ORIGINAL ARTICLE

Effects of the Medium Type, Serum Amount and Dexamethasone Induction on the Cell Proliferation and Alkaline Phosphatase Activity of Twice-passaged SD Rats' Bone Marrow Stromal Cells

Masayuki TAIRA[1], Naoyuki CHOSA[2], Setsuo SAITOH[1], Kaori SASAKI[1], Nobuko SATO[2], Junzo TAKAHASHI[3] and Yoshima ARAKI[1]

[1]Department of Dental Materials Science and Technology, Iwate Medical University School of Dentistry, 1-3-27, Chuo-dori, Morioka, Iwate 020-8505, Japan
[2]Department of Biochemistry, Iwate Medical University School of Dentistry 1-3-27, Chuo-dori, Morioka Iwate 020-8505, Japan
[3]Department of Oromaxillofacial Regeneration, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan
Corresponding author: Dr.Masayuki Taira
e-mail: mtaira@iwate-med.ac.jp

SYNOPSIS

The purpose of this investigation was to examine the effects of medium type (α -MEM or DMEM); serum amount (5, 10 or 15%); and dexamethasone (Dex) induction (Dex (-) or Dex (+)) on the proliferation and alkaline phosphatase (ALP) activity of twice-passaged SD rat's bone marrow stromal cells. (1) After 6 days culture (before confluence), cell number and total protein concentration were measured. It was found that the serum amount played the dominant role in increasing the proliferation. (2) After 24 days culture (long after confluence), total protein concentration and ALP activity were measured. It was found that α -MEM was superior to DMEM in producing larger total protein concentrations. Incrementing the serum amount tended to the increase in total protein concentrations. Dex induction (Dex(+)) decreased values of ALP activity / total protein concentration, reflecting the advance of the osteogenic differentiation stage. The use of Dex (+) α -MEM medium was preferred to produce greater protein production with higher osteogenic differentiation level

***Key words**: Bone marrow stromal cells, Cell proliferation, Osteogenic differentiation*

INTRODUCTION

Recently, tissue engineering[1)] using bio-absorbable scaffold materials such as collagen[2)] and polylactide[3)] has attracted the attention in dentistry and medicine. Primary cells such as bone marrow[4)] and growth factors such as bone morphogenetic protein (BMP)[5)] have been incorpo-

rated into these scaffold materials. The resultant constructs consisting of scaffold materials, primary cells and growth factors, when inserted surgically, allow damaged oral tissues such as lost mandibular bones to be quickly and firmly restored[6].

Bone marrow contains multi-potent stem cells, which can be recruited into the osteogenic lineage[7]. It is now highly expected to collect tiny amounts of bone marrow and to expand bone marrow-derived stromal cells quickly *in vitro*, followed by culturing in scaffold materials[8]. The culture method of bone marrow stromal cells has not been, however, standardized, yet. The choice of medium (often, α-MEM or DMEM) and the serum amount (10% or 15%) are not obvious and often empirical[9]. Expression of a particular cell phenotype in culture (e.g. osteoblast) depends on the culture medium[9], culture time[10] and the presence of compounds that influence the osteogenic differentiation such as Dex-containing supplements[11-13]. At present, it seems quite important to specify the medium type, serum amount and the need of Dex induction (Dex(+)) suitable (1) for quick cell proliferation and (2) for long differentiation - extra-cellular matrix secretion of bone marrow stromal cells, respectively.

The purpose of this study was, therefore, to examine the effects of medium type (α-MEM or DMEM); serum amount (5, 10 or 15%); and Dex induction (Dex (-) or Dex (+)) (1) on the cell proliferation of twice-passaged SD rat's bone marrow stromal cells cultured for short 6 days and (2) on the total protein concentration and ALP activity of these cells cultured for long 24 days. ALP activity was evaluated as one initial marker of osteogenic differentiation[12].

MATERIALS AND METHODS

Two 5-week-old Sprague-Dawley (SD) male rats were sacrificed by overdose of anesthesia (Nembutal, Abbott Laboratory, Chicago, IL., U.S.A.) and four femora were aseptically removed. The surgery and animal care conformed to "Guideline for the Care and Use of Animals of Iwate Medical University". Bone marrow was then flushed out from femora and cultured in two 75cm^2 tissue culture flasks (Corning, NY, U.S.A) containing 10ml α-MEM medium (α-minimum essential medium, Cat. No.=12571-063, Lot No.=1115465, Life Technologies, Rockville, MD, U.S.A) supplemented with 10% fetal bovine serum (Cat. No. 10099-141, Lot No.=A0249984, Life Technologies, Rockville, MD, U.S.A.) and 2% antibiotics (penicillin-streptomycin-amphotericin, Cat. No.=15240-096, Life Technologies, Rockville, MD, U.S.A.) in a 5% CO_2 incubator (MCO-17AIC, Sanyo Electric,

Osaka, Japan) at 37℃ for 10 days while the medium was exchanged every 2 days. Stromal cells were collected by trypsinization with 3ml phosphate buffered saline solution (PBS(-)) containing 0.08% trypsin (Cat. No. 15090-046, Life Technologies, Rockville, MD, U.S.A.) and 0.14% EDTA (Cat No. 15576-010, Life Technologies, Rockville, MD, U.S.A.) and sub-cultured twice by 1:3 ratios for additional 10 days. Twice-passaged cells were then collected by trypsinization and suspended in the medium at the cell density of 10^5/ml.

Twelve culture media were prepared by the combination of two original media (α-MEM or DMEM (Dulbecco's modified Eagle medium, Cat. No. 11885-084, Lot No.=1117645, Life Technologies, Rockville, MD, U.S.A.)); three serum amounts (5, 10 or 15%); and absence or presence of 10^{-8}M dexamethasone (Sigma Chemical, St. Louis, MO, U.S.A.) supplemented with 50 μg/ml vitamin C (L(+) ascorbic acid, Code 012-04802, Wako Chemical, Osaka, Japan) and 10mM Na β-glycerophosphate (Sigma Chemical, St. Louis, MO, U.S.A.) (Dex (-) or Dex (+)). Two original media examined had different formulations in the following fashion. α-MEM had fewer nutrients with less amino acids and vitamins but contained vitamin C at the concentration of 50 μg /ml, while DMEM had more nutrients with more amino acids and vitamins but included no vitamin C.

The subsequent two culture tests of twice-passaged rat bone marrow stromal cells were performed in this study.

(1) Short-term 6 days culture for the evaluation of cell proliferation: The twice-passaged cells at the cell number of $2x10^4$ were plated in 24-well microplates, and cultured for 6 days employing above 12 prepared media while the medium was exchanged every two days. Four wells were used to each prepared medium. Then, cells in two wells were collected by trypsinization and their number were directly counted by a haemocytometer on an inverted phase-contrast light microscopy. The cells in the remaining two wells were also dissolved in 120 μl solubilizing buffer which was 20mM Tris-HCl solution containing 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 1% Triton X (pH=7.4) following twice wash with PBS (-). The dissolved total protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL, U.S.A.) and a microplate reader (MPR-A4i, TOSOH, Tokyo, Japan).

(2) Long-term 24 days culture for the evaluation of protein production and ALP activity: The twice-passaged cells at the cell number of $2x10^4$ were plated in 60mm culture dishes, and cultured for 24 days using above prepared 12 media while the

medium was exchanged every two days. Two dishes were used to each medium. Cells were then dissolved in 240 µl solubilizing buffer. The total protein concentration in the half of the solubilizing buffer (120 µl) was measured by BCA protein assay kit, and ALP activities in the other half solubilizing buffer (120 µl) was determined by ALP test B kit (Wako Chemical, Osaka, Japan) employing a microplate reader. One BL scale of the ALP activity labeled in this study represented 1 mmol of p-nitrophenol liberated from 1L sample after 1 hour's incubation at 37℃.

Statistical analysis was performed by a computer software (StatView-J 5, SAS Institute Co., Cary, N.C., U.S.A.) with unpaired t-test.

RESULTS

(1) Short-term culture for the evaluation of cell proliferation:

Fig. 1 indicates cell numbers and total protein concentrations of twice-passaged SD rat's bone marrow stromal cells cultured for 6 days with 12 prepared media. There existed a strong positive linear relationship between cell number and total protein concentration (r=0.905). It became apparent that the serum amount dominantly determined the short-term cell proliferation expressed by both cell number and total protein amount in the manner that more serum amount lead to more cell proliferation for four typed media such as Dex (-) α-MEM, Dex (+) α-MEM, Dex (-)

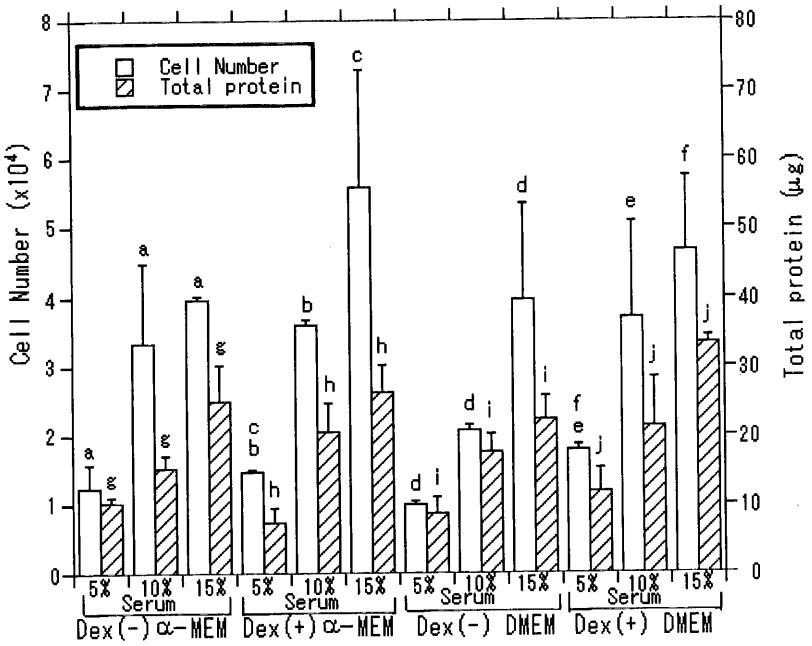

Fig. 1 Cell numbers and total protein concentrations of twice-passaged SD rat's bone marrow stromal cells cultured for 6 days with 12 prepared media. (n=6, 2 wells x 3 repetition of the measurements) Note: The group labeled by same alphabetical letters had statistically significant difference ($P<0.05$).

DMEM and Dex (+) DMEM. It was also found that the serum amount of more than 10% was required to exceed the original plated cell number ($2x10^4$). The medium type (α-MEM or DMEM) did not produce the difference in the cell proliferation as can be seen from the comparison between Dex (-) α-MEM and Dex (-) DMEM and those between Dex (+) α-MEM and Dex (+) DMEM over three different serum amounts. Dex induction (Dex (+)) produced a slight increase in the cell proliferation, as can be seen from the comparison between Dex (-) α-MEM and Dex (+) α-MEM and those between Dex (-) DMEM and Dex (+) DMEM over three different serum levels.

(2) Long-term culture for the evaluation of protein production and ALP activity:

Cells lived beyond the confluent stage and tended to be embedded in apparent extra-cellular matrix, and were hard to count directly.

Fig. 2 shows total protein concentrations and ALP activities of twice-passaged SD rat's bone marrow stromal cells cultured for 24 days with 12 prepared media. It became clear that the medium type had a significant influence on the total protein concentration in that α-MEM produced more total proteins than DMEM as can be

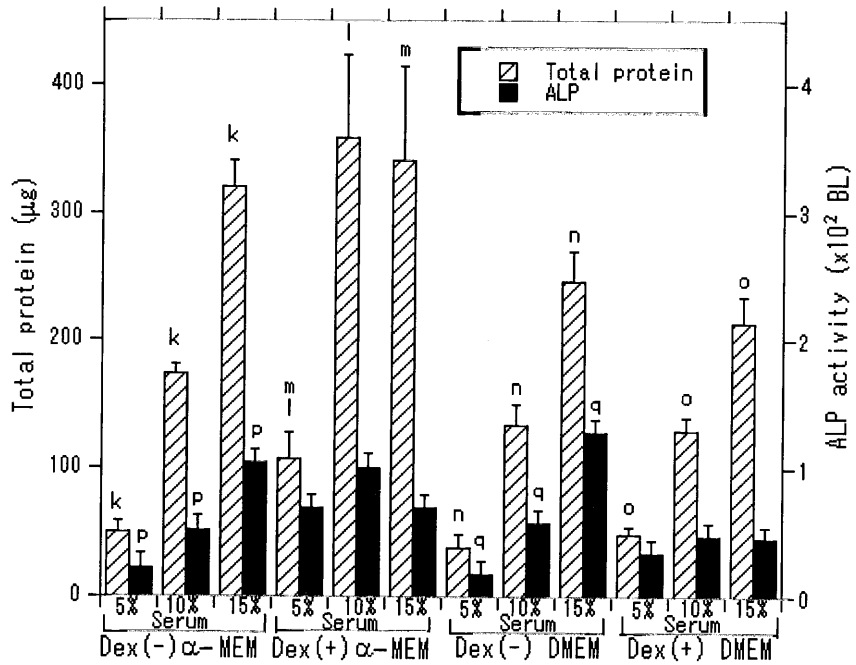

Fig. 2 Total protein concentrations and ALP activities of twice-passaged SD rat's bone marrow stromal cells cultured for 24 days with 12 prepared media. (n=6, 2 wells x 3 repetition of the measurements) Note: The group labeled by same alphabetical letters had statistically significant difference ($P<0.05$).

seen from the comparison between Dex (-) α-MEM and Dex (-) DMEM and those between Dex (+) α-MEM and Dex (+) DMEM over three different serum amounts. It was also noticed that the increment in the serum amount tended to produce more total protein concentrations for 4 typed media such as Dex (-) α-MEM, Dex (+) α-MEM, Dex (-) DMEM and Dex (+) DMEM. When cultured in α-MEM, Dex induction (Dex(+)) tended to bring about more total protein concentrations than Dex (-) control over three different serum amounts. When cultured in DMEM, however, Dex induction (Dex(+)) did not produce any increase in the total protein concentrations relative to those of Dex (-) control over three different serum amounts. As for ALP activities, the effect of Dex induction was significant. When cultured without Dex induction (i.e. Dex (-) α-MEM and Dex (-) DMEM media), more ALP activities were observed with the addition of the serum amount. On the other hand, when cultured with Dex induction (i.e. Dex (+) α-MEM and Dex (+) DMEM media), no systematic change in ALP activities was found with incrementing the serum amount. Fig. 3 depicts the values of ALP activity divided by corresponding total protein concentration. When cultured without Dex induction (i.e. Dex (-) α-MEM and Dex (-) DMEM media), these values remained quasi-constant

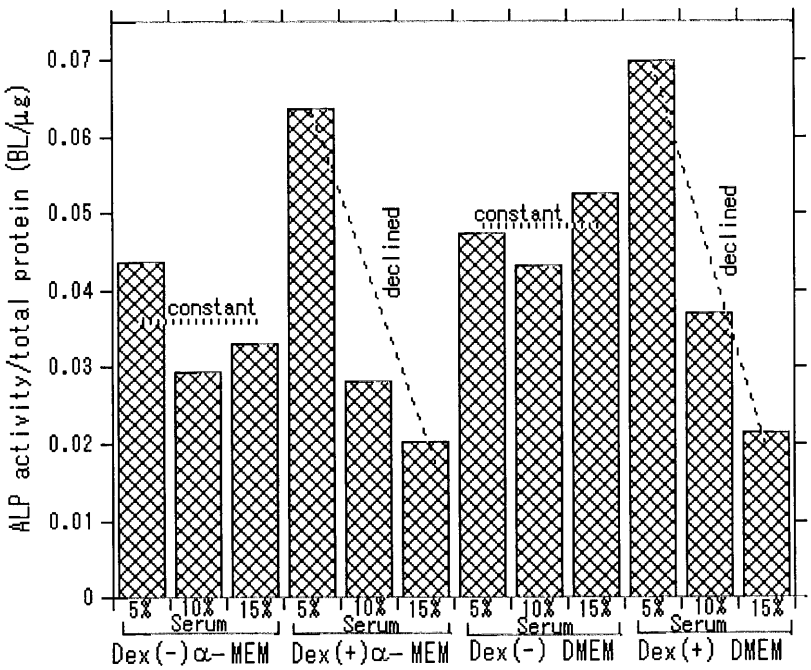

Fig. 3 The values of ALP activity divided by total protein concentration recorded when twice-passaged SD rat's bone marrow stromal cells were cultured for 24 days with 12 prepared media.

over three different serum amounts. On the other hand, when cultured with Dex induction (i.e. Dex (+) α-MEM and Dex (+) DMEM media), these values significantly declined with increasing the serum amount.

DISCUSSION

Bone marrow stromal cells are heterogeneous, but contain many osteoprogenitor cells including mesenchymal stem cells[14], and it is desirable to expand them from tiny amounts of bone marrow aspirates, to embed proliferating stromal cells into biodegradable scaffold materials and later to facilitate or initiate the osteogenic differentiation of the cells to certain extent concomitant with extra-cellular matrix protein secretion[2]. Because only limited amounts of bone marrow can be obtained in the clinical situation, twice-passaged cells were used in this study so that large amounts of cells could be collected and examined. It should be, however, noted that the cell passage number had the considerable influence on the osteogenic differentiation of bone marrow stromal cells. It has been reported that primary bone marrow stromal cells were capable of fully differentiating into matured osteoblast with its phenotype expression characterized by osteoblast-specific osteocalcin protein, while twice-passaged cells were somewhat limited in their ability of osteogenic differentiation[9]. With all the limitation of osteogenic differentiation, twice-passaged bone marrow stromal cells appear to be still useful for future therapeutic uses in osseous defects (i.e. tissue engineering for bone repair).

Serum used with a specific Lot number was selected here because the manufacturer recommended it as being suitable for the culture of osteoblasts. Because bovine fetal serum contains many unknown nutrition and growth factors, less amount of the serum (e.g. 10% instead of 15%) is generally probable for reliable and reproducible cell culture. In this regards, no serum culture and the cell culture using autologous (patient's own) serum might be future research topics.

Usually, α-MEM was employed for the culture of osteoblasts[12], whilst both α-MEM and DMEM were used for the culture of bone marrow stromal cells[9]. This study was, therefore, undertaken to distinguish the role of two media on the proliferation and osteogenic differentiation of twice-passaged bone marrow stromal cells. Concerning Dex induction, three additives were known to have the following roles. Vitamin C increases the secretion of collagen protein, a major constituent of the collageneous extra-cellular matrix[9]. It should be borne in mind here that concentrations of vitamin C in Dex (-) α-MEM, Dex (+) α-MEM, Dex (-) DMEM

and Dex (+) DMEM used in this study were 50, 100, 0 and 50 μ g/ml, respectively. Na β-glycerophosphate provides abundant phosphate ions so that the formation of non-collageneous extra-cellular matrix and calcified nodules (i.e. calcium-phosphate deposits) might be accelerated[11]. Dexamethasone promotes the osteogenic differentiation with the aid of vitamin C and Na β-glycerophosphate[11].

Considering the aforementioned generalized idea, the experimental results are reviewed here.

For short-term 6 days culture, the degree of cell proliferation was visualized by two parameters such as cell number and total protein concentration that were linearly correlated. Because the cells were fibroblastic and spread on the culture dishes, the detected total protein might come from the cells themselves with little extra-cellular matrix secretion. The cell proliferation was significantly increased with incrementing the serum amount in the medium, was slightly increased by Dex (+) supplement, and was not influenced by the medium type (i.e. α-MEM or DMEM) (Fig. 1). For quick proliferation of bone marrow stromal cells, therefore, it appears reasonable to use Dex (-) α-MEM or DMEM media with the largest serum amount of 15%. For quick proliferation with less differentiation, however, Dex (-) DMEM with 15% serum is preferred to Dex (-) α-MEM with 15% serum since the former does not include vitamin C which has the potential to slightly accelerate the differentiation process. Dex (+) media are excluded here because of the risk to facilitate undesirable early differentiation.

For long-term 24 days culture, the situation dramatically changed. Cells were embedded in extra-cellular matrix. The majority of total protein might originate from collagenous and non-collagenous extra-cellular matrix proteins. The medium type and the serum amount played major roles in determining the total protein concentration in that α-MEM produced more proteins than DMEM and that more serum amount lead to increased total protein concentrations (Fig. 2). It is reasonable to see that more serum amount in the medium resulted in more total protein concentrations due to increased nutrition and growth factors. It appears that α-MEM could produce more total proteins than DMEM partly due to larger Vitamin C contents and resultant larger collageneous ECM secretion, as can be seen from the comparison between Dex (-) α-MEM and Dex (-) DMEM and those between Dex (+) α-MEM and Dex (+) DMEM. Although Dex (-) α-MEM and Dex (+) DMEM had the same vitamin C concentration (50 μ g/ml), the former exhibited the larger total protein concentration at the serum

amounts of 10 and 15% (Fig. 2) probably because the former was not susceptible to the strong differentiation movement exerted by Dex induction (Dex(+)). Dex induction in α-MEM increased total protein concentrations over three different serum amounts, but Dex induction in DMEM did not increase the protein (Fig. 2). The reason for this contradiction has not been well understood and is still under evaluation. In Dex (+) DMEM, the role of vitamin C might be retarded by other biological system. Referring to ALP activity, all cells were ALP positive with their magnitude varied in 12 prepared culture media (Fig. 2). The values of ALP activity / total protein concentration (Fig. 3) appear to hint the real state of osteogenic differentiation level. Without Dex supplemented with vitamin C and Na β-glycerophosphate (i.e. Dex (-)), values of ALP activity / total protein concentration were quasi-constant high with irrespective to the serum amount and the medium type (Fig. 3), suggesting that the osteogenic differentiation was in the similar beginning stage and stagnated. On the other hand, with Dex induction (Dex(+)), these values of both α-MEM and DMEM declined with increasing the serum amount (Fig. 3). This phenomenon might reflect the shift of the osteogenic differentiation of bone marrow stromal cells from the earlier stage to the middle-later stage, in which the values of ALP activity / total protein gradually declined. When the osteogenic differentiation stage progressed, non-collageneous extracellular matrix proteins such as osteopontin and bone sialo protein might be produced with relatively less secretion of ALP and collageneous protein[15].

For long-term culture of bone-marrow stromal cells in bio-absorbable scaffold materials prior to re-implantation *in vivo*, the use of Dex (+) α-MEM with 10% serum might be recommended because it produced large protein production (Fig. 2) with relatively high differentiation level (Fig. 3).

For the near future, the culture of human mesenchymal stem cells will be an important research interest. After successful cell collection from bone marrow stromal cells, it will be necessary (1) to expand these stem cells without differentiation for short time and (2) to culture theses cells in absorbable scaffold materials with controlled differentiation direction and extra-cellular matrix secretion.

This study appears to provide basic data for the selection of the medium, serum and Dex induction supplemented with vitamin C and Na β-glycerophosphate (Dex (+)) when culturing not only bone marrow stromal cells but also mesenchymal stem cells.

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