RESEARCH

Genotoxicity of Endosseous Implants Using Two Cellular Lineages In Vitro

Mariza Matsumoto, DD, PhD[1]
Hugo Nary FIlho, PhD[2]
Raquel Ferrari, PhD[3]
Kristianne Fernandes, PhD[3]
Ana Claudia Renno, PhD[4]
Daniel Ribeiro,, PhD[4]*

Downloaded from http://meridian.allenpress.com/joi/article-pdf/40/1/25/2039443/aaid-joi-d-10-00112.pdf by guest on 31 March 2023

The genotoxic potential of corrosion eluates obtained from a single dental implant using murine fibroblasts or osteoblasts cells in vitro by the single-cell gel (comet) assay was examined. A single commercially available dental implant (Biotechnology) was eluted in a solution consisting of equal amounts of acetic acid and sodium chloride (0.1 M) for 1, 3, 7, 14, and 21 days. Murine fibroblast or osteoblast cultures were then exposed to all corrosion eluates obtained from endosseous dental implants for 30 minutes at 37°C. The results suggest that none of the eluates produced genotoxic changes in murine fibroblasts regardless of the length of exposure to the eluate. Similarly, no genotoxicity was found in osteoblasts. The results suggest that the dental implant eluates tested in this study did not induce genetic damage as depicted by the single-cell gel (comet) assay. Because DNA damage is an important event during oncogenesis, this study represents a relevant contribution to estimate the real risks to the cellular system induced by the corrosion products of a dental implant.

Key Words: endosseous implant, genotoxicity, mouse fibroblast cells, osteoblasts

INTRODUCTION

The implant dentistry area has expanded rapidly in recent years. The recognition that oral implants could attain high success rates provided the basis for a large number of studies.[1] Specifically, the recognition that titanium is a biocompatible material led some authors to study its surface properties, such as chemical composition, micro- and macrostructure, contamination, cleanliness, and surface properties of interaction with biomolecules.[2]

Although titanium is superior to previously used implant materials, titanium implants release metal ions into surrounding tissues that can form a corrosion zone impeding direct bone to implant contact.[3] These disadvantages reduce the clinical success rate of pure titanium implants and highlight the need for further improvement. In addition, there is concern about metal release from titanium devices to adjacent tissues, such as bone, muscles, and soft tissue, and of the increased metal concentration in parenchymal organs, such as the lungs, liver, and spleen.[4,5] However, slight biological relevance is given to this condition because of the lack of clinical evidence for cytotoxicity in most of the studies and the accumulation of metal concentration in the organism.[6,7] Thus, further biocompatibility data are needed to evaluate all risks of these compounds. Indeed, the limited data existing on the genotoxicity of titanium devices appear to be insufficient.

Taking into account biocompatibility tests available in general field, genotoxicity assays are of special concern as genotoxicity has gained widespread acceptance as an important and useful

[1] Department of Oral and Maxillofacial Surgery, University of Sacred Heart, USC, SP, Brazil.
[2] Private clinic, Bauru, SP, Brazil.
[3] University Nove de Julho, SP, Brazil.
[4] Federal University of São Paulo, UNIFESP, Santos, SP, Brazil.
* *Corresponding author,* e-mail: daribeiro@unifesp.br
DOI: 10.1563/AAID-JOI-D-10-00112

indicator of carcinogenicity.[8] This is because these assays are able to measure an initiating event in tumorigenesis. To date, a variety of assays can assess genotoxicity, including those that assess genetic damage, DNA repair capacity, metaphase chromosomal aberrations, micronuclei, and sister chromatid exchanges.[9]

Singh et al[10] introduced a microgel technique for detecting various forms of genetic damage that involved electrophoresis under alkaline conditions. As developed, this assay permits the sensitive detection of DNA damage at the level of single cells. In the microgel electrophoresis technique, a small number of cells, such as cultured cells, isolated peripheral lymphocytes, or cells isolated from various tissues are suspended in a layer of agarose gel on microscope slides.[9] The cells are lysed with detergent and treated with high salt. Nucleoids are formed, containing non-nucleosomal by still supercoiled DNA. After electrophoresis and staining with a fluorescent DNA-binding dye, cells with increased DNA damage display increased migration of chromosomal DNA from the nucleoid toward the anode, which resembles the shape of comet.[11] Herein, the DNA strand breaks, alkali-labile sites become apparent, and the amount of DNA migration indicates the amount of DNA damage in the cell.[9] The single-cell gel (comet) assay combines the simplicity of biochemical techniques for detecting DNA single-strand breaks and/or alkali-labile sites with the single-cell approach typical of cytogenetic assays.[12]

The advantages of the single-cell gel (comet) test include its simple and rapid performance, its sensitivity for detecting DNA damage, the analysis of data at the level of the individual cell, the use of small cell samples, and the usability of virtually any eukaryotic cell population.[9] Apart from image analysis, which greatly facilitates and enhances the possibilities of comet measurements, the cost of performing the assay is low.[9] This assay has already been used in many studies to assess DNA damage and repair induced by many agents in a variety of cells in vitro and in vivo. The test has been widely applied in DNA damage and repair studies, environmental biomonitoring, and human population monitoring.[12] In particular, the assay has been revealed as a promising tool for detecting DNA damage induced by compounds used in dental practice.[13]

The purpose of this study was to investigate whether some endosseous implants can induce DNA breakage in mouse fibroblasts and osteoblasts by the single-cell gel (comet) assay in vitro.

Materials and Methods

Cell culture

Murine fibroblast cells (lineage 3T3-L1) were obtained from the American Type Culture Collection and cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in a growth medium containing the following constituents: Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, Calif) with 25 mmol/L glucose, 1 mmol/L pyruvate, 4.02 mmol/L L-alanyl-glutamine, and 10% fetal calf serum (Sigma Chemical Co, St Louis, Mo).

Rat calvarial osteoblast-like cells (Osteo-1 lineage) were grown at 37°C in DMEM (Invitrogen) supplemented by 10% fetal bovine serum (Cultilab, Campinas, São Paulo, Brazil) and 1% antibiotic-antimycotic solution (Sigma) in a humidified air/5% CO_2 atmosphere. Confluent cells were detached with 0.15% trypsin (Invitrogen) for 5 minutes after that, 2 mL complete medium was added, and cells were centrifuged at 1000 rpm (180*g*) for 5 minutes. Cell suspension was counted using a Neubauer chamber and seeded in 96-well microtiter plates (Corning, Corning, NY) at a density of 1×10^4 cells per well (at a concentration of 1×10^6/mL).

All the procedures in this study were conducted ethically according to the procedures described by the Ethics Committee of Federal University of São Paulo, Brazil.

Treatment

For this study, the following dental implant was used: Bonelike implant 4.0 × 1.0 mm hexagonal (ASTM F-136; Lot number 5885KB, Biotechnology, São Paulo, Brazil). The dental implant was submitted to a corrosion process in a solution containing equal amounts of acetic acid and sodium chloride (Merck, St Louis, Mo), at 0.1M concentration, for 1, 3, 7, 14, and 21 days as established elsewhere. A volume of 10 uL of cells (approximately 10 000 cells) was then added individually to each final solution of eluate obtained for 30 minutes at 37°C. The negative control was treated with the same solution used for

Downloaded from http://meridian.allenpress.com/joi/article-pdf/40/1/25/2039443/aaid-joi-d-10-00112.pdf by guest on 31 March 2023

corrosion process for 30 minutes at 37°C. An independent positive control was performed with methyl methanesulfonate, a known genotoxic agent (Sigma) at 1 μg/mL for 1 hour in to ensure the reproducibility and sensitivity of assay. After completing the respective experimental periods, all individual treatments were centrifuged at 1000 rpm (180*g*) for 5 minutes, washed twice with fresh DMEM medium, and resuspended with fresh DMEM medium. Each individual treatment was repeated 3 times consecutively to ensure reproducibility.

Genotoxicity assay

The protocol used for single-cell gel (comet) assay followed the guidelines purposed by Tice et al.[9] Briefly, a volume of 10 μL of cells (~l × 10^4 cells) was added to 120 μL of 0.5% low melting point agarose at 37°C, layered onto a pre-coated slide with 1.5% regular agarose in duplicate, and covered with a coverslip. After brief agarose solidification in a refrigerator, the coverslip was removed and slides were immersed to lysis solution (2.5 M NaCl, 100 mM ethylenediamine tetra-acetic acid, 10 mM Tris-HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% dimethyl sulfoxide) for about 1 hour. Before electrophoresis, the slides were left in alkaline buffer (pH >13) for 20 minutes and electrophoresed for another 20 minutes, at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5), fixed in absolute ethanol, and stored at room temperature until analysis. To minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination.

A total of 50 randomly captured comets per treatment (25 cells from each slide)[12] were examined blindly by one expert observer at ×400 magnification using a fluorescence microscope (Olympus, Optical Co, Ltd, Tokyo, Japan) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Haverhill, UK) calibrated according to manufacturer's instructions. A computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and then evaluates the range of derived parameters. Undamaged cells have an intact nucleus without a tail; damaged cells have the appearance of a comet. To quantify the DNA damage, tail moment was evaluated. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. The comet tail moment is positively correlated with the level of DNA breakage in a cell. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample.

Statistical methods

Parameters from the comet assay was assessed by one-way analysis of variance followed by Tukey's test using SigmaStat software, version 1.0 (Jadel Scientific, Chicago, Ill). The level of statistical significance was set at 5%.

Results

The single-cell gel (comet) assay was used to measure DNA damage in murine fibroblast cells in vitro. DNA strand breaks were represented by the mean tail moment for 50 comets/sample. As seen in Table 1, none of the eluates induced DNA strand breaks in any periods tested. In the same way, corrosion eluates did not show any signs of genotoxicity, regardless of time of exposure adopted in this setting.

Discussion

The aim of this study was to evaluate the genotoxic damage induced by endosseous implant using murine fibroblasts or osteoblasts in vitro. The investigation was conducted using the single-cell gel (comet) assay. To the best of our knowledge, the approach has not been demonstrated so far.

In vitro studies are simple, inexpensive to perform, provide a significant amount of information, can be conducted under controlled conditions, and may elucidate the mechanisms of cellular toxicity.[14] Cell culture studies are commonly used to evaluate genotoxicity. The results obtained from these assays observed in vitro might be indicative of the effects observed in vivo.[14] Our choice of this cell line, that is, fibroblasts and osteoblasts cells, permits an accurate evaluation of the changes, excluding such factors as age and metabolic and hormonal states of the donor, which may influence the cell in primary culture.

In the past, titanium has been the material of choice for fixation devices and endosseous implants. However, its accumulation in soft and hard peri-implant tissues and in parenchymal organs

Downloaded from http://meridian.allenpress.com/joi/article-pdf/40/1/25/2039443/aaid-joi-d-10-00112.pdf by guest on 31 March 2023

Table 1

DNA damage (tail moment) in fibroblasts or osteoblasts exposed to corrosion eluated obtained from endosseous implant

Days	Cellular Type Tested	
	Fibroblasts	Osteoblasts
Negative control (zero)	0.8 ± 0.4	0.9 ± 0.5
1	0.5 ± 0.3	0.7 ± 0.3
3	1.0 ± 0.3	0.6 ± 0.3
7	0.4 ± 0.7	0.4 ± 0.2
14	1.2 ± 0.5	1.0 ± 0.4
21	1.3 ± 0.5	1.2 ± 0.8
Positive control†	3.7 ± 1.2*	4.3 ± 1.5*

*$P < 0.05$ when compared with a negative control (zero).
†Methylmethanesulfonate at 1 μg/mL for 1 hour.

after placement of titanium devices has been reported.[6,7] Bessho et al,[15] in their study using titanium miniplates in the treatment of rabbit mandibular body fracture, found a marked accumulation of titanium concentration in the lungs and liver compared with a nonfractured group. This decreased over the course of 2 years. The behavior was attributed to cracking in the miniplates caused by corrosion pits found in the material before its use, under stress caused by the fractured bone.

Regarding comet parameters, the tail moment represents a simple descriptor measured by the computerized image analysis system considering the length of DNA migration in the comet tail and the tail intensity. This parameter is one of the best indicators of induced DNA damage among the various parameters calculated by this method.[9] The statistical analysis of tail moment data confirm that corrosion eluates obtained from endosseous implant did not induce genetic damage in murine fibroblasts for all periods evaluated in this setting. The same result occurred for osteoblasts, because no genotoxicity was found. By comparison, Schliephake et al[5] observed particles of titanium deposited on bone surface during the preparation of implant beds and a higher concentration of the metal in the lungs after 5 months of implant placement in mini pigs. Such results suggest that abrasion of microparticles and subsequent hematogenous transport to locations away from the implantation site are possible. Despite these results, the biological relevance of this process was not considered once no clinical evidence of toxicity was detected and because the titanium concentration decreased in the referred organs over the years. Nevertheless, it cannot be excluded that even nontoxic concentrations might be sufficient to induce important biological effects in cellular systems. A recent study conducted by Tavares et al[16] revealed that a titanium surface induces genetic damage as assessed by comet assay and micronucleus test. It is important to stress that experimental and epidemiologic studies suggest that exposure to nickel compounds, for example, is associated with lung and nasal cancer, and cobalt or cobalt compounds are possibly carcinogenic to humans.[17] The mechanisms are not completely known, but pathways might involve the metals with DNA interaction, either directly or indirectly. Several metals have been shown to have cogenotoxicity; mechanisms seem to be predominant regarding the generations of oxidative DNA damage and interference with the DNA repair system and DNA replication process.[18] This requires further study.

In this study, we have always excluded comets without clearly identifiable heads (ie, comets with most of their DNA in the tails after electrophoresis) during image analysis. Although it should be emphasized that it is still not completely understood what these "clouds'" actually represent, this type of comet was excluded on the basis of the assumption that these cells represent dead cells, resulting from putative cytotoxic effects of corrosion eluates rather than primary DNA damage after a direct interaction between DNA and a genotoxic agent.[19] The approach of excluding comets with practically all DNA in the tail after electrophoresis when evaluating potential genotoxicity in the single-cell gel (comet) assay has also been used.[20]

Taken together, these results support the notion that corrosion eluated from endosseous implant did not induce genetic damage in murine fibroblasts or osteoblasts in vitro. This is an area that warrants investigation as the risk assessment of these substances with respect to genotoxicity will help improve oral health and prevent oral carcinogenesis.

Abbreviation

DMEM: Dulbecco's modified eagle's medium

Acknowledgments

The authors would like to thank Professor Márcia M. Marques, PhD, Universidade de São Paulo, Facul-

Downloaded from http://meridian.allenpress.com/joi/article-pdf/40/1/25/2039443/aaid-joi-d-10-00112.pdf by guest on 31 March 2023

dade de Odontologia, for the donation of Osteo-1 cells. This study was supported by FAPESP (Fundacao de Amparo a Pesquisa do Estado de São Paulo). Daniel Ribeiro is a recipient of the CNPq fellowship.

References

1. Adell R, Lekholm U, Rockler B, Branemark PI. A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. *Int J Oral Surg*. 1981;10:387–416.

2. Albrektsson T. The response of bone to titanium implants. *Crit Rev Biocompat*. 1985;1:53–84.

3. Morais LS, Serra GG, Muller CA, et al. Titanium alloy mini-implants for orthodontic anchorage: immediate loading and metal ion release. *Acta Biomater*. 2007;3:331–339.

4. Osborn JF, Willich P, Meenen N. The release of titanium into human bone from a titanium implant coated with plasma sprayed titanium. In: Heimke G, Soltesz U, Lee AJC, eds. *Advances in Biomaterials*. Amsterdam: Elsevier; 1990:75–80.

5. Schliephake H, Reiss G, Urban R, et al. Metal release from titanium fixtures during placement in the mandible: an experimental study. *Int J Oral Maxillofac Implants*. 1993;8:502–511.

6. Jorgenson DS, Mayer MH, Ellenbogen RG, et al. Detection of titanium in human tissues after craniofacial surgery. *Plast Reconstr Surg*. 1997;99:976–979.

7. Acero J, Calderon J, Salmeron JI, et al. The behavior of titanium as a biomaterial: microscopy study of plates and surrounding tissues in facial osteosynthesis. *J Craniomaxillofac Surg*. 1999;27:117–123.

8. Ribeiro DA. Do endodontic compounds induce genetic damage? A comprehensive review. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2008;105:251–256.

9. Tice RR, Agurell E, Anderson D, et al. Single cell gel/comet assay. *Environ Mol Mutagen*. 2000;35:206–221.

10. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*. 1988;175:184–191.

11. Olive PL, Banath JP, Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the comet assay. *Radiat Res*. 1990;112:86–94.

12. Hartmann A, Agurell E, Beevers C, et al. Recommendations for conducting the in vivo alkaline comet assay. *Mutagenesis*. 2003;18:45–51.

13. Ribeiro DA. Single-cell gel (comet) assay as a promising tool for the detection of DNA damage induced by compounds used in dental practice: the oral cancer risk assessment. *Crit Rev Oncog*. 2008;14:165–175.

14. Geurtsen W. Substances released from dental resin composites and glass ionomer cements. *Eur J Oral Sci*. 1998;106:687–695.

15. Bessho K, Fujimura K, Iizuka T. Experimental long-term study of titanium ions eluted from pure titanium miniplates. *J Biomed Mater Res*. 1995;29:901–904.

16. Tavares JC, Cornélio DA, da Silva NB, et al. Effect of titanium surface modified by plasma energy source on genotoxic response in vitro. *Toxicology*. 2009;262:138–145.

17. Merck O, Speit G. Detection of crosslinks with the comet assay in relationship to genotoxicity and cytotoxicity. *Environ Mol Mutagen*. 1999;33:167–172.

18. Fracasso ME, Perbellini L, Solda S, et al. Lead induced DNA strand breaks in lymphocytes of exposed workers: role of reactive oxygen species and protein kinase C. *Mutat Res*. 2002;515:159–169.

19. Ribeiro DA, Pereira PC, Machado JM, Silva SB, Pessoa AW, Salvadori DM. Does toxoplasmosis cause DNA damage? An evaluation in isogenic mice under normal diet or dietary restriction. *Mutat Res*. 2004;559:169–176.

20. Hartmann A, Speit G. The contribution of cytotoxicity to DNA-effects in the single cell gel test (comet assay). *Toxicol Lett*. 1997;90:183–188.

Downloaded from http://meridian.allenpress.com/joi/article-pdf/40/1/25/2039443/aaid-joi-d-10-00112.pdf by guest on 31 March 2023