Novel Application of Cytodetachment Technology to the Analysis of Dental Implant Surfaces

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Purpose: *The present study introduced the application of cytodetachment technology—the examination of single cell responses to implant material surfaces—to the analysis of implant surfaces with a view to significantly improving upon conventional methods of studying cell interactions with implant surfaces.* ***Materials and Methods:*** *With the new cytodetachment technology, osteoblasts (MG-63) were allowed to attach to a surface, and a nanoprobe was positioned adjacent to the cell. The probe was then moved using a piezo-actuator to completely detach the cell. The detachment forces were calculated and analyzed statistically for three different groups of implant disks: a titanium (Ti) grit-blasted (TiOblast) surface (n = 15, group 1), a fluoride-modified (OsseoSpeed) surface (n = 15, group 2), and a machined titanium surface (n = 6, group 3).* ***Results:*** *The detachment force was slightly higher for the OsseoSpeed surface than the TiOblast surface, but this difference was not statistically significant. The detachment force on the machined surface was statistically significantly lower than that seen in the other groups, thus supporting the rationale that changes in surface properties would be reflected in the measured detachment force. The OsseoSpeed and TiOblast surfaces demonstrated stronger osteoblast adhesion compared to the machined titanium surface.* ***Conclusions:*** *Within the limitations of this study, this report is a good proof-of-principle for the application of cytodetachment technology to testing implant surfaces. It might represent a new parameter to judge implant surface properties and might have broad applications in product development and research protocols for future implant surfaces.* Int J Oral Maxillofac Implants 2011;26:985–990

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The attachment of a cell to a specific substratum is integral to the function and morphology of the cell and has consequences for cell-to-cell signaling and cell differentiation. Additionally, the biochemical mechanisms related to cell attachment and spreading are important factors in determining the success of a biomaterial as a point of attachment for cells. The present study introduces a novel application of a technique called cytodetachment for use in analyzing dental implant surfaces. This involves the examination of single cell responses to implant material surfaces and promises a significant improvement over conventional techniques of studying cell interactions with implant surfaces.

Previous studies relating to cell adhesion and response on the implant surfaces[1–3] primarily involve cell attachment and washout and subsequent measurement of the number of cells remaining on the surface after washing. This yields a preliminary picture of adhesion strength, but it does not provide any additional information about how the cell might behave with the implant surface. Also, since the washout tests rely on the presence or absence of cells after washout, they do not provide quantitative data at the single cell level, which can be crucial, since controlling for confounding factors can be easier at the single cell level. In the past, centrifugation was used to determine the average force needed to remove cells from a solid support.[4] These studies measured an average detachment force but were unable to localize and calculate the force required to detach a single cell. Other authors have utilized atomic force microscopy to evaluate the adhesion force

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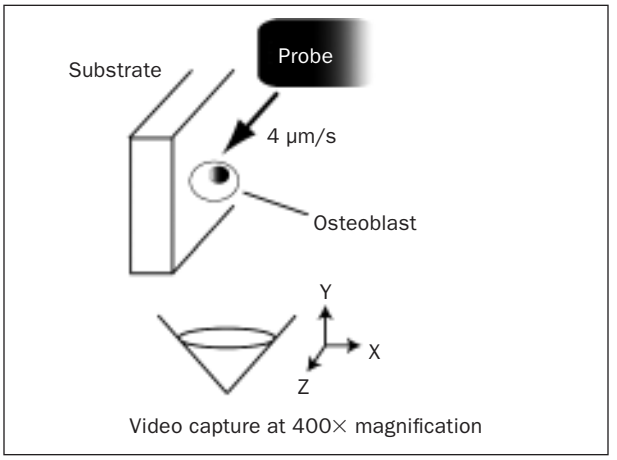

Fig 1 Diagram of the cytodetachment machine.

Fig 2 Exterior view of the cytodetachment machine.

between cells and mineral crystals or glass beads,[5,6] but to the best of the authors' knowledge, there is no published report of atomic force microscopy being used to quantify the interactions of single cells with implant surfaces.

With cytodetachment technology, osteoblasts are allowed to attach to a surface, and a nanoprobe is positioned adjacent to the cell. The probe is then moved using a piezo actuator to either nudge the cell or completely detach it. The displacement applied to the piezo, versus the actual observed displacement, gives an indication of how much the cell is resisting detachment, such that the cell detachment force can be calculated. Various models can then be developed for both nudging and complete detachment and to provide quantitative parameters that describe cell mechanics and, in turn, the cellular response to an implant surface.

Prior studies of cytodetachment technology done by the present authors tested adherence of chondrocytes[7] to differing substrates and found that the detachment force differed based on the surface properties of the substrate. As an additional test of the system's resolution, the mechanical adhesion of single cells as a function of seeding time has also been previously tested and proven.[8,9] The cytodetacher has been previously validated with respect to its accuracy using both a cantilever and a hydrogel of known material properties.[8] In previous published reports,[10–13] its repeatability and accuracy have been validated using cells, and each of three force displacement curves was curve-fitted linearly and the slope and quality of fit were quantified to yield an r^2 value above 0.97. The cytodetachment system's noise has been previously calculated to be less than 1%. Although the cytodetachment technology has been used by the authors' group since 1999, this paper represents the first application of this technology for the analysis of dental implant surfaces, and, to the best of the authors' knowledge, this is the first report of single cell analysis to compare cell responses to different dental implant surfaces.

The primary objective of this project was to compare the detachment force necessary to detach osteoblasts from three implant surfaces: a nanohydroxyapatite-blasted surface (TiOblast, Astra Tech), a fluoride-modified surface (OsseoSpeed, Astra Tech), and a machined titanium surface. The null hypothesis was that the detachment force necessary for the machined surface would be much lower than that needed for the treated implant surfaces, since the treated surfaces should theoretically encourage osteoblast attachment. The validation of this theory would in turn support the rationale that a change in the detachment force reflects an underlying change in the surface properties of the implant surface and serve as a good proof-of-principle of the application of cytodetachment to implant surface testing.

METHODS AND MATERIALS

Cytodetachment Hardware

The cytodetachment machine consists primarily of a tungsten nanoprobe (Advanced Probing Systems) suspended with a piezo actuator, a motion-stabilizing base, a charge-coupled distributor camera mounted on an inverted microscope, and a temperature-regulating hood (Figs 1 and 2). As seen in Fig 1, the osteoblasts can be seeded on a given substrate (ie, an implant disk), and the nanoprobe can be moved with a predetermined speed and distance, causing cell detachment. The camera captures this video footage, which is analyzed to provide quantitative measurements of cell displacement, probe deflection, and detachment force. A computer algorithm then calculates the value for the detachment force per cell.

Titanium Disks

Two sets of sterilized titanium treated disks (6.5 mm in diameter, 15 disks in each group) were obtained from Astra Tech and were subsequently coded to conceal the disk treatment (group 1: TiOblast surface; group 2: OsseoSpeed surface). In addition, six machined titanium disks were used to compare detachment forces (group 3).

Preparation of Cells

MG-63 osteosarcoma cells were chosen; this cell line is widely used and would enable comparisons with previous studies of implant surfaces.[1–4] The cells, once received, were thawed and counted for viability. The count yielded about 2 million cells, which were divided between two 75-mL flasks with 10 mL of media (88% Dulbecco modified eagle medium, 10% fetal bovine serum, 1% minimum essential medium/nonessential amino acid solution, and 1% phenosafranine [PSF]) and incubated at 37°C. Cells were passaged four times over to ensure that enough cells would be available for testing. Passage consisted of adding 4 mL of 0.05% trypsin–ethylenediaminetetraacetic acid per flask for 7 minutes for detachment from the flask. Then, 4 mL of media were added to neutralize the effect of the trypsin. Sample counting was performed with a hemocytometer, and the solution was centrifuged for 5 minutes. The supernatant was removed and the pellet resuspended in media for testing and passaging or freezing media for storage. Cells were frozen in media (70% media, 20% fetal bovine serum, and 10% dimethyl sulfoxide) at –80°C and later moved to a nitrogen tank for storage (2 × about 30 million cells/mL; 25 × about 1 million cells/mL).

Cells were counted and suspended in media prior to each experiment to determine the amount of viable cells in the solution. Typical experiments consisted of about 400,000 cells/4 mL of media seeded on a round 6- × 2-mm titanium disk in a six-well plate and incubated at 37°C. Pilot studies were conducted to calculate the appropriate seeding time (30 to 45 minutes). Thus, it was known that beyond 45 minutes, the cells would lose their circular appearance and start spreading out, and testing would become unfeasible. The disk was removed and submerged perpendicularly in a large Petri dish. The cytodetacher was then placed to view the nanoprobe (50.8 μm in diameter; Advanced Probing Systems) and the cells adhered to the disk.

Video capture was performed with standardized magnification on an inverted Olympus microscope, with a constant distance maintained between the probe and the substrate. The videos were extracted and analyzed in a video analysis program (VideoMach, Gromada). The videos were formatted into 640 × 480-pixel frames and broken down to two frames/second, with the images calibrated and marked using Microsoft Paint. The size of the cell, the force applied to the cell, cell displacement, and probe deflection were calculated by measurement of the locations of the probe and cell positions on each frame. Each titanium disk was used only once to rule out modification of its surface from a previous experiment.

Calculation and Calibration of Detachment Force

The video analysis of cell displacement was combined with the deflection of the tungsten probe and the predetermined displacement distance (40 μm) and speed (4 μm/s) and was fed into a previously established and validated computer algorithm,[7–9] which then returned a value for detachment force. This current experiment was run by one operator, who was blinded to the identity of the disks. A correlation coefficient of 0.93 was obtained for intraexaminer reliability, indicating a high degree of reproducibility.

Statistical Analysis

A priori sample size calculation revealed a requirement of 10 samples for the two test groups (Tioblast and OsseoSpeed), assuming an alpha level of .05 and an anticipated difference of 15% in a two-tailed test to yield a statistical power of 0.95. A one-way analysis of variance (ANOVA) was performed to compare the three groups. A Tukey Highly Significant Difference (HSD) test was run for post hoc analysis of the data. $P < .05$ was chosen to indicate statistical significance.

RESULTS

The cytodetachment readings were obtained for the two groups of test disks (OsseoSpeed and TiOblast) and for the machined-surface disks. In all, 45 data readings were done on disks from both the OsseoSpeed and TiOblast groups, and video analysis was done to confirm cell detachment. Of these 45 readings, 15 complete detachments were recorded for the TiOblast group and 11 were observed for the OsseoSpeed group. The remaining data readings showed cell displacement, but not detachment, and hence were not considered in the data summary. When applying a force to the probe, the aim is to completely detach the cells, but it is common for some cells to remain tethered after being displaced by the probe. If the cells were displaced but not detached, the force reading would underestimate the actual detachment force, making the surface seem less conducive to cell attachment. Video analysis was utilized to distinguish displacement from true detachment. Six complete detachment readings were obtained from the machined-surface group as a comparative reading. Figure 3 shows the tungsten

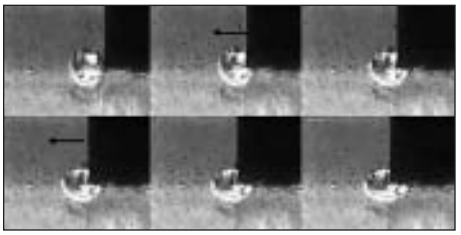

Fig 3 Representative cytodetachment with MG-63 osteoblasts (cell displacement prior to complete cell detachment).

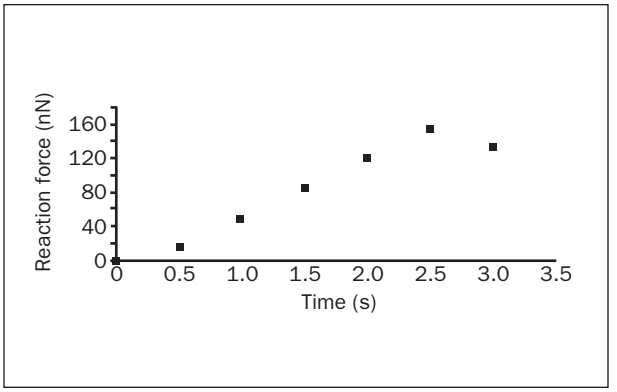

Fig 4 Representative force curve.

Table 1 Data Summary

Group	n	Mean force (nN)	SD	SE
Group 1 (TiOblast)	15	672.6	379.3	97.9
Group 2 (OsseoSpeed)	11	801.8	345.9	104.3
Group 3 (machined)	6	136.1	41.4	16.9

Table 2 ANOVA Summary

Source	SS	df	MS	F	*P*
Between groups	1809717.7	2	904858.8	8.15	.001554
Within groups	3219912	29	111031.4		

SS = sum of squares; df = degrees of freedom; MS = mean square.

Table 3 Comparison of Groups via Post Hoc Tukey HSD Test

Comparison	*P*
Group 1 (TiOblast) × group 3 (machined)	< .01
Group 2 (OsseoSpeed) × group 3 (machined)	< .01
Group 1 (TiOblast) × group 2 (OsseoSpeed)	NS

NS = not significant.

probe moving a predetermined distance (40 µm in this case) and the osteoblast being displaced until it is finally detached (final detachment not shown).

The detachment force readings over the time span of the displacement (Fig 4) demonstrated a gradual increase over time, with a logical drop after the point of actual detachment. The highest force plotted on the graph thus represents the detachment force.

The three groups showed differences in the mean detachment force required (Table 1), and a one-way ANOVA was run (Table 2) with post hoc Tukey HSD test (Table 3) to test for significant intergroup differences. There was a statistically significant difference in attachment force between the machined surface (group 3) and the other groups. This would validate the assumption that the OsseoSpeed and TiOblast surface treatments increase the affinity for attachment of the osteoblasts and represent a dramatic improvement over the untreated surface. Between the two test groups, although the mean detachment force for the OsseoSpeed group was slightly greater than that for the TiOblast group, this difference was not statistically significant, probably partly as a result of the large standard deviations in the data within the groups. The possible explanations for the large standard deviations might be variability between disks, as well as possible variability in cell properties within the same cell line. The variability between the disks refers to the possible slight variations in the surface treatments rendered in spite of standard company quality assurance protocols, which the cytodetachment technology might have picked up. However, the authors tried to control for these factors by doing pilot testing, using a standardized protocol, achieving high intraexaminer reliability, and using each disk only once to eliminate alterations of surface properties as a possible cause of differences. The intergroup differences are diagrammed in Fig 5.

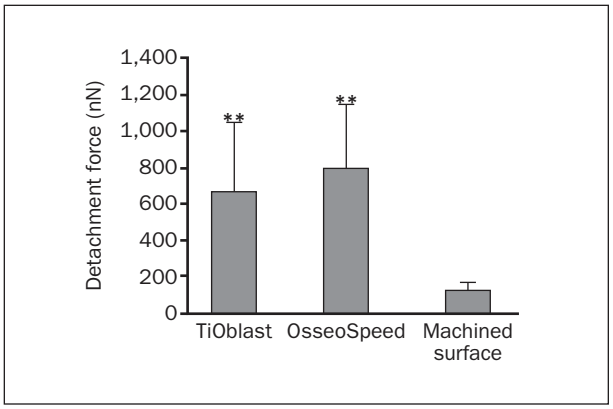

Fig 5 Diagram of data comparison. **Detachment forces for the TiOblast and OsseoSpeed surfaces were statistically superior to those for the the machined surface.

DISCUSSION

Cytodetachment technology provides a new perspective on cell attachment to implant surfaces. The present authors chose to use the MG-63 cell line to enable comparison with previous studies of cell–implantsurface interactions, many of which have used the same cell line. Several factors are crucial in the experiment, with the length of seeding time being one of the foremost considerations. Calculation of seeding time for osteoblasts is crucial, since, beyond a certain threshold, the osteoblasts have a tendency to spread out, making detachment testing with a probe unfeasible.

A distinct correlation between cell detachment force and implant surface properties has been investigated in prior studies,[9] and detachment force has been shown to be highly reflective of even slight changes in surface properties. This cell adhesion force has also been shown to be related to the ionic properties of the substrate.[6] Hence, it would logically follow that surface modifications of implant surfaces have a profound effect on the cytodetachment force measured. Thus, the measurement of this force might provide a broader perspective about the effect of surface treatments on cell integration, serving as a research tool for further development of implant surfaces.

The OsseoSpeed and TiOblast surfaces were compared in a prior publication[14] with regard to surface topography, pull-out tests, and histologic analysis after a period of healing. Surface evaluation included topographic analyses with interferometry, morphologic analyses with scanning electron microscopy, and chemical analyses with x-ray photoelectron spectroscopy. The fluoride-blasted group (OsseoSpeed) revealed fluoride on its surface, and the nanohydroxyapatite-blasted group (TiOblast) showed calcium and phosphorus with simultaneous decreases in titanium and oxygen. Removal torque values revealed increased retention for chemically modified implants that exhibit specific nanotopography. Another recent study[15] examined the osteogenic marker behavior of human bone marrow–derived mesenchymal stem cells on fluoride-modified grit-blasted (OsseoSpeed) titanium surfaces and compared this with grit-blasted ones (TiOblast). The mesenchymal stem cells showed a similar expression of early and late osteogenic markers on both surfaces. The gene expression profile for the fluoride-modified OsseoSpeed surface has also been compared with the results of pull-out tests.[16] The authors found that, after 8 weeks of healing, pull-out values, volumetric bone mineral density, and expression of osteocalcin, runx2, and type 1 collagen were higher for the fluoride-modified implant surfaces.

The current study complements previous studies by providing information on the single-cell/implant surface interaction across three implant surfaces. A substantial difference in detachment forces was found between the machined surface and the two treated surfaces, possibly indicating a better affinity or attachment for the osteoblasts to the TiOblast and OsseoSpeed surfaces. It is also possible that the OsseoSpeed surface shows overall better affinity than the TiOblast surface, since a slightly higher mean detachment force was observed for the OsseoSpeed group; however, this did not reach a statistically significant level.

How might the data from this study be important from the point of view of implant research and product testing? To date, the vast majority of information has been obtained with tests such as washouts or gene expression profiles. Cytodetachment technology adds a new perspective to this spectrum. With the emerging demand for implants that can be loaded at an earlier time point, implant surfaces that promote early osteoblast attachment, migration, and proliferation have assumed greater significance. In turn, there is a growing need for a test that can quantify osteoblast attachment to an implant surface and thus guide product development. The emergence of an in vitro test, such

as single-cell cytodetachment, to quantify cell attachment might therefore play an important role in filling the current void in research and testing tools. The addition of other tools to the cytodetachment test, such as fluorescence imaging to identify integrin attachment, would provide even greater potential to identify and quantify the cell–implant surface interaction. In addition, the dimensions of the disk used for the present test are identical to those of the titanium disks currently used for most routine product testing; hence, the cytodetachment technique is easily applicable.

The present study does have some limitations, which must be kept in mind when interpreting the results. There is an inherent learning curve for the operation of the cytodetachment machine, as is the case with any new technology; however, a high correlation coefficient for intraexaminer reliability was achieved for this experiment, pointing to reproducibility of the results. The cytodetachment technology primarily involves two-dimensional visualization, not a three-dimensional view. However, for this experiment, the two-dimensional visualization with the microscope was felt to be adequate and highly reproducible. The MG-63 cell line is immortalized; hence, direct extrapolations about the behavior of implants after placement in human bone cannot be made from this in vitro test alone. However, since this cell line is one of the most widely used experimental cell lines for this purpose, comparison with prior studies should be possible and might pave the way for future product development protocols.

CONCLUSION

Cytodetachment technology is a novel technique to quantify cell detachment from an implant surface, thus providing a new perspective on implant surface properties. Within the limitations of this study, this report provides good proof-of-principle for the application of cytodetachment technology in the analysis of implant surfaces. The average detachment force required for the OsseoSpeed surface was greater than that for the TiOblast surface, but this difference was not statistically significant. However, the mean detachment force was statistically significantly different between the machined surfaces and both treated surfaces; the latter showed much greater detachment forces. This implies that the osteoblasts showed greater attachment to the OsseoSpeed and TiOblast surface compared to the machined titanium surface. This technology might represent a new parameter to judge implant surface properties and might have broad applications in product development and research protocols for future implant surfaces.

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