Effects of DMEM and RPMI 1640 on the biological behavior of dog periosteum-derived cells

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Abstract Periosteum-derived cells (PDCs) are being extensively studied as potential tissue engineering seed cells and have demonstrated tremendous promise to date. There is convincing evidence that culture medium could modulate the biological behavior of cultured cells. In this study, we investigate the effects of DMEM (low glucose) and RPMI 1640 on cell growth and cell differentiation of PDCs in vitro. PDCs isolated from Beagle dogs were maintained in DMEM and RPMI 1640, respectively. Then, the cell migration rate of periosteum tissues was analyzed. PDCs of the third passage were harvested for the study of proliferation and osteogenic activity. Proliferation was detected by MTT assay. Alkaline phosphatase activity and mineralized nodules were measured to investigate osteogenic differentiation. Our data demonstrated that DMEM induced alkaline phosphatase activity and strongly stimulated matrix mineralization in cell culture, while similar cell migration rates and proliferation behaviors were observed in the two culture conditions. Interestingly, the osteogenic differentiation of PDCs could be enhanced in DMEM compared with that in RPMI 1640. Thus, it can be ascertained that DMEM may serve as a suitable culture condition allowing osteogenic differentiation of dog PDCs.

Keywords Periosteum · Cell culture · Culture medium · Alkaline phosphatase · Tissue engineering

Instruction

New techniques based on tissue engineering have provided opportunities to new formation or regeneration of periodontal deteriorated tissues and its goal is the reconstruction of the support tissues destroyed during the disease process. For periodontal tissue engineering, sufficient supply of autologous cells is of paramount importance for the preparation of transplants. In animal experiments, periodontal ligament fibroblasts (PDLFs) (Zhang et al. 2007; Lin et al. 2008) and bone marrow stroma cell (BMSCs) (Hasegawa et al. 2006; Li et al. 2008) have been two main cell sources for periodontal tissue engineering. Recent studies have showed that grafted periosteum or periosteum-derived cells (PDCs) could enhance periodontal wound healing, and they promote alveolar bone repair, as well as facilitate periodontal membrane and cementum regeneration (Steiner et al. 2007; Mizuno et al. 2006).

Periosteum is attached in the form of a bilayered tissue membrane to the bone that consists of an outer fibrous layer and an inner cambium layer (Youn et al. 2005). The outer layer is comprised of fibroblasts and their progenitor cells immersed in the collagenous matrix. Research showed that these cells could

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produce cementum with integrated collagen fibers when placed over dentin (Groeneveld et al. 1994). The inner cambial layer contains a variety of cellular types including osteoprogenitor cells, osteoblasts (Youn et al. 2005), which seem to possess osteogenic ability that can give rise to bone. De Bari found PDCs, regardless of donor age, possessed high self-renewal capacity and expressed markers of mesenchymal stem cells (MSCs) at the single-cell level (De Bari et al. 2006; Choi et al. 2007). With these in mind, we think PDCs could be another cell source for periodontal tissue engineering.

For successful engineering of an organ or tissue, it is indispensable to provide the cells an suitable culture microenvironment for supporting their proliferation and differentiation. Now various media have been used to culture PDCs. However, limited information is available about the potential effects of culture media on the growth and osteogenic differentiation of cultured PDCs. In our context, we chose the widely used media-DMEM and RPMI 1640 as our two different types of culture conditions, which exhibit different characteristics. DMEM contains a higher concentration in calcium (1.8 mM) and a lower concentration in phosphate (1 mM) than RPMI 1640, a less nutrient-rich medium containing 0.8 mM of calcium and 5 mM of phosphate (Robert and Leslie 1997; Lopez-Cazuax et al. 2006). In addition, DMEM (low glucose) has a lower concentration of glucose (1 g/L) than RPMI 1640 (2 g/L). It has been confirmed that the components of applied media can influence cell proliferation and cell viability (Lopez-Cazuax et al. 2006; Hou et al. 2007).

In order to better determine the culture condition which favors the growth and differentiation of PDCs, we sought to compare DMEM and RPMI 1640. This study underlines the importance of culture conditions and provides the data to select an optimal culture medium for PDCs.

Materials and methods

Materials

Cell culture

Low-glucose DMEM (Gibco, Cat no. 11885), RPMI 1640 (Gibco, Cat no. 31800), Trypsin (Amresco, Cat

no. 0458), Fetal bovine serum (Hao Yang Biological Manufacture, Cat no. TBD31HB, China), Tissue culture dishes (Corning, New York, USA).

Assays

MTT (Sigma, cat no. M5655), Triton-X100 (Amresco, Cat no. 0694), Alkaline Phosphatase Detection Kit (Nanjing Jiancheng Bioengineering Institute, Cat no. A059), Dimethyl Sulfoxide (DMSO, Shanghai Chemical, China), Auto Microplate Reader (Humareader, German), IX70 inverted fluorescence microscope (Olympus, Japan), SPSS 11.0 (SPSS Inc, Chicago, USA).

Methods

Isolation and culture of PDCs

Periosteum explants were harvested from the maxillary bone of 4 adult Beagle dogs. With the dogs under general anesthesia with ketamine (1–2 mg/kg), partial-thickness incisions were made on the alveolar mucosa of premolars, and mucosa flaps were elevated, then periosteums of about 5 mm × 4 mm were carefully stripped off mechanically using forceps, washed in phosphate-buffered saline (PBS), dissected into pieces of 1 mm × 1 mm, and placed into 6-well tissue culture plates pre wetted with culture medium (3–4 periosteum pieces per well). The explants were then covered with sterilized glass cover slides of 25 mm × 20 mm and kept in DMEM or RPMI 1640, containing 10% fetal bovine serum, 100 IU/mL penicillin G and 100 mg/mL streptomycin. The fresh medium was changed twice a week and explants were checked for the outgrowth of cell daily. When 80–90% confluence was attained, the cells were digested by using 0.25% trypsin/1 mM EDTA solution. PDCs from the third passage were used in the following tests and hematoxylin-eosin staining (HE staining) was carried out to observe cell morphology.

Cell migration rate

In primary culture, the cell migration rates of periosteum tissues in DMEM and RPMI 1640 were compared, using the cell migration rate(%) = (the number of pieces with cell growth/the total number of periosteum pieces cultured) × 100%.

Cell proliferation (MTT assay)

Assessment of cell proliferation was measured in terms of optical absorbance (OD) per well by using MTT. Cells were seeded at 3,000 cells per well (96-well plates) and cultured in DMEM or RPMI 1640. On day 1 to day 7, 20 μ L of MTT was added to each well to be assayed, and the cells were further incubated at 37 °C for an additional 4 h. Supernatants were then removed from the wells and 150 μ L of Dimethyl Sulfoxide was added to each well. The solutions were mixed thoroughly for 10 min to dissolve the dark blue crystals of formazan. Proliferation was determined by a Auto Microplate Reader at the wave length of 492 nm.

Alkaline phosphatase activity (ALP)

Cells were seeded in 12-well plates at a density of 5×10^4 cells per well and cultured in complete DMEM or RPMI 1640. After 4 days and 8 days, cell layer was rinsed with PBS, lysed by adding 100 μ L of 1% Triton X-100 buffer into each well, and was then subjected two cycles of freeze-thawing to dissociate the ALP from the cell membrane. According to the kit, the lysed sampled solution was then mixed with 50 μ L buffer solution and 50 μ L substrate liquid. After 15 min reaction, 150 μ L colored solution was added to stop the enzymatic reaction. The enzymatic activity was quantified by absorbance measurement at 492 nm.

After cells became confluent, an ALP assay kit (Kaplow method) was used for the staining of osteoblast-like cells. The culture dishes were fixed with 4% formaldehyde in PBS, then washed with distilled water and allowed to dry for 15 min. Afterwards, they were incubated with alkaline phosphatase staining solution for 15 min at 37 °C and a washing step followed. The solution was prepared according to the manufacturer's instructions. Finally, the cultures were observed under microscopy.

Mineralization of the extracellular matrix (Von Kossa)

The presence of calcium phosphate deposits was analyzed histochemically by Von Kossa staining in which phosphate deposits are stained black.

Cells were seeded in 24-well plates at a density of 5×10^4 cells per well, cultured in DMEM or RPMI

1640, and observed daily. The time when nodules could be seen by naked eyes on the bottom of plates and opaque cells nodules could be observed under inverted microscope should be recorded. After the cells had been cultured for 15 days, the cultures were rinsed with PBS and fixed with 4% formaldehyde. After washed with the cold distilled water, the fixed cultures were covered with a 3% silver nitrate solution and kept for 30 min in dark. Afterwards, they were rinsed with distilled water and a 5% Sodium Thio-sulfate solution was added for 2 min. The cultures were rinsed in distilled water thoroughly, and then incubated with Neutral Red for 1 min. The areas of mineralized nodules were measured when the diameter of the nodule was $\geq 50 \mu\text{m}$ under the inverted microscope (Hildebrandt et al. 2009).

Statistical analysis

The results of the cell migration rate were analyzed by Chi-square method. Student's unpaired *t*-test was used for individual group comparison. Data of MTT, ALP and mineralization were expressed as mean $\pm$ s. The significance of the difference between means was determined by SPSS 11.0. The level of significance was set at $p < 0.05$.

Results

Effect of media on cell migration rate

In primary culture, no cell contamination or the other unexpected things happened. Cells were in good conditions in each well. The cell migration rate showed no statistical difference between the two culture media, although it is slightly higher in DMEM (88.57%) than that in RPMI 1640 (82.14%) (Table 1).

The morphology of PDCs in culture

In primary culture, cells migrated from the periosteum pieces within 3–7 days for both media (DMEM and RPMI 1640). At first, cells had spindle-shaped, triangle, round or irregular morphology, and later they became more homogeneous, mainly spindle-shaped (Fig. 1a, b). Cells near periosteum pieces reached confluence within 15–20 days. Several nodular-like

Table 1 Comparison of cell migration rate of periosteum pieces

Group	The total number of cultured periosteum pieces	The number of pieces with cell growth (cell migration rate)	The shortest time of cells migrating from pieces (days)
DMEM	35	31 (88.57)	3
RPMI 1640	28	23 (82.14)	3

The cell migration rate was not significantly different between DMEM and RPMI 1640 groups

structures were observed under inverted microscope (Fig. 1c, d). At this time, cells were digested in the same well. Passage cells grew fast and cell morphology was similar to the primary cells (Fig. 1e, f).

Effect of media on cell proliferation

Cells in DMEM and RPMI 1640 represent similar cell growth curve. As shown in Fig. 2, the OD value increased gradually from day 1 to day 3, significantly increased on day 4 and reached a peak on day 7.

Fig. 1 Morphology of dog PDCs maintained in DMEM (a, c, e) or RPMI 1640 (b, d, f). In primary culture, Standard phase contrast photomicrographs illustrated various cellular features, cell retraction with a reticular organization (a, b) and

multicellular nodules (c, d). In passage culture, HE staining showed cell morphology was more homogeneous (e, f) and no difference was found between DMEM (e) and RPMI 1640 group (f). Phase contrast microscopy. Scale bars: 100 μ m

Fig. 2 Growth curve of PDCs in DMEM and RPMI 1640. Results were the mean of duplicate cultures ($n = 6$)

From day 4, the OD value in DMEM was a little higher than that in RPMI 1640, and the difference increased as time progressed, however, there was no statistical difference ($p > 0.05$) (Fig. 2).

Effect of media on cell differentiation

Expression of ALP is an useful marker for osteoblast and for the regulation of bone formation. The result showed that cells in both culture media expressed ALP activity (Fig. 3). After 4 days in culture, cells in both media expressed ALP activity at similar level ($p > 0.05$). In further culture, the ALP activity of both media increased. On day 8, the cells cultured in DMEM expressed higher ALP activity than cells in RPMI 1640 ($p < 0.05$) (Fig. 4).

Effect of media on cell mineralization

On day 4, the first cell nodule was observed in DMEM, however, in RPMI 1640, the nodule was

Fig. 4 Comparison of ALP activity between PDCs in DMEM and in RPMI 1640. ALP activity increased in a time-dependent manner with significant upregulation on day 8 in both DMEM and RPMI 1640. No significant difference was found on day 4 between the two media. However, on day 8, the PDCs cultured in DMEM showed higher ALP activity than that in RPMI 1640. Results were the mean of duplicate cultures ($n = 6$). * $p < 0.05$

formed at 10 days after cell harvest. After further culture, new nodules were formed, and the previously formed nodules turned bigger and more sharply margined. After 15 days in culture, the areas of mineralized nodules that were stained with Von Kossa positive were quantified (Fig. 5). Both the area and area ratio of mineralized nodules were significantly increased in DMEM group ($p < 0.01$) (Table 2).

Discussion

In the field of periodontal research, tissue engineering has a number of advantages. The reconstruction of periodontal supportive tissue, especially the regeneration of alveolar bone can prevent the loss of teeth.

Fig. 3 The osteogenic differentiation of PDCs was determined by ALP staining. The ALP-positive cells with dense cytoplasmic staining was seen in DMEM (a) as well as in RPMI 1640 (b). Scale bars: 100 μ m

Fig. 5 PDCs cultured in DMEM (a, c) and RPMI 1640 (b, d) for 15 days both showed mineralized ability in vitro, meanwhile, nodules formed in DMEM showed bigger and more

densely distributed than that in RPMI 1640. Phase contrast microscopy. Scale bars: 100 μ m

Table 2 Area and area ratio of mineralization in DMEM and RPMI 1640

Group	Area of mineralized nodules (μm^2)	Area ratio of mineralized nodules (%)
DMEM	51,081.98 $\pm$ 13,209.04*	8.94 $\pm$ 2.34*
RPMI 1640	18,031.95 $\pm$ 10,802.33	3.29 $\pm$ 1.97

Compared with RPMI 1640, DMEM induced mineralization in cell culture. Both the area and the area ratio of mineralized nodules was much higher in DMEM than that in RPMI 1640

* $p < 0.01$

Lots of studies have showed periosteum is easier to obtain from patient than are bone marrow and periodontium, and periosteum is a sufficient supply of osteogenic cells (Declercq et al. 2005a, b), chondrogenic cells (Jansen et al. 2008) and mesenchymal progenitor cells (De Bari et al. 2006). An important step in tissue engineering is to find a suitable culture medium for expanding PDCs. The cells are usually cultured in DMEM (De Bari et al. 2006; Zhang et al. 2006; Zhu et al. 2006), and other culture media such as RPMI 1640 (Chen et al. 2003), α -MEM (Youn

et al. 2005; Agata et al. 2007), Ham'F-12 (Perka et al. 2000), (DMEM)/Ham'F-12 (Zheng et al. 2006) and Medium 199 (Mizuno et al. 2006; Kim and Kim 2005) has also been used for PDCs' culture. Lots of studies have revealed that different types of culture media seem to have different effects on cell proliferation and cell differentiation. Related reports can be found about PDLCs (Hou et al. 2007) and dental-derived cells (Lopez-Cazuax et al. 2006) and so on. However, few studies have attempted to find which medium could favor growth and differentiation of PDCs.

DMEM and RPMI 1640 used in this study, are generally available for supporting the growth of different types of cells. However, there are some differences in their composition, and this could potentially affect cell proliferation, cell viability and cell differentiation. RPMI 1640, at first designed for lymphocytes, is simple in ingredients and rich in amino acids. DMEM, modified from Basal Medium Eagle, is rich in nutrition and exhibits different characteristics. Glucose is a central source of energy for all cells. Common media contain varying concentrations of glucose ranging from 1 to 4.5 g/L

(5.6–25 mM) (Li et al. 2007). DMEM (low glucose) contains a lower concentration of glucose (1 g/L) than RPMI 1640 (2 g/L). In addition, DMEM has a higher concentration in calcium (1.8 mM) and a lower concentration in phosphate (1 mM) than RPMI 1640 which contains 0.8 mM of calcium and 5 mM of phosphate. So far, there have been a couple of studies about the effects of calcium and glucose on cell culture (Liu et al. 2009; Li et al. 2007).

Because it was described that the medium can influence cell growth and survival, we firstly sought to investigate the effects of two different media (DMEM and RPMI 1640) on cellular morphology and cell proliferation. Interestingly, cells derived from periosteum exhibit comparable morphological features and nodules formed in primary culture. Moreover, the rate of cell migration was not influenced by the different conditions. Based on MTT assay, our results found no statistical differences between DMEM and RPMI 1640 groups in terms of cell proliferation. The results are inconsistent with the findings of Lopez-Cazuax's and Maeno's studies, in which they found rich calcium induced cell proliferation (Maeno et al. 2005; Lopez-Cazuax et al. 2006). However, some reports also demonstrated that lower calcium concentration did not affect proliferation (Youshimura et al. 1996). The contradiction may result from different choices of cell types and calcium concentrations. In addition, glucose may also affect cell proliferation. A lot of studies have demonstrated that high glucose induced cellular senescence, while reduction of glucose enhanced proliferation (Stolzing et al. 2006; Kim et al. 2006). In our study, the concentration of glucose in RPMI 1640 is two times as high as that in DMEM, but this had not influenced the growth of PDCs. Thus, further studies are needed to understand the effect of calcium or glucose on cell growth and even the cooperation effect of calcium and glucose.

To investigate whether media affects cell differentiation, we first assessed ALP activity. ALP, as a widely used enzyme marker for PDCs, plays an important role in osteoblast differentiation and bone mineralization, both in vitro and in vivo. Our results demonstrated that PDCs in the two different media both expressed ALP activity. In the same experimental conditions, cells cultured in DMEM induced higher ALP activity at day 8. Because the OD of MTT assay was not different between the two culture

condition, the difference found in ALP activity was likely to be related to the specific activity of the enzyme. The elevated calcium and reduced glucose in DMEM could be responsible for the enhancement of the enzyme. On the one hand, extracellular Ca^{2+} could be of great importance in the regulation of osteoblastic proliferation and differentiation (Eklou-Kalonji et al. 1998). Although some previous studies evidenced that ALP activity increased in lower-calcium environment (Matsumoto 1995; Youshimura et al. 1996) or was not influenced by the different concentrations of calcium (Eklou-Kalonji et al. 1998), many studies reported that high- Ca^{2+} medium initiated a quite higher ALP activity in cell culture, and with gradual increase in calcium concentration, ALP-positive colonies was up-regulated (Maeno et al. 2005). It was also reported that rich calcium and poor phosphate medium promoted ALP activity (Lopez-Cazuax et al. 2006). On the other hand, extracellular glucose influences osteogenic differentiation (Kim et al. 2006; Li et al. 2007). In PDLCS a high glucose medium (4.5 g/L) inhibit the differentiation compared with the low glucose medium (1.1 g/L) (Kim et al. 2006). In MSCs obtained from rats high glucose induced cellular senescence, while reduction of glucose enhanced differentiation (Li et al. 2007). Thus, we can hypothesize that high glucose concentration may inhibit the osteogenic differentiation of PDCs. However, in light of different cells and different calcium or glucose concentration, a further study on the effects of calcium and glucose on the biological behavior of dog PDCs will favor our understanding of the mechanism by which DMEM favors dog PDCs' differentiation.

The most reliable parameter for final osteoblast differentiation is the formation in a controlled cell mediated way of a calcified extracellular matrix (Declercq et al. 2005a, b). To find out more about whether the media affect osteogenic differentiation, we used Von Kossa staining to analyze mineral deposition in the two media. In both media, nodules can spontaneously formed. However, nodules formed much earlier and obviously much more in DMEM than that in RPMI 1640, which meant cells cultured in DMEM had a higher nodule-forming potential, meanwhile, the mineralization in DMEM was more restricted to well-defined nodules, while the nodules in RPMI 1640 were formed in a more dispersed pattern. This further showed that DMEM promoted

the osteogenic differentiation of PDCs (Lopez-Cazuax et al. 2006). A recent study demonstrated that greater Ca^{2+} concentrations could enhance mineralization and the optimal extracellular Ca^{2+} and Pi for MSCs to proliferate and differentiate is 1.8 mM and 0.09 mM respectively, which are the concentrations supplied in DMEM (Liu et al. 2009). In addition, it was reported that reduction of glucose increased the number of colony forming units and high glucose inhibited the percentage of the calcified area (Kim et al. 2006), which is in accord with our results that the area and the area ratio of mineralized nodules were much larger in DMEM (low glucose) than that in RPMI 1640. In view of the above mentioned studies, it is reasonable to speculate that the higher concentration of calcium and lower concentration of glucose in DMEM could favor the mineralization of PDCs.

In conclusion, our study indicates that culture medium really modulates the biological behavior of dog PDCs. The choice of a suitable culture medium is an important key to cell culture. In light of our study, compared to RPMI 1640, DMEM has proved to be a more potent culture medium in terms of cell differentiation. However, in our study, both culture media investigated in this study are based on FBS-containing conditions. The use of FBS is still contradictory because of variations and possibility of xenogenic contamination (Hildebrandt et al. 2009). Therefore, serum-free conditions should be investigated for more defined culture conditions in successive test.

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