BIOMEDICAL AND ENVIRONMENTAL SCIENCES **20**, 91-98 (2007)

Three-dimensional Expansion: In Suspension Culture of SD Rat's Osteoblasts in a Rotating Wall Vessel Bioreactor[1]

KE-DONG SONG[*], TIAN-QING LIU[*,2], XIANG-QIN LI[*], ZHAN-FENG CUI[#], XIANG-YU SUN[*], AND XUE-HU MA[*]

[*]*Stem Cell and Tissue Engineering Laboratory, State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian 116024, Liaoning, China;* [#]*Department of Engineering Science, Oxford University, Parks Road, Oxford OX1 3PJ, U. K.*

Objective To study large-scale expansion of SD (Sprague-Dawley) rat's osteoblasts in suspension culture in a rotating wall vessel bioreactor (RWVB). **Methods** The bioreactor rotation speeds were adjusted in the range of 0 to 20 rpm, which could provide low shear on the microcarriers around 1 dyn/cm^2. The cells were isolated via sequential digestions of neonatal (less than 3 days old) SD rat calvaria. After the primary culture and several passages, the cells were seeded onto the microcarriers and cultivated in T-flask, spinner flask and RWVB respectively. During the culture period, the cells were counted and observed under the inverted microscope for morphology every 12 h. After 7 days, the cells were evaluated with scanning electron microscope (SEM) for histological examination of the aggregates. Also, the hematoxylin-eosin (HE) staining and alkaline phosphatase (ALP) staining were performed. Moreover, von-Kossa staining and Alizarin Red S staining were carried out for mineralized nodule formation. **Results** The results showed that in RWVB, the cells could be expanded by more than ten times and they presented better morphology and vitality and stronger ability to form bones. **Conclusions** The developed RWVB can provide the culture environment with a relatively low shear force and necessary three-dimensional (3D) interactions among cells and is suitable for osteopath expansion *in vitro*.

Key words: Osteoblast; Bioreactor; Microcarrier; Tissue engineering; Laminar flow

INTRODUCTION

The great demand for a feasible method to restore skeletal defects has stimulated a large number of physicians and medical researchers to investigate novel therapies. A new method that could overcome the drawbacks of the existing therapies would remarkably improve the quality of life for people suffering from bone loss as a result of trauma, tumor resections or skeletal abnormalities[1-3]. Tissue engineering has been defined as the application of principles and methods of engineering and life sciences for the development of biological substitutes, to restore, maintain or improve tissue function[4]. Bone tissue engineering has a variety of advantages over conventional approaches: i) It can greatly reduce the limitation of donor shortages. ii) Immunologic rejection may be avoided even with allotransplantation. iii) The shapes and dimensions of artificial biomaterials can be designed and moulded on the basis of different tissues and practical requirements. The purpose of bone tissue engineering is to offer a sort of ideal biotic active bone tissue with many virtues, such as widespread source, convenient application and definitive curative effect[5]. Osteoblasts, as the most reliable seeded cells for bone tissue engineering, must be expanded *in vitro* before being seeded into scaffolds. Therefore, the large-scale culture and expansion of osteoblasts in bioreactor are one of the most essential issues in tissue engineering for treating bone coloboma.

Bioreactor technology has become the most common and effective method for large-scale expansion of animal and plant cells since Van succeeded in culturing animal anchoring cells in suspension cultures on the microcarrier in the 1960s[6]. To date, several kinds of bioreactors have been applied to various types of animal cells, such as

[1]This work was supported by grants from the Int. Cooperation Project for National & Abroad Lab. of the National Natural Sciences Foundation of China (No. 2002008) and The Science & Technology Foundation of Liaoning Province (No. 20022140).
[2]Correspondence should be addressed to Prof. Tian-Qing LIU, Stem Cell and Tissue Engineering Laboratory, State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian 116024, Liaoning, China. Tel/Fax: 86-411-84706360. E-mail: liutq@dlut.edu.cn
Biographical note of the first author: Ke-Dong SONG, male, born in 1978, Ph. D., majoring in the field of bone tissue engineering.

0895-3988/2007
CN 11-2816/Q

stirred-tank reactor[7], airlift bioreactor[8], hollow-fiber membrane bioreactor[9], fluidized bed bioreactor[10], fixed bed bioreactor[11], and packed bed bioreactor[12]. However, cells cultured in these bioreactors would inevitably suffer from shear stress and bioreactors mentioned above have difficulties in providing suitable microenvironment for cell communication similar to that inside the human body.

RWVB is a novel device for cultivating cells and tissues simulating the microenvironment *in vivo*[13]. In this bioreactor, shear stress acting on cells is low and can be well controlled. The maximum fluid shear stress is estimated by assuming uniform flow passing a single microcarrier and using the Stokes equation described by Botchwey and his co-workers[14]:

$\sigma = -3\mu V/2a$, where σ is shear stress, μ is viscosity, V is flow velocity, and a is the diameter of the microcarrier. There are great deals of 3D communications among cells. Therefore the functions of cells and tissues cultured in RWVB may be much more close to natural ones.

The efficacy of RWVB for the generation of tissue equivalents has been demonstrated using endothelial cells[15], cardiac cells[16], chondrocytes[17], prostate[18], and melanoma cancer-derived cells[19]. On the basis of these studies, it has been proposed that RWVB can support the engineering of tissue development and function[20].

In this paper, the growth and expansion of osteoblasts were investigated and compared to the three different culture systems: static culture, spinner flasks and RWVB. The function of all the expanded osteoblasts was estimated with relative technologies including morphology observation and histological staining.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% neonatal bovine serum and 1% antibiotics, Trypsin/EDTA (ethylenediamine tetraacetic acid), phosphate-buffered saline (PBS) and type II collagenase was purchased from Gibco Company. Other reagents including lactic acid and glucose reagent detection kits were purchased from Nanjing Jiangcheng Bioengineering Institute. Microcarrier is cytodex-3 (Solohill Engineering, INC.). Spinner flask was purchased from Bellco Company. Several systems were used in the experiments, including inverted phase contrast microscope (IX70-131 Olympus, Japan), scanning electron microscope (SEM, JEOL-1200EX, Japan), UV/VIS spectrophotometer (V-560 SCOJΛ), osmometer (VAPRO, USA), microplate reader (Sunrise, Australia), digital color camera (Sony 3) and image analyzer (Image-pro-plus, Cold Spring, USA) systems. RWVB developed by our team is shown in Fig. 1.

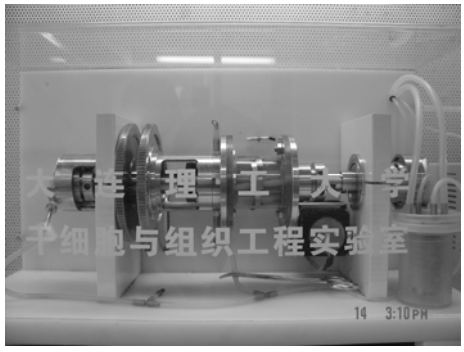

FIG. 1. Rotating Wall Vessel bioreactor (RWVB) culture system.

Primary and Passage Culture of Osteoblasts

Osteoblasts, isolated from the calvaria of neonatal SD rats by an enzymatic digestive process, were pooled, pelleted, and resuspended in a known amount of media. The cells were counted with a haemacytometer and diluted to either 1×10^6 cells·mL^{-1} or 0.5×10^6 cells·mL^{-1} in fresh media. Cells were grown in an incubator, which was maintained at 37℃, 5% CO_2. The medium was changed on day 3. After 2 passages, the cells were ready to be used in the experiments.

Cell Culture in T-Flask, Spinner Flask, and RWVB

Suitable amount of cytodex-3 microcarriers was weighed under the concentration of 10 mg·mL^{-1}, and dipped into PBS for 24 h, then sterilized for 30 min at 121℃ in autoclave.

The diluted osteoblasts were inoculated into T-35 culture flasks and 100 mL-spinner flask respectively at the concentration of 4×10^4 cells·mL^{-1}. One hundred mL fresh medium was supplemented into spinner flask. The above flasks with osteoblasts were moved to the incubator at 37℃ in an atmosphere containing 5% CO_2 with 85% relative humidity.

The spinner flask was rotated at a speed of 20 rpm in the initial 6 h, and then at 40 rpm. The medium was changed about 10 mL every 2 days. For every 12 h, the cell suspension was collected and centrifuged at 1000 rpm for 5 min to separate the cells adhering to macrocarriers from medium. Then the cells were counted with a haemacytometer after being separated from macrocarriers. For glucose and lactic acid determinations, the supernatants as samples were collected and stored at -20℃ for later analysis.

The cells adhering to microcarriers were inoculated in RWVB after being pre-cultured in T-flask for 24 h. Meanwhile, the RWVB system was controlled at 37℃ in an atmosphere containing 5% CO_2 and the medium was circulated with a peristaltic pump. Then RWVB was adjusted to 10 rpm for the purpose that the aggregates of cells and microcarriers could rotate in a relative ideal trajectory to avoid collide attachment to the bioreactor walls. The rotating RWVB was speeded up gradually with the aggregates' size increasing. During the experiment, one third of the medium was changed every 2 days and the subsequent cell counting was carried out with the same method as above.

Aggregate Morphology Observation

The samples were collected everyday and the cell morphologies on the microcarriers were observed under the inverted phase contrast microscope.

Scanning Electron Microscopy Analysis of Aggregates

Cultures were fixed and dehydrated through ascending ethanol solutions (50%, 70%, 80%, 90%, and 100%) for 15 min each time, and then immediately dried with hexamethyldisilazane solution for 5 min. Critical point drying of the samples was followed by gold sputtering. The preparations were examined under SEM at 40 Kv[21].

Glucose Consumption and Lactic Acid Production

Ten μL samples were collected to determine the glucose and lactic acid concentrations with UV/VIS Spectrophotometer.

Glucose consumption was calculated according to the following equation:

$C_{glucose}$ (mmol/L) = (Sample OD/Standard OD)×$C_{calibrating\ liquid}$ (mmol/L)

Lactic acid concentration was calculated with the equation as follows:

$C_{lactic\ acid}$ (mmol/L) = ((A1-A2)/(A3-A2))× $C_{normal(3mmol/L)}$×Diloting multiples

Where, A1, A2, and A3 are the OD of assay tube, vacant tube and standard tube respectively.

Measurement of Ph Value and Osmolarity in Media

The osmolarity and pH value of the medium were measured with osmometer and pH meter respectively.

HE Staining and Alkaline Phosphatase Staining

The cell morphologies were observed by HE staining, the cell phenotype and differentiation were marked by ALP staining. Osteoblasts were seeded onto the cover slips at the concentration of 1×10^5 cells·mL^{-1} and cultured in DMEM, supplemented with 10% neonatal bovine serum in incubator. After 24 h, the cover slips were rinsed slightly three times in PBS to remove the media and fixed in 10% formalin solution for 30 min. Then the cover slips were rinsed slightly again with ultrapurified water to remove the formalin solution, air-dried and placed into the incubator. After 4 h, the cover slips were rinsed with ultrapurified water again. Then the samples were dipped into 2% cobaltous nitrate solution for 5 min. Subsequently, the cover slips were rinsed 3 times with ultrapurified water. Then the samples were dipped into 1% ammonium sulphide solution for 2 min and mounted with neutral gum.

Finally, the samples were placed under the inverted microscope as previously described[22] and the cell morphologies were observed with the objective of 10 times. Ten random pictures of the adhesion samples were taken by digital color camera system (Sony 3 CCD) and the cell number in every picture was counted by the image analyzer system (Image-Pro-Plus).

Calcium Staining (Von-Kossa Staining and Alizarin Red S Staining)

Usually, the mineralization of nodules in cultures could be assessed with von-Kossa staining for that calcium usually co-precipitates with phosphate ion *in vitro* culture conditions. The staining procedure was as follows: The specimens were prepared with the same method as the above. The cover slips were rinsed slightly in PBS and treated with 5% silver nitrate solution (100 μL/well) at 37℃ for 30 min. The excess silver nitrate solution was rinsed away with ultrapurified water and the culture plate was dipped into formaldehyde solution for 5 min. The mineralization of von-Kossa stained cover slips was observed under the inverted microscope.

Alizarin Red S staining is a standard method for visualization of nodular and calcium deposition of osteoblast cultures *in vitro*. Here, the mineralization of extracellular matrix was marked with Alizarin Red S staining with slight modifications as described previously[23]. The procedure was as follows: The osteoblasts were rinsed slightly in PBS twice and fixed in 95% ethanol at -20℃ for 15 min followed by several rinses with ultrapurified water. Then the cells were stained with 0.1% Alizarin Red S for 15 min at room temperature followed by incubation with PBS for 30 min. Thereafter, the cell culture plates were air-dried and red staining of Alizarin Red S was identified by light microscopy.

Statistical Methods

The data were expressed as $\bar{x} \pm s$. Statistical

differences were analyzed with *t* test or analysis of variance. $P<0.05$ was considered statistically significant.

RESULTS

Cell Morphology Observation Under Inverted Microscope

The cell morphologies on the microcarriers cultured in different fashions is shown in Fig. 2. Few aggregates were viewed after cultivation for 112 h in T-flask and a few in spinner flask; while in RWVB, large amount of the microcarriers aggregating through extracellular matrix could be seen.

The aggregates in RWVB clearly demonstrate the good communication of cells due to the 3D environment offered by RWVB, displaying its advantage over the conventional static culture and spinner flask.

Aggregate Topography Examination Under Scanning Electron Microscope

SEM examination of the aggregates was performed to ensure the regular growth of osteoblasts on the microcarriers. As shown in Fig. 3, osteoblasts were well-settled around the microcarriers and the cells readily adhered to the microcarriers and formed floating cell-microcarrier aggregates.

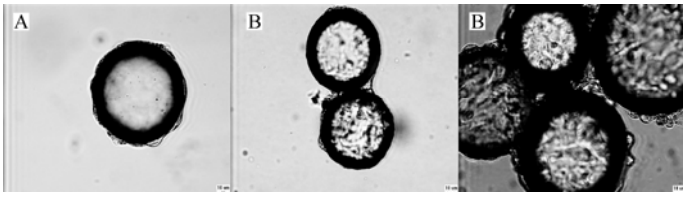

FIG. 2. Sizes and morphologies of aggregates of osteoblasts growing on cytodex-3 microcarriers cultured in different fashions for 112 h. (A) T-flask, (B) Spinner flask, (C) RWVB; Magnification, 200×.

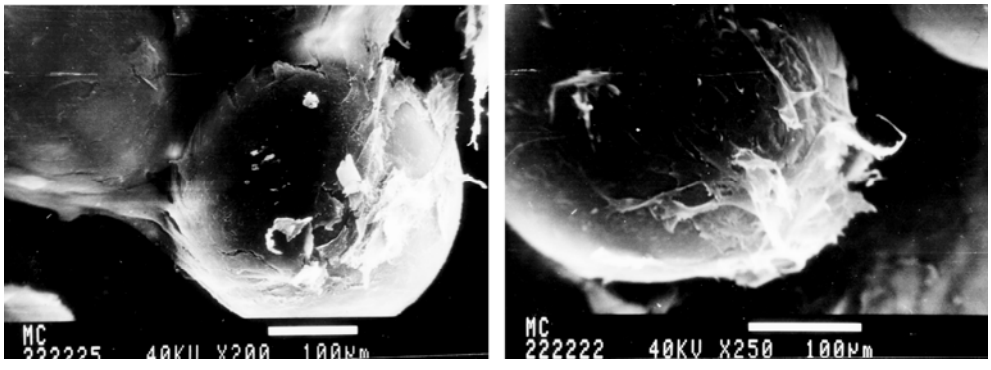

FIG. 3. SEM micrographs showing co-culture of osteoblasts and microcarriers in a 7-day culture in RWVB. (A) A portion of an aggregate formed by osteoblasts and microcarriers; (B) The extracellular matrix on monomicrocarrier indicating early stage of mineralization.

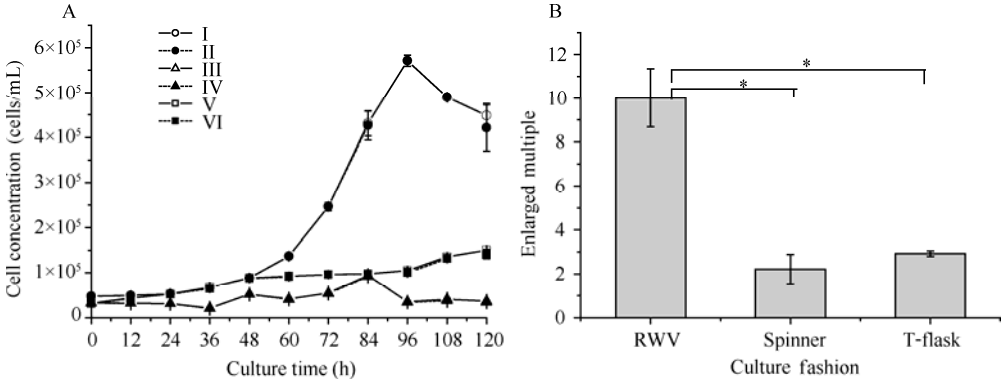

FIG. 4. Growth and expansion of osteoblasts in different culture systems. (A) The growth curves of osteoblast in different culture fashions. (B) The comparison of osteoblast expansion in different culture fashions. I, II: Total and viable cell concentration in RWVB; III, IV: Total and viable cell concentration in spinner flask; V, VI: Total and viable cell concentration in T-flasks. $^{*}P<0.01$.

Expansion of Osteoblast

The growth curves and the enlarged multiple of osteoblasts in the three culture systems are shown in Figs. 4A, 4B respectively, where the error bars correspond to the standard deviation and $P<0.01$ (compared to T-flask and spinner flask).

As Fig. 4A shows, osteoblast concentration in T-flasks increased slowly during the whole culture process. The reasons were that contacting restraint would appear after the cell concentration achieved a specific degree, causing the restricted expansion.

The osteoblast concentration in spinner flask presented a slight decrease during the first 36 h due to the fact that when the osteoblasts cultivated in static conditions were inoculated into the spinner flask, they needed to be adaptive to the dynamic environment. Consequently, a fraction of the cells would drop off from the microcarriers and even some cells suffering from shearing force would die. After this phase, the osteoblasts exhibited a slight expanding trend.

The concentration of osteoblasts in RWVB increased quickly after the cells were adapted to the 3D environment and numerous aggregates were present in the medium. Furthermore, the osteoblast metabolism in RWVB was active for the medium color changed continuously (carmine → nacarat → saffron).

The direct columnar diagram of cell expansion was obtained, indicating that cells in RWVB expanded much more than in the other two culture systems (Figs. 4A and 4B).

pH and Osmolarity of Medium and Metabolism of Glucose and Lactic Acid

The nutrient metabolism in the three culture systems was measured and compared (Fig. 5). The concentration of glucose in RWVB was higher than in the other two culture fashions (Fig. 5A), and the concentration of lactic acid in T-flask was the highest and minimum in spinner flask (Fig. 5B).

The above finding could be explained by the fact that in RWVB, the medium was partially changed through peristaltic pump and supplemented with oxygen through oxygen pot continuously, which provided the cells with relative fresh nutrient and partial metabolites could be removed, as lactic acid and ammonia. While in spinner flask, although the medium was well-distributed, the higher shearing force and the worse oxygen permeation were disadvantageous for the cell growth, which made the cells much inactive and slow in metabolism. Additionally, in T-flask, contacting inhibition would occur among the anchoring cells and less fresh medium could be got. Once the osteoblasts grew into monolayer, they would compete with others for

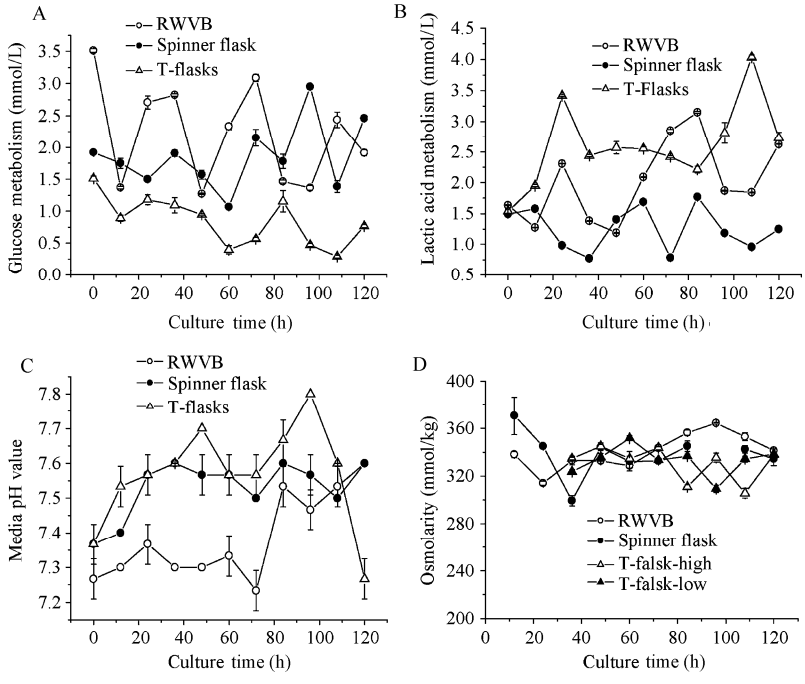

FIG. 5. Nutrient metabolism, osmolarity, and pH values of medium in different culture systems. (A) Glucose metabolism; (B) Lactic acid metabolism; (C) pH value; (D) Osmolarity value.

nutrient. So the metabolites would be accumulated more and more with time and the lactic acid concentration rose to the highest in the three culture systems.

The pH and osmolarity values of medium were measured every 12 h during these experiments and the results are shown in Figs. 5C-D respectively.

As Fig. 5C shows, the pH values of the medium ranged 7.3-7.8 in spinner flask and T-flask during the whole culture process, but in RWVB, the pH values were almost maintained at 7.3 in the exponential growth period of cells except for a rise in the later period of culture. Since the optimal range of pH value in culturing animal cells was 7.2-7.4, the culture conditions in RWVB were relatively good in the three culture systems.

Medium osmolarity for the culture of osteoblasts *in vitro* should also be maintained within a suitable range around 340 mmol·kg^{-1}. From Fig. 5D, it can be seen that all the medium osmolarities in the three culture systems could meet the cell growth and the osmolarity changed least.

HE Staining and ALP Staining

The histological differences of osteoblasts expanded in the three culture systems were observed under the inverted microscope with HE and ALP staining (Fig. 6).

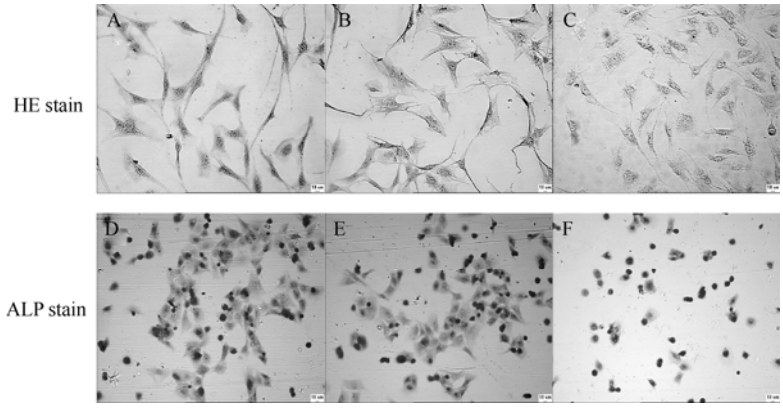

FIG. 6. HE and ALP staining of osteoblasts cultured in different conditions. (A) RWVB; (B) T-flasks; (C) spinner flask with HE; and (D) RWVB; (E) T-flasks; (F) spinner flask with ALP. Magnification, 100×. RWVB significantly increased the active expression of ALP.

The osteoblasts incubated in RWVB and T-flask grew better and presented better morphologies. However, the cells incubated in spinner flask grew inactively and secreted less extracellular matrix (ECM).

ALP, a transient early marker of osteoblasts[22] is usually used to assess the osteoblastic characteristics of the isolated cells[24-25]. The cells cultured in RWVB and T-flasks presented higher ALP active expression. Normal phenotype, biggish bulk, numerous pseudopodia and rich cytoplasm and bulky nuclei were all situated on one side. Many blue-black granules of ALP positive reactions existed in cellular matrix and the majority of the positive cells presented squamate. Especially, ALP positive reactions were enhanced in the cell aggregates. These osteoblasts with high expression of ALP showed high activity,while the osteoblasts cultured in spinner flask presented weak ALP positive reactions, indicating that the cells were inactive and had a worse spread.

Mineralization of the Extracellular Matrix (Von-Kossa and Alizarin Red S Staining)

Von-Kossa staining of mineralized nodules could present the end differentiation of osteoblasts[22]. Mineralized and non-mineralized nodules could be distinguished (Figs. 7A-C). The mineralized nodules mainly covering the surfaces of the sheets were quite different in the different culture systems.

Mineralization and nodule formation could be also determined with Alizarin Red S staining (Figs. 7D-F). Alizarin Red S staining increased obviously in aggregates cultured in RWVB and T-flask compared with that in spinner flask, where there was little staining in the aggregates.

Nodule Formation and Distribution

Figure 8 shows the total number and area of nodules. Here, the nodule area was defined as the dark brown-black stained area.

As Fig. 8A shows, the number of nodules in RWVB

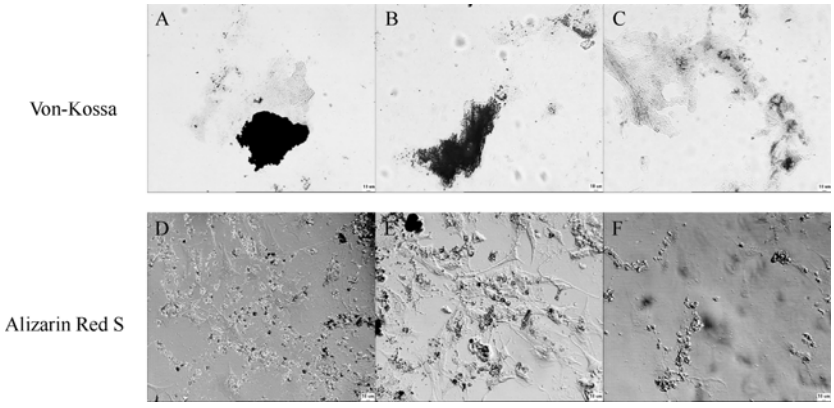

FIG. 7. Staining of calcium with von-Kossa and Alizarin Red S of osteoblasts cultured in different conditions (A) RWVB, (B) T-flasks, (C) spinner flask with von-Kossa; and (D) RWVB, (E) T-flasks, (F) Spinner flask with Alizarin Red S, Magnification, 100×. RWVB significantly increased the number and size of nodules on day 30.

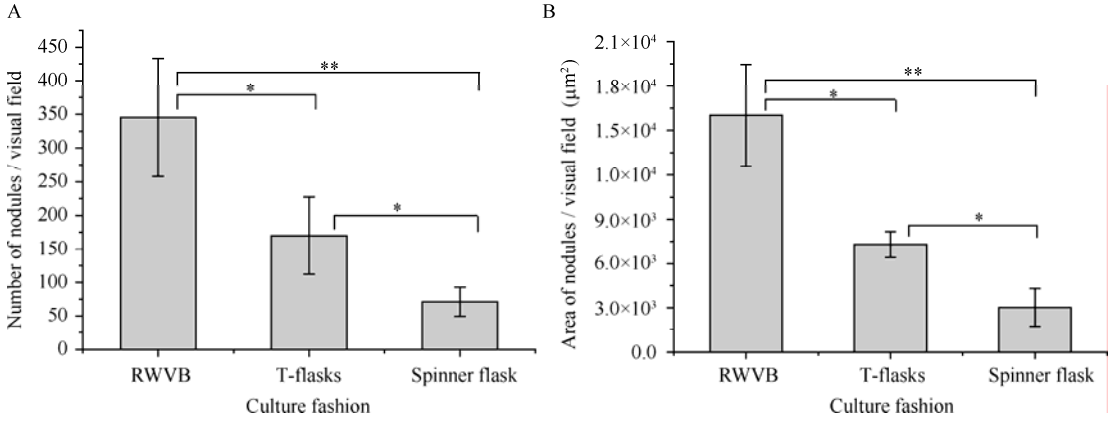

FIG. 8. Nodule Formation and Distribution with Alizarin Red S staining of cells cultured in different fashions (A) Total number of nodules, (B) Total area of nodules; Data are expressed as $\bar{x} \pm s$; *Different from T-flasks and spinner flask, $P<0.05$; **significant difference under $P<0.01$.

was significantly larger then in T-flask and least in spinner flask. The state about calcified nodular area of the cells in Fig. 8B was the same as that in Fig. 8A.

DISCUSSION

The concentration of microcarriers must be fit for the inoculated concentration of osteoblasts to achieve the ideal cell expansion. Hu[26] expounded the conception of critically inoculated quantity of cells and he considered that anchoring cells could grow only when the number of them on one microcarrier exceeded a constant value. This study has proved that the osteoblasts can grow and expand better in RWVB at the inoculated concentration of 4.8×10^4 cells·mL^{-1}.

There is evidence that the osteoblasts cultured in RWVB can maintain their phenotype and are better differentiated compared with conventional cultures. For example, the traditional static culture in T-flask maintains the cells unilaminar and allows cell-cell and cell-matrix interactions. Although osteoblasts cultured in T-flask could form nodules, the amount and densification of these nodules are less than those of cells cultured in RWVB. Additionally, the cells hardly adhere to the substratum and the cell-cell and cell-matrix relationships are poor though the cells are maintained in suspension culture.

The whole culture vessel of RWVB is driven by generator and rotates along horizontal axis. The aggregates turn with the vessel in the same direction of circular cylinders and do not collide with the wall and other objects. Moreover, there are no bubbles and stirrer in the whole culture system, which causes the destructive stress to be abated. The fluid flow is laminar and dynamic in RWVB, so the aggregates have much more active nutrient and oxygen metabolism compared with the other two systems, and then the hydrodynamic stimulation of inner convection could enhance the nutrient transferring into aggregates formed by cells and microcarriers and accelerate the proliferation of osteoblasts and stimulate the synthesis of DNA distinctly. In contrast,

in the spinner flask, the flow is turbulent and shear force is much higher[27].

The pH and osmolarity values in RWVB can be adjusted quickly and retained at a relative steady state because the osteoblast metabolites can be partially discharged through the continuous medium change and the nutrients can be circulated effectively and consumed sufficiently.

This demonstrates that a dynamic laminar flow generated by a rotating fluid environment is an important and efficient way to provide hydrodynamic stimulation and reduce diffusional limitations of nutrients and wastes while producing low levels of shear[28]. By comparing the results in conventional static culture and spinner flask，the osteoblasts exhibit better ability to expand in RWVB owing to the better culture environment with low shearing force and good cell communication on 3D. With the stress stimulation inside the fluid in RWVB, the active expression of ALP can be increased, and the formation of mineralized nodules can be accelerated. The rapid proliferation and differentiation of osteoblasts are possible and the expansion of seeded cells on 3D in large-scale of tissue engineering could be realized. The 3D suspension culture fashions of RWVB results in better cell response than in the other two culture fashions, spinner flask and T-flasks.

Furthermore, if the expanded cells were seeded into a proper scaffold and cultured in RWVB, the tissue engineered bone might be well developed, which has been finished in the other part of our work. Therefore, RWVB is a relatively ideal 3D culture system for both osteoblast expansion and engineered tissue fabrication *in vitro*.

REFERENCES

1. Ren L, Tsuru K, Hayakawa S, *et al.* (2002). Novel approach to fabricate porous gelatin-siloxane hybrids for bone tissue engineering. *Biomaterials* **23**, 4765-4773.
2. Ishaug-Riley S L, Crane-Kruger G M, Yaszemski M J, *et al.* (2003). Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* **19**, 1405-1412.
3. Partridge K A, Yang X, Clarke N M P, *et al.* (2002). Adenoviral BMP-2 Gene Transfer in Mesenchymal Stem Cells: *In vitro* and *in vivo* Bone Formation on Biodegradable Polymer Scaffolds. *Biochem Bioph Res Co* **292**, 144-152.
4. Langer R (1993). Tissue engineering. *Science* **260**, 529.
5. Vacanti C A, Vacanti J P (1994). Tissue-engineered morphojenesis of cartilage and bone by means of cells transplantation using synthetic biodegradable polymer matrices. *J Clin plast Surg* **21**, 445-451.
6. Van wezel A L. (1967). Growth of cell-strains and primary cells on microcarriers in homogeneous culture. *Nature* **216**, 64.
7. Freed L E, Vunjak N G (1997). Microgravity tissue engineering. *J In vitro Cell Dev Biol-Animal* **33**, 381-385.
8. Tan W S, Liu J, Zhang Y X (1996). Application of the airlift bioreactor for hybridom a cell cultures. *Chin J Biotechnol* **12**, 477-481.
9. Herma G, Tarja J, Lemke H D (2001). Monitoring of cell viability and cell growth in a hollow-fiber bioreactor by use of the dye Alamar Blue. *J Immunol Methods* **252**, 131-138.
10. Xin J Y, Cui J R, Chen J B, *et al.* (2003). Continuous biocatalytic synthesis of epoxypropane using a biofilm reactor. *Process Biochemistry* **38**, 1739-1746.
11. Meißner P, Schröder B, Herfurth C, *et al.* (1999). Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells. *J Cytotechnology* **30**, 227-234.
12. Jose' Manuel Go'mez, Domingo C (2003). Kinetic study of biological ferrous sulphate oxidation by iron-oxidising bacteria in continuous stirred tank and packed bed bioreactors. *Process Biochemistry* **38**, 867-875.
13. Schwarz R P, Goodwin T J, Wolf D A (1992). Cell culture for three-dimensional moldeling in rotating-wall vessels: an application of simulated microgravity. *J Tissue Cult Method* **14**, 51-58.
14. Botchwey E A, Pollack S R, Levine, *et al.* (2001). Bone Tissue Engineering in a Rotating Bioreactor Using a Microcarrier Matrix System. *J Biomed Mater Res* **55**, 242-253.
15. Sanford G L, Ellerson D, Melhado-Gardner C, *et al.* (2002). Three-dimensional growth of endothelial cells in the microgravity-based rotating wall vessel bioreactor. *In vitro Cell Dev Biol Anim* **38**, 493-504.
16. Carrier R L, Papadaki M, Rupnick M, *et al.* (1999). Cardiac tissue engineering: cell seeding, cultivation parameters, and tissue construct characterization. *Biotechnol Bioeng* **64**, 580-589.
17. Vunjak-Novalovic G, Martin I, Obradovic B, *et al.* (1999). Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue engineered cartilage. *J Orthop Res* **17**, 130-138.
18. Rhee H W, Zhau H E, Pathak S, *et al.* (2001). Permanent phenotypic and genotypic changes of prostate cancer cells cultured under a three-dimensional rotating wall vessel. *In vitro Cell Dev Biol Anim* **37**, 127-140.
19. Licato L L, Prieto V G, Grimm E A (2001). A novel preclinical model of human malignant melanoma utilizing bioreactor rotating wall vessels. *In vitro Cell Dev Biol Anim* **37**, 21-126.
20. Unsworth B R, Lelkes P I (1998). Growing tissues in microgravity. *Nat Med* **4**, 901-907.
21. Osanoa E, Kishib J, Takahashic Y (2003). Phagocytosis of titanium particles and necrosis in TNF-a-resistant mouse sarcoma L929 cells. *Toxicology in vitro* **17**, 41-47.
22. Sun J S, Chang Walter H S, Chen L T, *et al.* (2004). The influence on gene-expression profiling of osteoblasts behavior following treatment with the ionic products of sintered beta-dicalcium pyrophosphate dissolution. *Biomaterials* **5**, 607-616.
23. Wong G L, Cohn D V (1975). Target cells in bone for parathormone and calcitonin are different: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. *Proc Natl Acad Sci USA* **72**, 3167-3171.
24. Chaudhary L R, Hofmeister A M, Hruska K A (2004). Differential growth factor control of bone formation through osteoprogenitor differentiation. *Bone* **34**, 402-411.
25. Doherty M J, Schlag G, Schwarz N, *et al.* (1994). Biocompatibility of xenogeneic bone, commercially available coral, a bioceramic and tissue sealant for human osteoblasts. *Biomaterials* **15**, 601-608.
26. Hu W S (1982). Large-scale mammalian cell culture. *Biotech and Boeings* **24**(2), 365-369.
27. Goldstein A S, Juarez T M, Helmke C D, *et al.* (2001). Effect of convection on osteoblastic cell growth and function in biodegradable polymer foam scaffolds. *Biomaterials* **22**, 1279-1288.
28. Martin I, Wendt D, Heberer M (2004). The role of bioreactors in tissue engineering. *Trends Biotechnol* **22**, 80-86.

(*Received October* 10, 2005 *Accepted August* 4, 2006)