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Culturability of rat bone marrow stromal cells and evaluation for osteoblastic formation

Sıçan kemik iliği stromal hücrelerinin kültüre edilebilirliği ve osteoblastik formasyonun değerlendirilmesi

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Amaç: Bu çalışmada, genç yetişkin sıçan kemik iliği stromal hücrelerinin kültüre edilebilirliği ve kültür hücrelerinin genel histomorfolojik ve osteoblastik formasyon yönünden değerlendirilmesi amaçlandı.

Çalışma planı: Yüksek doz eter ile yaşamları sonlandırılan 16 adet 2.5 aylık Wistar cinsi genç erkek sıçanın arka femur ve tibiaları çıkarıldı, metafiz ve medullaları taşıyıcı serumla yıkandı ve toplanan aspirat DME/F12 serumuyla kültüre edildi. Kültür flasklarında medium içindeki ve tabandaki hücreler, fotoğraf ataçmanlı invert mikroskopla incelenerek görüntülendi. Pikro-tionin boyama tekniğiyle boyanan flask tabanındaki hücreler ışık mikroskobuyla incelenerek resimleri çekildi. Yapışık kültür hücreleri tripsinize edilerek çözüldü ve yayma preparatlar hazırlanarak stromal hücrelerin genel histomorfolojik yapıları incelendi.

Sonuçlar: Sıçan kemik iliği hücrelerinin kültürde üreme yüzdesi %93.8 (15/16) oranında yüksek bulundu. Kültür flasklarında, zamanla birbirine yapışarak çoğalan farklı morfolojide stromal hücreler ve aralarında pikro-tionin ile boyanan çok nükleoluslu büyük çekirdekli osteoblastlara benzeyen hücreler saptandı.

Çıkarımlar: Bulgularımız, kemik iliği hücre kültürlerinde, elde edilen hücrelerin osteoblastlara çok benzediği ve bu hücrelerin çok yönlü farklılaşma yeteneği olan osteoprogenitör hücrelerden köken alabileceği öngörüsünü desteklemektedir.

Anahtar sözcükler: Kemik iliği hücresi/sitoloji; hücre kültürü; osteoblast/sitoloji; sıçan; kök hücre/sitoloji.

Objectives: We investigated culturability of bone marrow stromal cells of young mature rats and evaluated cultured cells with regard to histomorphologic features and osteoblastic formation.

Methods: Sixteen mature Wistar rats were sacrificed under high dose of ether anesthesia. Their femurs and tibias were removed, medullas and metaphyses were washed with carrier serum and the collected material was cultured in DME/F12 serum. The cells in the test medium and at the bottom of Petri dishes were studied under inverted microscope and were photographed. Then, the cells at the bottom were stained with the picro-thionin technique, examined under light microscope, and photographed. Following trypsinizing, cluster cells were dissolved and smears were prepared for histomorphologic evaluation of stromal cells.

Results: Rat bone marrow cells in the culture showed a high percentage of reproducibility (93.8%; 15/16). The stromal cells in Petri dishes were of different morphology, and were interspersed with large multinucleated osteoblast-like cells following staining with the picro-thionin technique.

Conclusion: Our findings favor the opinion that cells harvested in bone marrow cell culture are very similar to osteoblastic cells and that they may stem from osteoprogenitor cells which have multifacet differention ability.

Key words: Bone marrow cells/cytology; cells, cultured; osteoblasts/cytology; rats; stem cells/cytology.

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Advanced studies have been carried out for in vitro production of the bone tissue cells in human beings. One of the objectives is to proliferate osteoblasts on molds biologically compatible with the bone, using them in the repair of extensive bone tissue defects on the same living organisms. It is expected that the receiving live bone tissue become united with the cells on the mold, reproducing sufficient number of bone cells in a short period of time in the defective area. It was proposed that culture can be harvested from the calvarium of a fetal mouse,[1] however search for other source tissues is required as it is very hard to prepare this culture. Bone marrow includes pluripotent mesenchymal cells, which have potential to differentiate to osteoblasts. In the present study, we investigated the culturability of bone marrow stromal cells of rats, and morphologically evaluated whether these cells are similar to osteoblasts or not.

Material and method

Sixteen Wistar type of male rats, aged 2.5 months were used for the study. They were sacrificed under high dose of ether anesthesia; the femur and tibia of their back extremities were removed surgically under sterile conditions. The medulla and metaphysis of each rat removed with a transverse cut from the subchondral region using the DME-F12® (Biological Industries, Israel) medium containing a 10% fetal calf serum (FCS®) by injection were washed in the medium and stored. The cell suspension containing the bone marrow cellular components was washed twice with serum-medium by centrifuging for five minutes in the tissue culture laboratories. The cells were counted, and then cultivated in 25 cm2 sterile flasks as to have 1x106 cell in 5 ml medium. Growth and proliferation of cells were controlled by using an inverted microscope every 24 hours. The mediums were replaced with fresh serum-mediums, and some non-adherent cells and red blood cells were isolated. Growth of cells proliferating by adhesion to the bottom inside the medium and flask at different time periods was photographed using the inverted microscope.

Histochemical fixation: Considering that the cultures covering approximately 75% of the flask surface were successful, they were fixated in a 10% formalin solution buffer phosphate for 15 minutes for histochemical analysis.

Successful cultures were stained with the picrothionin technique, which was developed by Schmorl in 1934 and used in staining the frozen or celloidin embedded tissues. Before these procedures, one drop of concentrated ammonium was added into each 100 ml of the 0.125% thionin solution. This solution was transferred into flasks in 5 ml, which were slightly washed with distilled water, and then kept for 15 minutes; afterwards, the solution was spilled, and slightly washed by re-washing with distilled water for two minutes. Then, the flasks were kept in a saturated aqueous picric acid 1.22% for 0.5-1 minute. After removing the extra blue dye by keeping in 70% alcohol for 5-10 minutes, they were shaked with distilled water, and examined under inverted microscope with a camera attachment. A portion of successful cultures was treated with trypsin preparation in a 25% calcium- and magnesium-free phosphate buffer, being centrifuged and washed, followed by staining with Giemza stain, examination under light microscopy and photographing at various magnifications.

Results

Fifteen (93.8%) of the bone marrow suspensions obtained from the tibial and femoral bones of 16 Wistar rats were successfully cultured in the DME medium. One of them developed infection; therefore, it was excluded as a failure.

The cells started to proliferate in the culture flasks in 24 hours; in general near-confluence was observed in seven days, and the confluence was reached in nine days (Figure 1a-c). Photographs of the seven-day cell cultures showed cells of various morphologies (Figures 2, 3).

It was observed that the cells in the smear preparations, made by tripsinizing from the cells adhering to the bottom of the cultured flasks were rounder, and they looked like osteoblasts in terms of nucleus and structure of the nucleus (Figure 4a, b).

Multi-cornered osteoblast-like thionine-picric-positive cells were found in the flasks stained with the thionine-picric acid (Figure 5a). They were giant, multi-cornered, multinucleated hyperactive cells with large cytoplasms (Figure 5b).

Discussion

One of the reasons why we selected rats for our experiment is that surgical procedures are easier to perform on rats, and also they are the most common animals used in the studies for tissue culture. In the bone culture studies, use of the fetus cavaria of 12-day old rats is the most common.[2] In the present study, we evaluated the culturability of the rat bone marrow stromal cells, which are difficult to isolate and define histomorphologically due to their varieties. Although there are publications stating that non-fetal tissues are less successful,[1] we obtained high percentages of successful cultures.

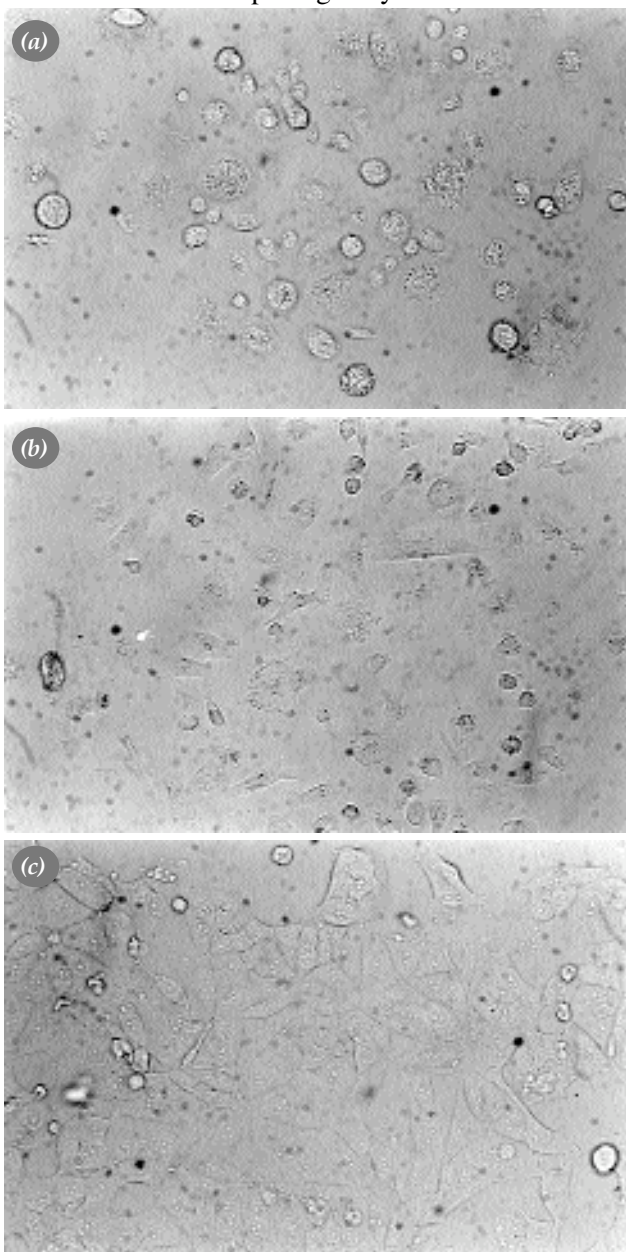

Figure 1. (a) One-day old, **(b)** three-day old, and **(c)** seven-day old microphotographs (magnification x 90) of the healthy cell cultures proliferating in the culture flasks obtained under inverted microscope.

While no information was available if the bone marrow cell suspension can be obtained without sac-

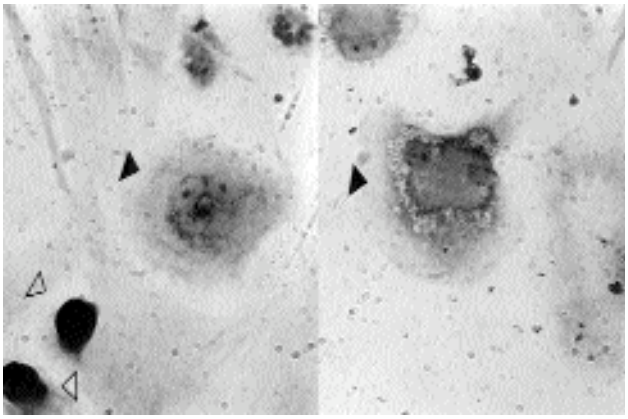

Figure 2. A microphotograph of the seven-day culture bone marrow cells under light microscope. On the left, cells with active mitosis (marked with blank arrowhead), and next to it multinucleated and nucleated large cytoplasmic cells (marked with arrowheads) (Giemza x magnification 175).

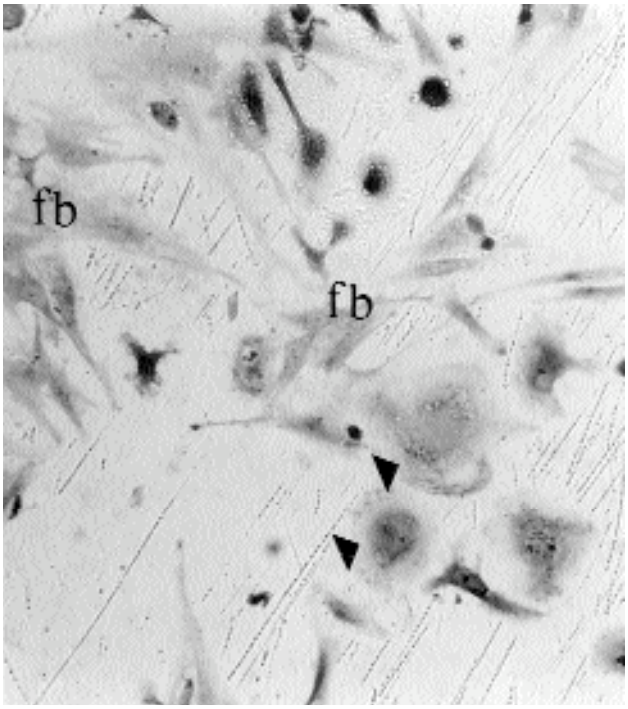

Figure 3. A microphotograph of the successful seven-day bone marrow cultures. Fibroblast-like (marked with fb) cells with their various appearances as well as rounded, large cytoplasmic cells, which are likely to be multipotent (marked with arrowhead) (Giemza x 90).

rificing the rats, we believe that it can be succeeded with a surgical technique to be developed. In the future, it is likely to demonstrate that the bone defect established in an animal, which is kept alive after obtaining the cell suspension for culture, can be replaced with the culture cells obtained in the experimental studies.

The amount of the bone arrow cell suspension should remain at an optimum level of 1-2 ml, and it has been reported that when it is more, the rate of blood cells in the suspension is increased, resulting in reduced progenitor cells in the culture.[3] We kept the amount of cell suspension from the animals under this limit in our experiment. The cells, which we couldn't characterize were components of the connective tissue other than the osteoblast (Figure 2-4).

It was demonstrated that the adult mouse bone marrow includes mesenchymal progenitor cells, which are characterized by phenotypic changes to four different types; chondrocytes, adipocytes, stromas (supporting the osteoclast formation), and osteoblasts.[4] Again, it was found that the bone marrow stromal cells have the potential to differentiate into many connective tissue cells, including the cartilage.[5] In cultures treated with dexamethasone, multi-nucleated muscle cells, lipid-containing-adipocytes, chondrocyte nodules, and mineralized bone modules were found at days 9-10, day 12, day 16 and day 21, respectively.[6] Osteoblasts, fibroblasts and chondroblasts are similar cells. No red blood cell can be found in the culture cells. As they have no adherence features, they are washed away from the culture medium.

Endosteal cells (osteoprogenitors) are regarded as mesenchymal stem cells undifferentiated during the embryonic development in the living.[7-9]

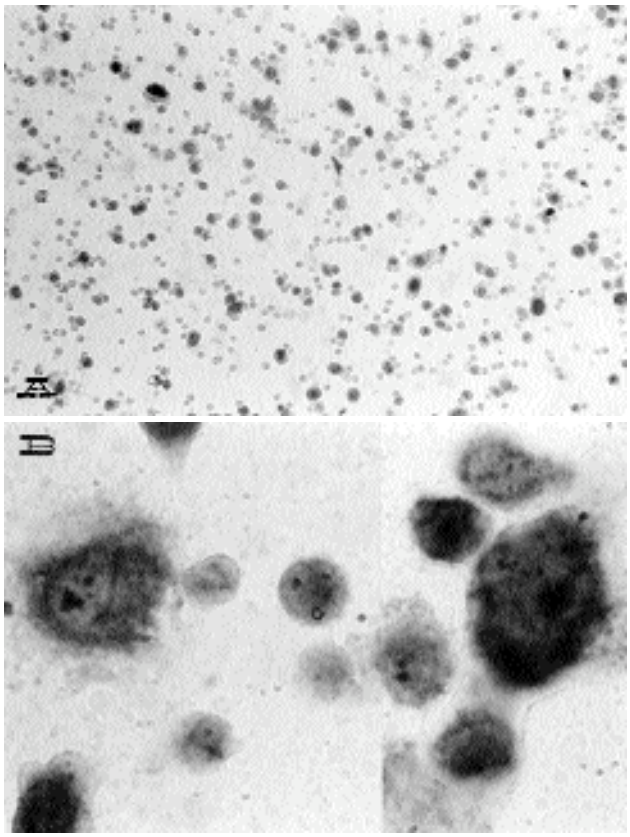

Figure 4. Microphotographs at different magnifications from the smear preparations made by trypsinizing from the seven day old cultured bone marrow cells. **(a)** General appearance of the cultured bone marrow cells (Giemza x 46). **(b)** Cells of various morphologies are evident with dark-stained cytoplasms (Giemza x 435).

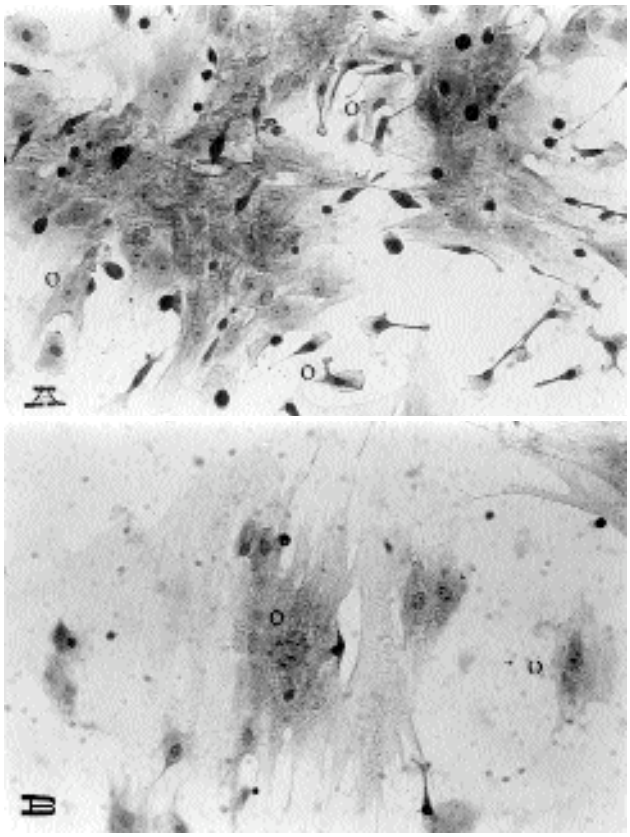

Figure 5. (a) Regular, multicornered, osteoblast-like thionin-picric acid cells in the successful bone marrow cells obtained from the seven day old rat bones (marked with "o") are evident (x 20). **(b)** Giant hyperactive, multicornered and multinucleated cells with large cytoplasm (marked with "o") are evident (Thionin-picric acide x 85).

Osteoprogenitors are resting cells, which have the potential to differentiate into chondroblast, fibroblast or osteoblast cells, however their differentiation phase has not been elucidated. It is likely that the sequence is as follows; firstly expression of colony forming cells (CFCs), followed by growth of specialized colony forming cells (SCFCs). In the future, it would be possible to demonstrate these phases using advanced histological techniques. The colony forming cells are lymphocyte-like pluripotent stem cells, rarely found in the bone marrow, but much rarely in the peripheral circulation. Specialized colony forming cells can be found in several tissues as periostium, peritrabecular tissue and vascular pericytes. A group of SCFCs are planned to differentiate into osteoblasts.[2] The source where the osteoblast SCFCs are most frequently found and easily obtained is the bone marrow.[2] That is why we used bone marrow in our study.

The cultured stromal cells undergo an osteoblastic differentiation when osteogenic inductors are added into the growth medium. For osteoblastic differentiation of pluripotent cells, chemicals with mitogenic effect such as dexamethasone, beta-glycerophospate (b-GP) and b-FGF should be added into the culture medium.[10] Also, it has been reported that mature osteoblasts added into the culture medium secrete paracrine growth factors, which stimulate the osteoblastic cell formation from the osteoprogenitors.[11] Our study is distinguished from others due to the absence of any osteogenic inductor used during the study.

In order to define the osteoblastic characteristics, histochemical fixation and pciro-thionine staining technique were used. It has been reported in the literature that parameters like alkaline phosphatase activity, type 1 collagen formation, activity of adenylcyclase stimulated by parathormone, bone gla protein stimulated by Vitamin D3 or osteocalcin can be used in order to define the osteoblastic characteristics.[12]

In our future studies, we would like to make use of the above mentioned osteogenic inductors, and proliferate and smear the osteoblasts on poly (L-lactic acid) and polyethylene moulds, transferring them onto the established bone defects for biological repair.

In conclusion, our findings support that cells obtained by histochemical determination and staining method are similar to the osteoblast cells in our previous study[1], and that these cells can stem from the osteoprogenitor cells, which have multifacet differentiation ability. Those cells can be used during in vivo analysis of the biological effect of biomaterials for orthopedic use.

References

1. Bilir A, Ceyhan T, Altınoz MA, Guneri AD, Bayrak I, Altug T. Culturability of osteoblast cells extracted from mature and fetal BALB/c mice calvaria. [Article in Turkish] Acta Orthop Trauvmatol Turc 2000;34:389-95.
2. Ross MH, Romrell LJ, Kaye GI, editors. Histology: a text and atlas. 3rd ed. Baltimore: Lippincott Williams & Wilkins; l995.
3. Muschler GF, Boehm C, Easley K. Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. J Bone Joint Surg [Am] 1998; 80:302.
4. Dennis JE, Merriam A, Awadallah A, Yoo JU, Johnstone B, Caplan AI. A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse. J Bone Miner Res 1999;14:700-9.
5. Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol 1998;176:57-66.
6. Grigoriadis AE, Heersche JN, Aubin JE. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J Cell Biol 1988;106:2139-51.
7. Encina NR, Billotte WG, Hofmann MC. Immunomagnetic isolation of osteoprogenitors from human bone marrow stroma. Lab Invest 1999;79:449-57.
8. Islam A, Steiner R. Primary culture of marrow core in collagen gels: modulation and transformation of endosteal cells. I. Morphologic observations. J Med 1989;20:241-50.
9. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg [Am] 1998;80:985-96.
10. Bellows CG, Ciaccia A, Heersche JN. Osteoprogenitor cells in cell populations derived from mouse and rat calvaria differ in their response to corticosterone, cortisol, and cortisone. Bone 1998;23:119-25.
11. Hughes FJ, McCulloch CA. Stimulation of the differentiation of osteogenic rat bone marrow stromal cells by osteoblast cultures. Lab Invest 1991;64:617-22.
12. Yamamoto T, Ecarot B, Glorieux FH. In vivo osteogenic activity of isolated human bone cells. J Bone Miner Res 1991;6:45-51.