The experimental study of cryopreserved osteoblasts combined with bioactive glass repairing mandibular defects in rabbits

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Purpose: The purpose of this study was to observe the biological character of cultured periosteal-derived osteoblasts (POBs) preserved in liquid nitrogen in vitro, and to have a preliminary study on the osteogenetic capability of bioactive glass ceramics (BGC) combined with POBs.

Materials and Methods: The POBs were acquired from New Zealand big white rabbit and after several generations culture some of them were kept in liquid nitrogen. The cryopreserved cells for six months were cultured in DMEM medium and examined by morphological and histological observation. Then those growing well in vitro were seeded into the porous BGC materials. A week later, the combined materials were implanted into the bone defects of rabbits' mandible, the control groups were implanted into the single BGC (no cells). After two, four, eight, and 12 weeks of operation, the specimens were respectively excised and examined by X-ray and histological chemistry observation.

Results: The cryopreserved POBs grew well in vitro and also had the typical characters of mature osteoblasts. Cultured with BGC materials, the cells could attach grow and proliferate well on the surface of most endopores. After four weeks of operation the transplanted osteoblasts began to form new osteoid or bone-like tissue in most pores of implanted BGC, and the bone defects were repaired better and earlier.

Conclusion: It was practical to use the cryopreserved osteoblasts as seeded cell for further study on bone tissue engineering. It suggested that the "living" bone substitute would get more application and play a more important role in bone restoration and reconstruction. **(Int Chin J Dent 2003; 3: 91-99.)**

Clinical Significance: The results of this study demonstrate that the porous bioactive glass ceramics combined with cryopreserved osteoblasts can repair the bone loss of rabbits' mandible, and what we have done will do good to future clinical application.

Key Words: bioactive glass ceramics, bone tissue engineering, bone defcents, cell culture, cryopreserved osteoblast.

Introduction

With the rapid development of cell biology and material biology in the past 10 years, great progress has been made on bone tissue engineering. It was an exciting direction to restore and reconstruct bone defects by using correlative techniques of tissue engineering. Many scholars[1-7] had been being engaged in such

studies as the osteoblasts culture and amplification, cell transplantation, mixed-cultivation between the cells and some bioactive materials. Most of the osteoblasts they used as the seeded cell were freshly acquired from the host body, and a repeated process of the cells' segregation, purification, amplification and identification would be done in every experiment. Then it became very important to establish an "osteoblast bank" and use the crypopreserved cells to do the studies on bone tissue engineering. In our study, we chose the cryopreserved periosteal-derived osteoblasts (POBs) as the seeded cell, the bioactive glass ceramics (BGC) as the cell carrier, and after having been mixed- cultured in vitro for one week, the combined BGC was transplanted into the mandibular defects of rabbit. Through some examinations we would observe the osteogenesis of the transplanted osteoblasts and discuss the possibility of cryopreserved osteoblasts transplantation.

Materials and Methods

Cell Isolation, Culture, and Cryopreservation

The POBs were obtained from newborn rabbits of the New Zealand strain. The tibia periosteum was excised aseptically, cleaned of other soft tissues, sheared into pieces, and digested in 0.25% trypsin for 30 minutes, 1% collagenase for two hours. The released cells were collected in plastic culture flasks, and the culture medium was Dulbecco modified eagle medium (DMEM) containing 20% fetal bovine serum (FBS), the culture condition was in a humidified atmosphere consisting of 5% CO_2 at 37˚C, and 100% relative humidity. When the cells in primary culture grew to approach confluence, they were digested by 0.25% trypsin and subcultured in new flasks. The cultured cells of fourth to sixth generations growing well were chosen to reduce the temperature step by step to -100˚C and then kept in liquid nitrogen (the freezing medium included 10% dimethyl sulfoxide, 40% FBS, 50% DMEM).

Cell Anabiosis and Identification

The cells preserved in liquid nitrogen for six months were revived and continued to subculture until to get enough cells. Some cells were examined by alkaline phosphatase (ALP) stain (using the method of modified Gomori's). Some were cultured in conditioned medium (concluding 10% FBS, 10 mmol/L ß-sodium glycerophosphate, 50 mg/L vitamin C) for 30 days, and then stained in situ with alizarin red S to observe the bone-like nodes forming. Other cells were used in the followed experiment.

BGC Preparation and Mixed-cultivation with Osteoblasts in Vitro

All the porous BGC materials were prefabricated nubbly shapes (10x8x3 mm), cleaned by ultrasound for 30 minutes, washed by distilled water three times, then sterilized for later use.

The anabiotic osteoblasts were diluted by DMEM concluding 20% FBS until a density of $(4\text{-}5)\text{x}10^6$/mL, and each 0.1 mL cell suspension was seeded into the porous BGC which had been put in the 12-well plastic culture plate. Then they were cultured in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37˚C, and 100% relative humidity. And 30 minutes later more DMEM was added to the wells until on the upper surface of BGC. The medium was changed every second day.

After mixed-cultured in vitro one week, some combined BGC species combining many osteoblasts were

examined by scanning electron microscope, others were used in the followed experiments.

Animal Experiment

A total of 12 mature New Zealand big white rabbits were chose and randomly divided into four groups, and each group had three rabbits. Bone defects were made in all the rabbits' bilateral mandible, and one side was implanted the combined BGC (as the experimental group), the other side was implanted the single BGC (as the control group).

The operation procedure as follows: Rabbits were anaesthetized by intravenous injection of 3% sodium pentobarbital (30 mg/kg weight). Under sterile conditions the skin on the mandibular infraborder before the masseter muscle attachment was incised, and the mandibular body was exposed. With the help of dentistry drill, bone defect was made the similar size as BGC implant (10x8x3 mm). The combined BGC implant were then fixed in the position of bone loss, and the host periosteum was well protected and sutured. The skin and other soft tissues were closed demixingly. The wound was open for air. The operation of control groups was as the above. All the experimental rabbits were given cidomycin for three days after operation, and feed routine diet.

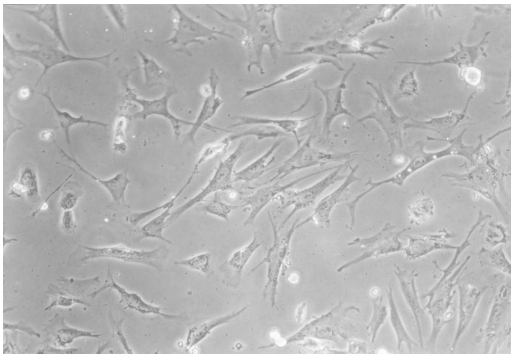
At two, four, six, and eight weeks after operation, three rabbits of one group were killed and the whole mandibles were harvested. The specimens including the implant and some surrounding bone tissue were carefully excised, and first of all examined by X-ray under same conditions with the dental digital X-ray imaging system, fixed in formalin, decalcified in 5% azotic acid for four days, and then embedded in paraffin. Sections of 5-7 μm in thickness were deparaffinized for hematoxylin and eosin staining, and then were observed under general light microscope.

Results

The primary POBs at the firstly beginning were round monocyte. One day later, they began to stick onto the floor of the culture flasks, then they extended and disfigured as the polygonal appearance. After cultured three to four days, the adherent cells began to grow and proliferate quickly, which exhibited morphologies ranging from the spindle-shape typical of fobroblasts to polygonal-shape. At eight to nine days, the adherent cells reached confluency and mainly shaped in spindle. The anabiotic cells had the similar morphologies as subcultured cells, and after growing in vitro for three days, they also expressed significantly high ALP activity, which showed mass gray or black deposit in the cytoplasm of most cultured cells. After cultured in conditioned medium for 30 days, the anabiotic cells formed mineralized nodes which took on the red, round shapes by stain of alizarin red S (Figs. 1-3).

When mixed-cultured in vitro with porous BGC, the osteoblasts could stick to the surface of most endopores of the material, and continue to grow and proliferate, even excrete extra celluar matrix (ECM) (Fig. 4).

The X-ray results and histological results of combined BGC repairing mandibular defects of rabbits in different time were showed in Tables 1 and 2, and Figs. 5 and 6.



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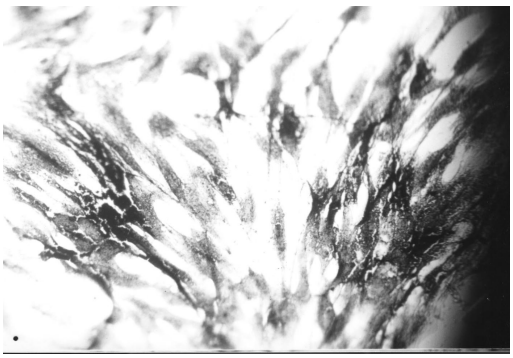

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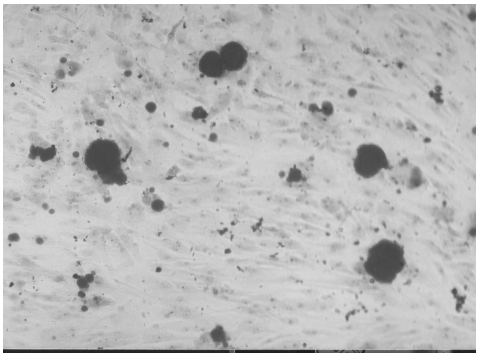
Fig. 1. After 24 hours cultured in vitro, the anabiotic osteoblasts were mainly spindle-shaped or polygonal-shaped (Inverted phase-contrast microscope, x200).

Fig. 2. After three days cultured in vitro, the anabiotic osteoblasts were stained black or brown in cytoplasm (Modified Gomori's method, x400).



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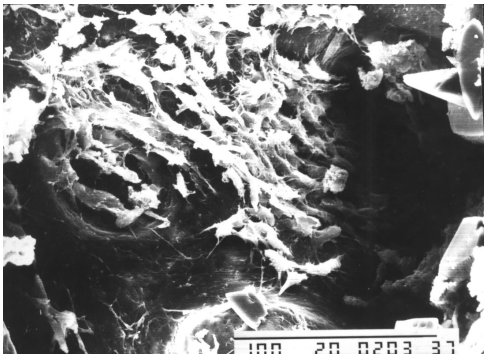

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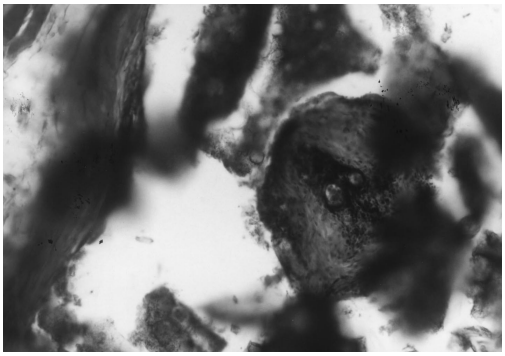
Fig. 3. After 30 days cultured in conditioned medium in vitro, the osteoblasts formed round minerlization nodes stained red (Red alizarin stain, x100).

Fig. 4. After one week of the cultured cells combined with BGC in vitro, many cells grew and proliferated on the surface of most endopores (SEM examination, x500).



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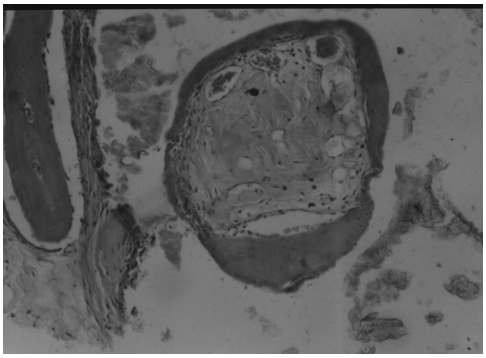

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Fig. 5. After eight weeks of the combined materials implanted into the bone defects, the transplanted osteoblasts formed osteoid tissue (HE stain, x100).

Fig. 6. After 12 weeks of the combined materials implanted into the bone defects, the transplanted osteoblasts formed new bone tissue (HE stain, x100).

Table 1. X-ray results of combined BGC repairing bone defects in different time.

Time (Post-operation)	Combined BGC (Experimental group)	Single BGC (Control group)
2 weeks	Apparent border between BGC and host bone, local density significantly lower than surrounding bone tissue.	Similar as the experimental group.
4 weeks	The border was clouding, synostosis began to form, local density was near to that of surrounding host bone.	The border was also distinct, less synotosis formed, lower density than host bone.
8 weeks	The border was unclear, synostosis completely formed, local density was as high as surrounding bone.	The border became clouding, local density was near to host bone tissue.
12 weeks	The border disappeared, BGC implant completely repaired the bone defects.	Nearly the same as the experimental group.

Table 2. Histological results of combined BGC repairing bone defects in different time.

Time (Post-operation)	Combined BGC (Experimental group)	Single BGC (Control group)
2 weeks	Fibrous tissue grew into surrounding pores, new blood vessel could be seen; the adherent cells in most endopores grew well ,excreted much ECM.	Fibrous tissue grew into the the surrounding pores of BGC; no cells could be seen in most endopores.
4 weeks	Osteoid formed on the border; new bone-like tissue could be seen in most endopores, a few of lymphocytes and acidophil granulation cells were seen around the new tissue.	Bone-like tissue could be seen only on the border, no new tissue formed in endopores, some lymphocytes and gigantocells could be seen.
8 weeks	Many fresh bone tissue formed in most endopores; synotosis began to form on the border.	Osteogenesis could also be seen in the pores of the border, no bone formed in endopores.
12 weeks	In most endopores many lamellar-like bone tissue formed, vasculogenesis was seen much.	Synotosis formed between the BGC and host bone,no new bone was found in endopores.

Discussion

The Choice of the Seeded Cell

The seeded cell of bone tissue engineering was the osteoblast, which could be acquired by many ways in vivo. Such as the bone marrow stromal cells (BMSCs) from bone marrow, the periosteal-derived osteoblasts (POBs) from periosteum, the osteoblasts from bone tissue and the heterotopic mesenchymal stem cells from other tissues. At present the BMSCs and the POBs were applied much more than others on

the experimental studies. We chose the young rabbit' POBs as the seeded cell, and found that they not only grew and proliferated well in vitro but also could be induced to differentiate into mature osteoblasts. We didn't see the aging change in all the passages (in our experiment the cultured cells were observed by 20th generation). These results were also verificated by other scholars.

The periosteum concluded such cells as osteoblasts, fibroblasts, blood cells, blood vessel endothelial cells and so on. After digested by pancreatin and collagenase, the periosteum were dissected into the above-mentioned cells, which were mixed in the primary cell culture. Blood cells were suspended cells, which could not stick to the bottle floor in vitro and were cleaned gradually in the process of the changing of culture medium. Blood vessel endothelial cells were much less than osteoblasts and couldn't grow for a long time without the stimulation of some definite growth factor. The fibroblasts were similar as osteoblasts in appearance but were less in quantities, and under some definite conditions they could be induced to the cells having some characters of osteoblasts. Thus through unceasing cell culture and passages, the periosteum cells were gradually converted into the more pure cell (osteoblasts, or osteoblast-like cell) line.

The Superiority of Using the Cryopreserved Osteoblasts

Many studies[8] had confirmed that the cultured cells in vitro could be kept in a low temperature circumstance for a long time, and the anabiotic cells also had the similar principal functions as before. Our experiment showed that the cryopreserved osteoblasts had not only a high ability of growth and proliferation but also the typical functions of mature osteoblasts such as the synthesis of ALP and bone-like tissue. This suggested that it was practical to establish an "osteoblast bank" preserving cultured cells and use the cryopreserved cells to work as the seeded cell of bone tissue engineering. We knew that the cultured cells grew quickly in vitro, and if we didn't take some measures to preserve them, there would be too many cells to use, which would result in much meaningless consumption. At the same time too many passages might make the cultured cells becoming aging, old, or even dead. Thus it was essential to choose the cultured cells growing well in vitro to preserve in liquid nitrogen for future use. The quick development of modern low temperature medicine could also make this being possible.

There were many advantages for us to use the anabiotic osteoblasts to carry on studies on the bone tissue engineering: (1) Timing: Whenever we had the demand for osteoblasts we could resuscitate the cryopreserved cells to culture and proliferate. (2) Saving: It needed only the liquid nitrogen jar for keeping the cells, which avoided much expense of the primary and secondary cell culture. (3) Repeating: The anabiotic osteoblasts from one same bottle had the similar growing behavior and biological function. And after several generations' amplification we could acquire enough cells and use them for the contrast experimental research.

Choice of the Cell Carrier

Cell carrier[3-4] was some material that could provide an enough space for cells' growth and transplantion. If the cultured osteoblasts were only injected into the body in the manner of liquid, new bone-like tissue would not form or form little, which was mainly because the transplanted cells couldn't keep the effective

concentration in the local region. While the cell carrier had a porous structure, and the cultured osteoblasts could attach and grow onto the surface of the most endopores of the material. Then after transplanted into the body, the carrier could make the adherent cells form new bone-like tissue in a relatively steady surrounding. This suggested that it was significant for the bone tissue engineering to select an appropriate cell carrier.

The bioactive glass ceramic[9-12] was a sort of degradable material, which had been widespreadly used as the substitution of bone to restore and reconstruct the bone defects, especially the cranofacial bone loss. Recently many scholars[4-7] had made use of the porous bioglass as the cell carrier. Our experiment showed that the BGC material had good bio-compatibility, less inflammatory reaction and foreign body rejection. It could composite large quantities of cultured osteoblasts in vitro which continued to proliferate in vivo and then formed new bone-like tissue. The degradation ratio of BGC was very low, and at the 12th week after transplantation we only found a little portion of material being in resorption. But some scholars considered that as long as the attaching osteoblasts could grow well and form new osteoid tissue, the cell carrier materials would have good clinical value even if they were absorbed so slowly.

The Osteogenesis Principles of the Combined BGC

After the combined BGC material was transplanted into the location of the mandibular defects of rabbits, the adherent osteoblasts could also maintain the ability of growth and proliferation with the host offered nutrien, which put up a satisfactory osteogenesis potential. The combined BGC was a "living" tissue-engineered bone substitution material, which combined many osteoblasts and could repair the rabbit' mandibular bone loss better and earlier. The principles were discussed as followed.

The BGC Material had Good Bone Conductibility and Bio-compatibility

When the porous BGC was transplanted into the body, the blood, bone marrow and ECM around the operation region began to aggregate and contact with the material, and then an ion exchange occurred on the surface of the implant. As a result a sort of biochemistry couple formed between the BGC and the surrounding bone tissue. Simultaneously a layer of hydroxyapatite (HA) containing some bicarbonate compound came into being on the surface of the BGC. The HA layer, which had the similar structure as the inorganic component of common bone tissue, could be discerned and accepted by the host body. Some of the osteoblasts and collagen fibers around the operation region could deposit and grow on the surface of the HA layer. With time went on, some new bone-like tissue gradually formed in the HA layer. All these above mentioned were the rudiment of the synostosis forming between the implant and the surrounding bone tissues. Our experiment also confirmed that there would be much satisfactory synostosis formed at the edge of the implant no matter what the BGC combined the cultured osteoblasts. The X-ray and histological examination results gave the detailed interpretation.

The Role of the Transplanted Osteoblasts Combined into Most Endopores of the BGC

After cultured in vitro with the porous BGC for one week, there would be large quantities of osteoblasts attaching onto the surface of most endopores. Some experiments[4-7] in vitro suggested that the combined osteoblasts not only grew well and excreted abundant ECM but also kept the fundamental biological

characters of mature osteoblasts. At the beginning of the combined BGC implanted into the body, the attached cells began to grow by sucking some nutrien component of the surrounding micro-environment. And some time later, blood capillary began to grow into the endopores of the porous BGC. Then an exchange of the nutrien matters and metabolism products between the transplanted cells and the host body estabilished. Thus in a new circumstance these transplanted osteoblasts could continue to grow and proliferate, synthesize and excrete ECM, even form bone tissue. In our study we observed that the density of two groups at fourth week was both lower than the surrounding bone tissue (because of the porous structure). At eighth week their density all became higher than before, but that of the experimental group, which was as high as the host bone tissue, increased much more than the control group. This was mostly because the transplanted cells could form new bone in the bosom of the implant, which made the implant take on a higher density by the X-ray examination, while the osteogenesis of the control group only limited on the border of the material. The histological results also showed in the experimental group many new bone-like tissue formed within most endopores, while there was no new tissue formed in the center of the control group at all time segments. In addition, we found that the synostosis time (as displayed in X-ray) of the experimental group was earlier (at eighth week) than the control group (at 12th week). We discussed the possible causes was as followed: (1) The synostosis of the experimental group was due to the osteogenesis of both the host osteoblasts and the transplanted cells, while the control group was only due to the host osteoblasts. (2) The transplanted cells were some mature osteoblasts and could excrete some bioactive factors, which might stimulate the differentiation of the host stromal cells and augment the osteogenesis ability. But the concrete principles should be confirmed by further studies.

Conclusion

In our experiment, we used the porous BGC as the cell carrier and the cryopreserved osteoblasts as the seeded cell to replace the bone loss of rabbits' mandible, and acquired successful results were obtained. It suggested that the bone tissue engineering would become a best choice to restore human bone defects in future.

References

1. Goldstein SA, Patil PV, Moalli MR. Perspectives on tissue engineering of bone. Clin Orthop 1999; 367 Suppl: 419-23.
2. Lo H, Kadiyala S, Guggino SE. Poly(L-lactic acid) foams with cell seeding and controlled-release capacity. J Biomed Mater Res 1996; 30: 475-84.
3. Brekke JH, Toth JM. Principles of tissue engineering applied to programmable osteogenesis. J Biomed Mater Res 1998; 43: 380-98.
4. Xynos ID, Hukkanen MV, Batten JJ. Bioglass 45S5 stimulates osteoblast turnover and enhances bone formation in vitro: implications and applications for bone tissue engineering. Calcif Tissue Int 2000; 67: 321-9.
5. Effah-Kaufmann EA, Ducheyne P. Evaluation of osteoblast response to porous bioactive glass (45S5) substrates by RT-PCR analysis. Tissue Eng 2000; 6: 19-28.
6. Loty C, Sautier JM, Tan MT, Obocuf M, Jallot E. Bioactive glass stimulates in vitro osteoblast differentiation and creates a favorable template for bone tissue formation. J Bone Miner Res 2001; 16: 231-9.

7. Price N, Bendall SP, Frondoza C, Jinnah RH, Hungerford DS. Human osteoblast-like cells (MG63) proliferate on a bioactive glass surface. J Biomed Mater Res 1997; 37: 394-400.
8. Cooper LF, Harris CT, Bruder SP, Kowalski R, Kadiyala S. Incipient analysis of mesenchymal stem-cell-derived osteogenesis. J Dent Res 2001; 80: 314-20.
9. Low SB, King CJ, Krieger J. An evaluation of bioactive ceramic in the treatment of periodontal osseous defects. Int J Periodontics Restorative Dent 1997; 17: 358-67.
10. Heikkila JT, Aho HJ, Yli-Urpo A, Happonen RP, Aho AJ. Bone formation in rabbit cancellous bone defects filled with bioactive glass granules. Acta Orthop Scand 1995 66: 463-7.
11. Norton MR, Wilson J. Dental implants placed in extraction sites implanted with bioactive glass: human histology and clinical outcome. Int J Oral Maxillofac Implants 2002; 17: 249-57.
12. Piattelli A, Scarano A, Piattelli M, Coraggio F, Matarasso S. Bone regeneration using bioglass: an experimental study in rabbit tibia. J Oral Implantol 2000; 26: 257-61.

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