were cultured during 7 more days. Then, Medium was changed to adipogenic induction medium. Adipogenic induction medium consisted of DMEM, 10% FBS, 1 μ M dexamethasone, 0.5mM methylisobutylxanthine, 10 μ g/ml insulin, 10mM

indomethacin. MSCs were cultured in adipogenic induction medium for 3 days. Then Medium was exchanged to adipogenic maintenance medium. Adipogenic maintenance medium consisted of DMEM, 10% FBS, 10 μ g/ml insulin. MSCs were cultured in adipogenic maintenance medium for 1 day. Three cycles of induction/maintenance stimulate Adipogenic differentiation. After 3 complete cycles of induction/maintenance, we cultured the MSCs for 7 more days in Adipogenic maintenance medium and replaced the medium every 2-3 days.

For staining, the cells were fixed in 10% formalin for 30-60 minutes temperature. After we removed the formalin, we washed the cells in the water. Oil Red O and hematoxin staining were used and we viewed the plates on a phase contrast microscope. Lipids appeared red and the nuclei appeared blue.

III. RESULTS

1. Morphology (Figure 1.)

The mesenchymal stromal cells(MSCs) from BM have been shown to be morphologically distinguishable from adherent hematopoietic cells, including monocytes. MSCs seemed like fibroblast-like

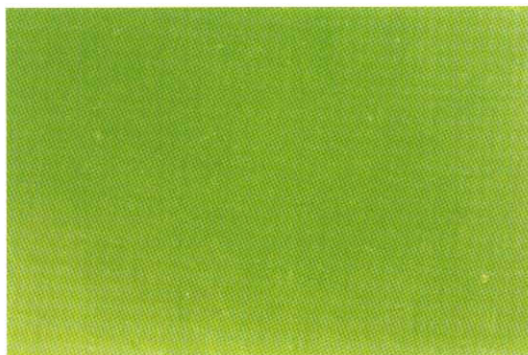


Figure 1. This figure shows morphology of MSCs which were spindle shaped and had long projection

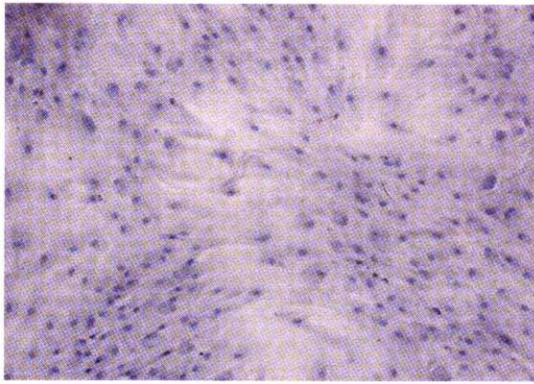


Figure 2. Oil red-O staining for un- differentiated MSCs

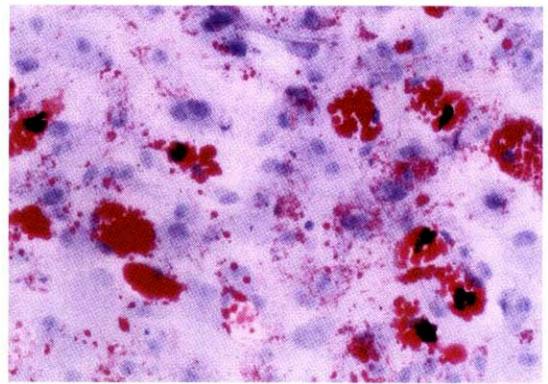


Figure 3. Oil red-O staining of adipogenic differentiated cells

Table 1. Comparison between osteogenic differentiated cells and undifferentiated MSCs in ALPase activity

	differentiated cells	undifferentiated MSCs
ALPase activity	6,5328	3,6824

and had long projection. MSCs proliferated well but more passaged, less proliferated.

2. Adipogenic differentiation (Figure 2,3)

We stained adipogenic differentiated cells with Oil red-O for confirmation of differentiation. Nucleoli was stained blue, lipid vacuoles were stained red. In high magnitude, we could see cells which were filled with many lipid vacuoles and ring-like morphology, traditional morphology of adipocyte by phase-contrast microscopy. The above features were not observed in MSC cultures grown in regular culture medium.

3. Chondrogenic differentiation (Figure 4)

We stained chondrogenic differentiated pellets with toluidine blue for confirmation of differentiation. After staining, proteoglycans in extracellular matrix which were made during chondrogenic differentiation were stained blue.

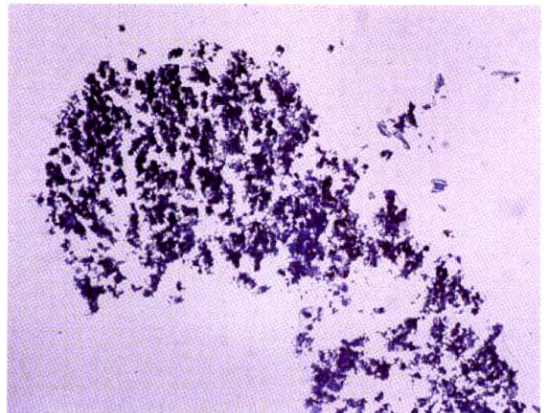


Figure 4. Toluidine blue staining for chondrogenic differentiated cell pellet

4. Osteogenic differentiation (Figure 5,6)

After switching MSC from the regular culture medium into an osteogenic medium, cells changed to osteoblast-like phenotype evolved, as judged by the expression of alkaline phosphatase and by the deposition of a mineralized matrix.

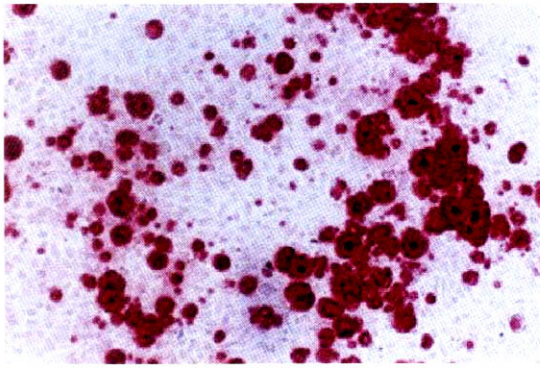


Figure 5. Alizarin red staining for osteogenic differentiated cells

After alizarin red staining, mineralized matrix was stained red. But mineralized matrix were not observed in MSCs grown in regular culture medium. And differentiated cells had twice alkaline phosphatase activity in comparison to undifferentiated MSCs. Osteogenic differentiated MSCs were seemed to be cuboidal by phase-contrast microscopy.

IV. DISCUSSION

As mentioned above, there were many methods for isolation of mesenchymal stromal cells, for example, centrifugation, magnetic-antibody complexes, fluorescence activated cell sorter and flow cytometry. When only centrifugation was used for isolation of MSC, the purity of MSC was low. Therefore we needed additional step which is cloning of single colony. Moreover, magnetic-antibody complexes, fluorescence activated cell sorter and flow cytometry needed additional expensive equipment, for example, magnetic, column and so on. But When we used antibody cocktail for isolation of MSC, we needed only one more step than centrifugation, addition of antibody complex.

RosetteSep is a novel system for the purification of cells directly from whole blood that combines the

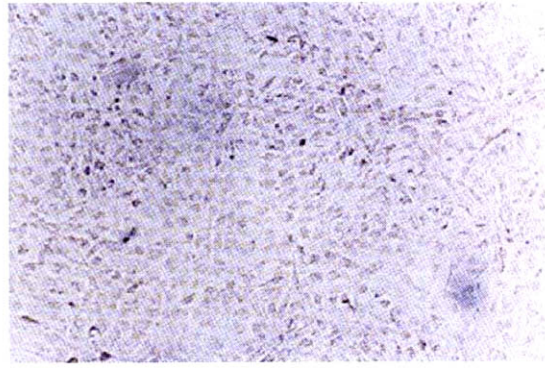


Figure 6. Alizarin red staining for undifferentiated MSCs.

ease of density gradient separation with the specificity of antibody-mediated enrichment. RosetteSep links unwanted nucleated cells (NC) to red blood cells (RBC) using a cocktail of biphasic tetrameric antibody complexes such that RBC rosettes form around targeted NC. During a standard density centrifugation, the rosettes pellet along with the free RBC, while the desired (non-targeted) cells are highly enriched at the interface between the plasma and the density separation medium. RosetteSep for isolation of MSC has antibody complexes against CD 3, 14, 19, 38, 66b and glycophorin A. Glycophorin A is surface marker of RBC. CD 3, 14, 19, 38, 66b are surface markers of T cell, monocyte and macrophage, B cell, early T and B cell and plasma cell, granulocyte.

MSCs from bone marrow seemed like spindle-shaped and had long projections. Elena et al. reported morphological types of MSCs adherent after 4 hours of culture and identified distinctive cells that were flat and large (up to 50 μm) and that had characteristic long projections.¹⁶⁾ MSCs were well proliferated during culture but more passages, less proliferated. We thought that small changes of micro environment during culture and passages made cells differentiate slightly. Pittenger reported

that population doublings and in vitro culture conditions may have caused some loss of multilineage potential that was originally present in these cells. Another interpretation is that some of these cells might represent progenitor cells, with restricted differentiation and proliferation potential, that were present in adult marrow.¹⁴⁾

We examined multipotential properties of MSCs. MSC differentiated to osteoblast, chondroblast, adipocyte successfully. But all cases have undifferentiated cells, which may be progenitor cells or differentiated cells.

There is a great deal of debate in the literature as to what constitutes a stem or progenitor cell as well as the degree of plasticity of various progenitor cells. Stem cells are capable of self-renewal and can differentiate into all cell types (pluripotential) or to more than one differentiated cell types (multipotential)¹⁷⁾. Progenitor cells are the intermediate step between stem cells and fully differentiated cells¹⁸⁾. Early progenitors can still be multipotential cells, but unlike stem cells, they are not capable of self-renewal¹⁸⁾. Because MSCs have been described as multipotential cells, many investigators have adopted the term mesenchymal stem cells, in one view somewhat premature, since no suitable assay to assess their self-renewal has yet been developed¹⁹⁾.

A broad range of alternative terms (such as "marrow stromal stem cells" or "osteogenic stem cells") historically used to define cells with MSC properties tends to add even more confusion to the contentious nature of this field. In addition, recent findings suggest that bone marrow may contain putative stem cells more primitive than MSCs²⁰⁾ as well as pluripotent cells capable of producing progeny with characteristics of mesoderm, neuroectoderm, and endoderm²¹⁾. Such cells can be copurified with MSCs from glycophorin A- cell fraction of human bone marrow, but their more detailed phenotype

has not yet been reported. It may be possible in the future to isolate these primitive cells using antibody cocktail strategy described in this report, with regard to the MSCs themselves, further investigation is required to determine whether CD3-, 14-, 19-, 38-, 66b- cells are a self-renewing cell population or whether they are derived from more primitive progenitors with a yet unknown phenotype.

V. CONCLUSION

Centrifugation in addition to antibody cocktail is easy, rapid, economical, effective method for isolation of human MSC. However all cells of isolated MSCs could not differentiate. So additional study to investigate the purity of isolated MSCs by antibody cocktails was needed.

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사람 Mesenchymal stromal cell(hMSC) 분리를 위한 간소화된 방법에 대한 연구

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많은 연구들에서 hMSC를 얻기 위해 centrifugation, fluorescence activated cell sorter(FACS), magnetic activated cell sorter(MACS)가 이용되어져 왔다. 그러나 centrifugation만을 이용한 경우 순도가 떨어지며 FACS나 MACS의 경우에는 비용, 시간이 많이 드는 단점이 있다. 따라서 이 연구에서는 antibody cocktail을 이용하여 hMSC를 좀더 쉽게 얻어내는 방법에 대해 알아보았다.

사람의 골반에서 12G의 바늘을 이용하여 골수를 흡입한 후 heparin이 들어있는 시험관에 넣고 처리과정을 시행하기 전에 냉장고에 보관하며 가능한 한 빨리 처리과정을 실시한다.

얻은 골수에 적당량의 RosetteSep(Stemcell Technologies)을 첨가한 후 실온에서 20분간 반응시킨다. 그 후 적당량의 Ficoll-paque위에 골수와 RosetteSep의 혼합물을 섞이지 않게 올리고 원심분리를 이용하여 원하는 세포층을 얻어낸다. 이 세포층을 따로 분리한 뒤 배양한다. 배양 시 세포가 80%이상 차기 전에 계속 passage를 시행하며 배양한다. 이는 세포가 밀도가 높아져 원치 않는 세포로 분화되는 것을 막기 위함이다.

배양된 세포가 다양한 분화능력을 가지고 있는지 알아보기 위해 세 가지로 분화를 유도하였다. 적절한 배지와 적절한 환경에서 배양함으로써 얻어진 세포를 osteoblast, chondroblast, adipocyte로 분화를 유도하였다.

분화된 세포가 원하는 형질의 세포로 분화되었는지를 확인하기 위하여 osteoblast의 경우 alizarin red staining, alkaline phosphatase activity, chondroblast의 경우 toluidine blue staining, adipocyte의 경우 Oil-Red-O staining으로 염색하여 분화를 확인하였다.

분리해낸 세포는 각각 세 가지 세포로 분화가 되었으며 이는 RosetteSep이 hMSC를 성공적으로 분리해냈다는 것을 보여준다. 그러나 모든 세포가 분화를 보이지는 않았으며 따라서 hMSC의 순도를 높이기 위한 연구가 더 필요하다. RosetteSep을 이용하면 다른 방법들 보다 쉽게 hMSC를 얻을 수 있으나 기존의 방법과 순도의 측면에서 더 비교할 필요가 있다.

주요어: 인간 줄기세포 분리, 골모세포, 연골모세포, 지방세포, RosetteSep