Stability of cultured dental follicle cells

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Received 7 November 2003; revision accepted 16 January 2004

Abstract. Because the dental follicle is required for tooth eruption, establishment of dental follicle cell (DFC) lines is needed for experimentation to determine how the cells regulate eruption. Thus, it is critical that the follicle cells in culture remain stable and neither become transformed nor differentiate. To determine the stability of rat DFC cultures in terms of exhibiting contact inhibition of growth when confluent (no transformation), DFC at different passages were analysed using flow cytometry. Gene expression of cyclin E was determined by reverse transcription polymerase chain reaction as a further method to determine if growth was occurring when the cells were confluent. Alkaline phosphatase and von Kossa staining were also performed as a means of determining stability in terms of differentiation; that is, are the DFC maintaining their phenotype or are they differentiating into osteoblasts and osteocytes? After plating cells of a given passage, they initially underwent a rapid phase of growth with 30–40% of the cells in S, G₂ and M (dividing track) as determined by flow cytometry. The number of such cells declined to only 7–15% at preconfluency. At late confluency, only 2 and 5% of the cells were in the dividing track in passages 6 and 9, respectively, but in passage 12 this had risen to 15%. For a given passage of cells, cyclin E gene expression significantly declined in late confluency as compared to the early growth phase. However, in passage 12, the gene expression of cyclin E at late confluency was higher than the expression at late confluency in passage 6. Thus, the DFC were remarkably stable through passage 9, but by passage 12 it appeared that a small percentage of the cells had become transformed and had lost their contact inhibition growth properties. Alkaline phosphatase and von Kossa staining were negative for all passages, suggesting that the cells remained stable in terms of differentiation and did not differentiate into either osteoblasts or osteocytes.

INTRODUCTION

Because the dental follicle plays a central role in regulating tooth eruption, establishment of dental follicle cell (DFC) lines is needed for *in vitro* studies of gene expression, signal transduction and protein secretion related to the initiation of tooth eruption. We have localized many molecules

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that appear to be involved in tooth eruption to the dental follicle (see review by Wise *et al.* 2002). In turn, we have also established the method for isolating and culturing DFC (Wise *et al.* 1992) and find that many genes related to eruption, such as *OPG*, *CSF-1* and *MCP-1*, are expressed both *in vivo* in the follicles and *in vitro* (see review by Wise *et al.* 2002).

The dental follicle is a loose connective tissue sac which surrounds the unerupted tooth. Primary cultures of DFCs isolated from it are sometimes slightly contaminated with epithelial cells from the stellate reticulum, which lies near the dental follicle *in vivo*. However, such epithelial cells die out by passage 3 because the culture conditions we use (Wise *et al.* 1992) support only the growth of fibroblast-like DFC, and do not support epithelial cell growth. Therefore, cells of passage 5 or higher are generally used for *in vitro* experimentation after the cell lines have been established from primary cultures, to ensure the purity of the DFC population. However, questions arise as to how many passages beyond this may be used for experimentation before the cells become transformed (immortalized) and lose their contact inhibition of growth and/or differentiate into another cell type, such as an osteoblast or osteocyte. Such information is of importance because either process would obviously affect the reliability of *in vitro* experiments using DFC, especially when attempting to extrapolate such results to the *in vivo* condition. Thus, we have attempted to measure several parameters related to the cell stability of DFC, including contact inhibition of growth, calcium deposition and alkaline phosphatase activity, as well as the expression level of cyclin E.

MATERIALS AND METHODS

Cell culture

The animal-use protocol was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Rat dental follicles were isolated from the first mandibular molar of rats that were 5–7 days of age, and then trypsinized to obtain DFC. The DFC were cultured in Eagle's minimum essential medium containing 10% newborn calf serum and 1 mM sodium pyruvate at 37 °C in an atmosphere containing 5% CO₂ (Wise *et al.* 1992). Cells were passed at confluency until the desired passage. Rat bone marrow cells were isolated from rat hind-limb tibia and cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, vitamin C and dexamethasone.

Cell cycle analysis and gene expression study

To analyse the cell cycle and gene expression of the DFC, approximately 3×10^5 cells from each of the passages 6, 9 and 12 were seeded per 75 cm² T-flask in 20 ml medium with a change of medium at 2-day intervals. Cell growth was monitored daily under an inverted microscope. The cells were collected from day 2 to day 14 of culture for cell cycle analysis using flow cytometry. Briefly, the cells were fixed in cold methanol for 30 min, then suspended in phosphate-buffered saline. Prior to the flow cytometry analysis, cells were treated with RNase A to eliminate RNA. Propidium iodide was added to each sample at a concentration of 25 µg/ml. DNA cell cycle samples were acquired on a Becton Dickinson FACScan flow cytometer (San Jose, CA, USA). Data analysis was performed using MODFIT LT software (Verity Software House, Inc., Topshame, ME). A total of 1×10^4 – 1.5×10^4 cells in each sample was sorted to determine the number of cells present in G₀ + G₁, G₂ + M and S phases. Cells in S and G₂ are committed to ultimately divide and thus, they were combined with cells in M phase and reported as dividing cells (dividing track).

Cells were collected at day 2, days 7–10 and day 14 to represent early growth, confluency and late confluency stages for gene expression studies. RNA was extracted from the cells using TRI REAGENT (Molecular Research Center, Cincinnati, OH, USA) and treated with DNase I to remove any contaminant DNA. RNA concentration was determined with a spectrophotometer at 260 nm. Gene expression of cyclin E was determined by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) using rat cyclin E-specific primers with the forward sequence 5'-CTGGCTGAATGTTTATGTCC-3' and reverse sequence 5'-TCTTTGCTTGGGCTTTGTCC-3' (Hur *et al.* 2000). Briefly, 1 µg RNA was reverse transcribed into cDNA, and then 2 µl cDNA was mixed with buffer, primers and DNA polymerase followed by a 28-cycle PCR amplification. The PCR product was electrophoresed in 1% agarose gel at 100 V for 1 h, and then viewed under ultraviolet light for the presence of the 386-base-pair cyclin E amplicon. Cyclin E gene expression was normalized to β -actin.

Von Kossa and alkaline phosphatase staining

To measure if the fibroblast-like cells of the follicle were differentiating, DFC of passage 12 were grown on coverslips until confluency or late confluency and were subjected to von Kossa staining to determine the deposition of calcium that would occur if the cells were differentiating into osteoblasts, osteocytes, or cementoblasts. Cells were also stained for alkaline phosphatase using naphthol AS-BI phosphate (Sigma, St Louis, MO, USA) as the substrate. The bone marrow cells grown on coverslips were used as the positive controls for both types of staining.

Statistical analysis

All of the experiments were repeated three or four times. Treatment effects were compared by analysis of variance with SAS version 8.1, and means were separated using least significant difference (LSD) at $P = 0.05$. Data were presented as mean $\pm$ SD.

RESULTS

The DFC underwent a rapid dividing period from day 1 to day 3 after culture initiation. Cell contact began after 4 days of culture and reached confluency around day 7 (Fig. 1). Within a given

Figure 1. Growth of passage 9 dental follicle cells in Eagle's minimum essential medium as shown at days 2, 4 and 7 after the culture initiation. Note the beginning of cell contact of the cultured cells at day 4 and confluency by day 7.

Figure 2. Change in the percentage of dividing cells over the culture period for passages 6, 9 and 12 as determined by flow cytometry. Note the sharp decrease in the percentage of dividing cells from early growth to preconfluency for all passages. By late confluency, the percentage of dividing cells in passage 6 (2%) is less than for passage 12 (15%).

passage, 30–40% of the cells were on a dividing track (S, G₂, M) during the first 2 days in culture (Fig. 2). After 4–6 days (preconfluency), the number of such cells declined to only 7–15% of the total. At confluency (day 7–10), 4% and 5% of the cells were in the dividing track in passages 6 and 9, respectively, but this increased to 15% in passage 12 (Fig. 2). The percentage of dividing cells further declined to 2% in passage 6 by late confluency.

The cyclin E gene was constitutively expressed in all passages and growth phases tested. However, within a given passage, a higher level of expression was seen at early growth (day 2) of a given culture and it declined at early confluency. The expression level was significantly decreased at late confluency (days 12–14), as shown for day 14 in Fig. 3. Comparing late confluency of passage 6 with passage 12, the level of cyclin E expression was less in passage 6 than in passage 12 (Fig. 3).

For von Kossa and alkaline phosphatase staining, no obvious staining was observed in the DFC (Fig. 4). In contrast, dark brown staining for von Kossa stain and red staining for alkaline phosphatase were seen in the cytoplasm of the bone marrow cells (positive controls), indicating the effectiveness of the staining methods (Fig. 4).

DISCUSSION

Regarding contact inhibition of growth (degree of cell transformation or immortalization), the DFC were quite stable through passage 9. For example, at passages 6 and 9, during the first 2 days following plating of the cells on the culture flask, 30–40% of the cells were on a dividing track (S, G₂ and M), as measured by flow cytometry. This would be expected as the cells had not yet become confluent and thus, should be growing and dividing. The percentage of dividing cells sharply declined at preconfluency as the cells began to touch each other, as observed by light microscopy (Fig. 1). When the cells reached late confluency at passages 6 and 9, only 2% and 5% were on the dividing track, respectively. In contrast, approximately 15% of the cells were in a dividing track for passage 12 at late confluency. These results were supported by the results of cyclin E expression. Cyclin E is a cell cycle protein that regulates cyclin-dependent kinase 2 to promote entry into and through the S-phase. Because the expression of cyclin E is maximal at the G₁–S transition (reviewed by Sherr 1996), increased expression of cyclin E indicates that

Figure 3. Cyclin E gene expression in the DFC of different passages and growth stages (E = early growth, C = confluency, Lc = late confluency). Agarose gel (upper) and expression ratio (lower) are shown. Note that cyclin E expression is significantly lower at late confluency (Lc) in all passages. Comparing passages, it is significantly lower at Lc at passage 6 than passage 12. Bars labelled with different letters are significantly different at $P = 0.05$.

more cells are in preparation for division. Thus, as seen by RT-PCR, the expression of cyclin E was far greater when cells were not confluent (dividing) as compared to when they were confluent (less-dividing) (Fig. 3). Accordingly, the expression of cyclin E in passage 6 was significantly lower than in passage 12 at late confluency; that is, only 2% of the cells were in division at late confluency in passage 6 versus 15% in passage 12 (Fig. 3).

Normal somatic cells also pass through a limited number of divisions before they die or cease division, probably because their chromosomes lose a short length of telomere at each cell division cycle. For cells to continue their proliferation, they must retain mechanisms preventing shortening of telomeres. Expression of telomerase preserves telomeres and maintains the correct telomere structure after cell division (Counter *et al.* 1992; Kim *et al.* 1994; Stewart *et al.* 2003). Such cells are immortal; that is, they may divide indefinitely and do not exhibit contact inhibition of growth (Bodnar *et al.* 1998). In long-term cultures, normal cells can reach this division limit and either die out or enter a senescent state. In contrast, immortalized cells continue to divide and grow. The studies of the DFC cultures described here suggest that there are very few transformed (immortalized) cells in our cultures through passage 9 but that by passage 12 a small portion of cells may be escaping contact inhibition of growth.

Regarding the differentiation of DFC in culture into another cell type, such as osteoblasts, osteocytes or cementoblasts, the absence of von Kossa staining suggests that this is not occurring and that mineral (calcium) deposition was not present. The absence of alkaline phosphatase staining in cultured DFC confirmed the result of the von Kossa test. Alkaline phosphatase has long been recognized as an enzyme active in bone apposition and bone resorption. Osteoblasts

von Kossa staining

Alkaline phosphatase staining

Figure 4. von Kossa (upper) and alkaline phosphatase (lower) staining of cultured DFC (left) and bone marrow cells (right). Note the dark brown (von Kossa) and red (alkaline phosphatase) staining in the cytoplasm of the bone marrow cells (positive controls) as well as some staining of the extracellular matrix. In contrast, there is no staining in the DFC or surrounding matrix showing that the cells are not depositing/secreting calcium and thus have not differentiated.

and newly formed osteocytes are rich in alkaline phosphatase and bone alkaline phosphatase is a marker for osteoblasts (Mājno & Rouiller 1951; Weinreb *et al.* 1990). Thus, the absence of staining for alkaline phosphatase in the DFC suggests that these cells had not differentiated into either osteoblasts or osteocytes.

Ideally, *in vitro* experiments should maximally reflect *in vivo* conditions. In that vein, it is probable that immortalizing DFC cultures with simian virus 40 (SV40) would not fully meet this goal. Immortalization can alter the physiological processes of cells, such as their mechanisms of cell death (Brezden & Rauth 1996) and, of course, loss of contact growth inhibition (Daya-Grosjean *et al.* 1984). Moreover, immortalization can affect gene expression and translation. For example, primary cultures of human and mouse fibroblasts do not express *B-Raf* kinase whereas immortalized cell lines do (Barberis *et al.* 2000). Thus, the non-immortalized cell cultures

we have established may more reliably reflect the state of the cells *in vivo* than would SV40-immortalized cells.

In terms of gene expression, the follicle cells in culture were comparable to the dental follicle *in vivo*. Numerous putative eruption genes such as *CSF-1*, *MCP-1*, *OPG*, *EGF* and *VEGF* are expressed both *in vitro* and *in vivo* in the follicle (reviewed by Wise *et al.* 2002; Wise & Yao 2003). Moreover, molecules that alter the expression of a given gene *in vitro* also do the same *in vivo*. For example, interleukin-1 α enhances *MCP-1* gene expression both in cultured DFC and in the dental follicle *in vivo* (Que & Wise 1998), as well as enhancing nuclear factor- κ B both *in vitro* and *in vivo* (Que *et al.* 1999).

In conclusion, these studies have demonstrated that the DFC in culture appear to be stable through passage 9 in terms of lack of transformation (maintaining their property of contact growth inhibition when confluent) and in terms of not differentiating into osteoblasts nor osteocytes. By passage 12, it appeared that a small percentage of the cells had become transformed or immortalized. Thus, it is recommended that DFC of passage 9 or less be used for *in vitro* experimentation to ensure reliable results. Such cells from passage 9 or less appear to be reliable and, as such, are normal DFC suitable for gene and protein expression analysis.

ACKNOWLEDGEMENTS

This research was supported by grant DE08911 from NIDCR to G.E.W. The authors would like to thank Ms Marilyn Dietrich for her assistance in flow cytometry analysis.

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