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Extracellular vesicle identification in tooth movement models

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Abstract

Extracellular vesicles (EVs) are 30–150 nm in diameter vesicles released by cells that serve important intercellular regulatory functions. EVs include exosomes and microvesicles. Exosomes form in multivesicular bodies and are released extracellularly as the multivesicular bodies fuse with the plasma membrane. Microvesicles bud directly from the plasma membrane. Here, we examine methods that are available or emerging to detect and study EVs during orthodontic tooth movement (OTM). EV's involvement in regulating bone remodeling associated with OTM may be demonstrated by adding isolated EVs to an animal model to change the rate of tooth movement. Exosomes in multivesicular bodies might be detected by immunogold labeling of markers in sections from the tooth and jaw and detection by electron microscopy. Gingival crevicular fluid (GCF) is enriched in EVs. Detection and characterization of EVs released by osteoclasts during resorption has been described and this information could be used to analyze EVs in OTM models. Regulatory EVs may be enriched in the GCF from teeth that are being moved or are undergoing root resorption. Emerging approaches, including nanoparticle tracking, ExoView and micro- and nanofluidics show promise for studying EVs in the GCF. Techniques that amplify signal, including polymerase chain reaction (PCR), provide the sensitivity necessary to utilize EVs from GCF as biomarkers. Studies of the role of EVs in OTM will provide fresh insight that may identify means for enhancing OTM procedures. EVs in GCF may include biomarkers for bone remodeling during OTM, orthodontic-associated root resorption, and other dental pathologies.

Keywords

tooth; orthodontics; exosome; microvesicle; biomarker

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Conflict of Interest Statement

The authors declare that they do not have a conflict of interest of any type.

1. Introduction

Orthodontic tooth movement (OTM) is a common process for subtly or radically improving the functionality of the dentition. In general, orthodontic procedures work well. However, the specialty still lacks vital data on which to base clinical procedures (1). Despite being used commonly for more than a century, fundamental questions regarding OTM remain unresolved. For example, what is the optimal force for OTM in a specific situation? How well do methods for accelerating OTM work? How can root resorption in response to orthodontic force be detected before irreversible damage to the tooth occurs? These questions are difficult to study. True quantitative markers of bone resorption associated with tooth movement or root resorption could help answer such questions and provide an evidence base from which orthodontists could draw while designing treatment plans and monitoring procedures.

We recently screened for EV-based biomarkers associated with root resorption. We utilized proteomic techniques to identify and quantitatively compare proteins in gingival crevicular fluid (GCF) associated with deciduous teeth being resorbed with matched control teeth (2). Our data suggested that there were many proteins that differed significantly between control teeth and teeth undergoing root resorption. In the same study, we found that more than half the proteins that were detected have been identified in EVs (2). These data raised the possibility that GCF is rich in EVs. This was confirmed by Atsawasuwan and colleagues (3). Their study showed by electron microscopy the presence of EVs in GCF. The EVs in GCF could be pelleted by ultracentrifugation and contained markers commonly found in EVs, including microRNAs, CD9 and CD63. They identified microRNA (miR)-29 as the first microRNA candidate biomarker for OTM. Taken together, it is now reasonable to conclude that GCF is rich in EVs and that EVs are a potential source of biomarkers in GCF.

Recent studies have also shown that EVs from bone cells (osteoclasts and osteoblasts) and other cell types, notably stem cells and cancer cells, can regulate bone remodeling (4–13). We believe that specific EV-based biomarkers for OTM or root resorption may exist because EVs are a subset of the regulatory molecules involved in triggering the tissue response to orthodontic force leading to OTM or root resorption.

2. Methods of detecting EVs during orthodontic tooth movement

2.1 Biological effects

Many studies have appeared during the last decade that support the idea that EVs are important intercellular communicators that can be used for therapeutic purposes (14). These include studies showing EVs enhancing bone generation and regeneration (9;15). It is very likely that OTM can be manipulated in animal models (and perhaps in humans) by EVs added locally or systemically. By labeling EVs genetically or with a physical tracking dye, it should be possible to determine the cells that EVs are targeting during orthodontics in animal models. Such experiments also allow the EVs to be manipulated, for example, by knocking out EV proteins or adding specific microRNAs, to test mechanistic hypothesis.

In a more general sense, progress in the field of EVs will depend on moving beyond simply isolating vesicles that are nanometer in diameter from conditioned media or biological fluid. We believe it will be crucial to identify assays for regulatory activities, and then purifying the activities following the traditions of classic biochemists. It will be crucial to identify activities that can be assayed simply and in small volumes. Unfortunately, despite the promise EVs have shown in therapeutic models, like bone calvarial regeneration (9;15), until the underlying regulatory mechanisms are firmly established, progress toward translational use of EVs will be limited.

2.2 Detection of the EVs *in vivo* that are regulating OTM

Several markers for EVs involved in regulating bone remodeling have been identified. These include receptor activator of nuclear factor kappa B-ligand (RANKL) (16;17), RANK (18), semaphorin 4B (19), ephrinA2 (20), microRNA (miR)-214–3p (19), miR-29 (3), and miR-146a-5p (21). Protein biomarkers could be detected by immunogold electron microscopy in alveolar bone associated with OTM models. The original detection of exosomes was by immunogold labeling of the transferrin receptor (22). RANK, for example, could be detected in multivesicular bodies in osteoclasts in alveolar bone on the pressure side of teeth in a rodent OTM model, and such data would be informative. Likewise, RANKL would be expected in multivesicular bodies in osteoblasts, lining cells or osteocytes. The timing and of the appearance of these markers in multivesicular bodies could provide new clues to the regulation of bone remodeling in response to mechanical force.

Biochemical assays of tissue associated with tooth movement would be meaningless with respect to EVs, as all EV components are also components of the cells of origin. EVs will like have to be isolated from a biological fluid for biochemical analysis. GCF is the most plausible source for dental related EVs.

3. Methods for isolation/characterization of EVs from the GCF

In this section, we do not attempt to comprehensively review the methods available that may hold promise for detecting and characterizing EVs in GCF. Instead, we focus on a few where we have direct experience, or which show particular promise (from our perspective).

3.1 Nanoparticle tracking

A preliminary account of our group's efforts to detect EVs by nanoparticle tracking in GCF are presented in another manuscript in this issue (23). Nanoparticle tracking has quickly emerged as a “gold standard” for the detection and enumeration of EVs (24). Our data suggest that EVs from GCF can be studied by this technique (23).

3.2 ExoView

Detection of EVs in GCF is challenging because of the small amount of material available. NanoView Biosciences (Boston MA) is pioneering one possible solution to this problem. It combines microfluidics with immunodetection and interferometric imaging to detect unlabeled EVs from crude biological fluids (serum, saliva, cerebrospinal fluid). The starting

30 μ l sample is injected into a chip that utilizes microfluidic techniques to pass the sample over successive silicon chips coated with antibodies to cell surface proteins on the EVs surface. After the sample is passed over all the chips in the array an NVDX10 reader (NexGen Arrays, LLC, Boston, MA) is used to visualize the bound EVs by interferometric imaging. The NVDX10 reader illuminates the sample with 420 or 535 nm wavelength light. Diffraction of the light occurs as it passes the EVs and the diffraction pattern is analyzed by the NexGen array software, which then calculates the size of the EVs from the interferometric data (25).

In addition to direct detection of unlabeled EVs through interferometric data, it is also possible to label the bound EVs using antibodies and detect them by fluorescence. In preliminary runs of GCF samples (courtesy of NanoView Biosciences, Boston MA) in an ExoView platform, both CD9 and CD63 containing EVs were detected in GCF (Figure 1). The EVs detected by fluorescence were mostly not detected by light scatter. This suggests that they were smaller than the detection limit, which is about 60 nm in diameter. This is consistent with the size of EVs we have detected by electron microscopy after isolation from conditioned media of osteoclasts (18).

3.3 Microfluidics and Nanofluidics

Various strategies can be used to separate EVs using nanofluidic devices (26). These may be passive or active. Active approaches include field flow fractionation (size), centrifugal (size and density), optical (size, refractive index, polarizability), affinity capture (antigenic site or other affinity ligand), electrophoresis (size, charge) dielectrophoresis (polarizability and size) magnetophoresis (size, magnetic properties), acoustophoresis (size, density, compressibility). Additional active approaches are ion concentration polarization (size, electrophoretic mobility), and electrohydrodynamic vortices (size, charge).

Passive approaches include electrostatic deterministic lateral displacement (DLD) (size, deformability and shape), hydrodynamic filtration (size), hydrophoretic filtration (size, shape), spiral microfluidics (size, shape), straight line inertial microfluidics (size, shape), and electrostatic sieving (size, charge). For the scientist interested in studying EVs during orthodontic procedures a wide variety of methods for separating nanoparticles, like EVs, from very small quantities of sample, are emerging. Collaboration between the micro/nanofluidic engineer and the craniofacial biologist will be crucial to bring these technologies into the scientific lab and the clinic.

Recently several groups have described using microfluidics combined with acoustic sorting (27–29). This approach uses microfluidic devices that are modified by using applied acoustic fields to exert forces on EVs that allow their separation from other biological components. Sorting is based on the size, density and compressibility of both the particles and the fluid. Various approaches have been tested for isolating EVs. A recent article described the use of creating a standing wave in a microfluidic chamber (30) (Figure 2). Polystyrene beads are used as seeding particles, which are retained in the standing wave. EV samples are then passed through the microfluidic chamber. They are attracted to the seeding particles due to secondary acoustic forces. The seeding particles and trapped EVs are then released and pass through the chamber when the acoustic wave is turned off. This specific technique was

compatible with relatively low volumes of sample and did not require pretreatment of conditioned media from cell culture, urine or blood plasma. It produced enough enriched EVs for downstream applications including ELISA analysis and quantitative Real Time PCR (qPCR).

4. Signals from EVs

4.1 RNA/microRNA PCR amplification of signal

PCR massively amplifies signals, a requirement for studying EVs during orthodontics. Conveniently the explosion of research in the EV field that has occurred in the past decade was detonated by the demonstration that active RNAs and microRNAs could be transferred from the cell of origin to the target cell in EVs (31). It is now clear that the RNA and microRNA content of EVs depends on the cells from which the EVs originated, and in addition, the physiologic state of the cell (14).

In their recent study, Atsawasuwan and colleagues showed the feasibility of detecting and quantitating microRNAs from the GCF of patients (3). They surveyed a small collection of microRNAs and identified miR-29 as significantly more abundant in the GCF of teeth being subjected to orthodontic force. They pooled GCF from 6 different periopaper dips. This is not practical to perform in the clinic. We have confirmed the ability to detect specific microRNAs in GCF, and have done this from two pooled samples, and from half of the two pooled samples (equivalent to one sample) (Figure 3). Our data suggest that with the microRNAs we examined the limit of quantitative detection is approached. However, we suspect microRNA-146a-5p may show dramatically-increased abundance during orthodontic treatment. EVs from osteoclasts were shown to have 80-fold more miR-146a-5p than precursors (21).

The number and identity microRNA markers for orthodontic force application is not known, nor is the relative abundance of the markers. Further research will be required to identify one or more abundant microRNAs to be used as “housekeeping” standards, and a panel of microRNAs that are abundant either before or after orthodontic force. It will also be vital to identify abundant microRNA markers associated with pathologies like root resorption. Ultimately the goal would be to identify a panel of microRNAs that are found at lower and higher levels after force application, or periodontal infection, root resorption or other instances where biomarkers may be sought, along with microRNAs that are expressed at constant levels, and then use ratios to achieve robust read outs.

4.2 Novel twists on old protein biomarkers

Recently GCF was assayed for a panel of bone markers, including RANKL (32). This and other studies using “soluble” RANKL as a biomarker raise the interesting question of why the RANKL was soluble. Until recently it was considered to be soluble either due to cleavage of the extracellular domain of the transmembrane protein by an exoprotease, or because of expression of a transcript lacking the transmembrane domain (33). Now it is clear that RANKL is also present on EVs (17). It is reasonable that RANKL embedded in an EV,

may have a different meaning as a biomarker than RANKL cleaved from the cell of origin's plasma membrane.

4.3 New EV protein biomarkers

As described above, recent studies of EVs released from osteoclast and osteoblasts have identified potential EV biomarkers. A recent report has already identified RANK-rich EVs as a biomarker of psoriatic arthritis (34). As we gain more information about the regulatory function of EVs associated with orthodontic tooth movement, root resorption, periodontal disease and other dental related concerns, it is likely additional protein biomarkers located on or in EVs will be identified.

4.4 Limitations to using EVs in GCF as Biomarkers

Despite extensive efforts, GCF has not yet proven to be a useful source of biomarkers for clinical applications. EVs are a newly identified component of GCF. Whether sufficient numbers of these molecules can be obtained, and whether they contain useful biomarkers for conditions like root resorption or periodontal disease remains unknown. Even if such biomarkers exist, it is likely that new technologies like microfluidic, chip based apparatus will be required to detect and analyze the biomarkers. In addition, the current process of collection of GCF using filter paper tips or micropipettes is too cumbersome and inconsistent to make routine GCF analysis commonplace in the clinic. New technology for the quick and accurate collection of GCF will be required. This seems unlikely to occur in the near future, but advances in science and technology are occurring very rapidly. As new technologies for collecting GCF and assaying for biomarkers become possible it is important to identify potential biomarkers. The development of EVs as GCF based tools in dentistry will involve an interplay between advances in understanding the basic science of EVs and the development of new tools.

5. Conclusions

During the past decade, EVs have emerged as important intercellular regulators. They also are currently being explored as biomarkers. The use of EVs as diagnostic biomarkers will in part depend on understanding their regulatory role during a relevant process (like OTM). This can be achieved by *in vitro* studies of EVs produced by bone cells, and by the effects of isolated EVs on the process, and by direct visualization and study of EVs during OTM by methods like immunogold labeling for electron microscopy. At the same time description of changes in EV contents during OTM, by studying EVs found in the GCF, may both identify biomarkers and generate hypothesis regarding the regulatory role of EVs. Such studies will require emerging technologies for analysis of nanoparticles derived from very small samples. Success in this challenging area should facilitate the understanding and use of EVs in all of biomedical science.

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B

Figure 1. Detection of EVs using the ExoView platform (NanoView Biosciences) utilizing specific labeling of EVs, bound to the surface with CD63 antibody, and detected with fluorescently-tagged anti-CD63 antibody.

A) Schematic of NanoView Biosciences detection system. Antibodies to EV surface antigens are attached to a signal enhancing surface. EVs are then incubated on the spots and unbound sample is washed free. Bound EVs can then be detected by interferometrics or by fluorescent labeling. B) GCF was collected from the buccal side of the left upper central incisor by a single Periopaper dip following the method described in detail by our group previously (2) and following a procedure approved by the University of Florida Internal

Review Board (IRB201600476). The GCF was analyzed using the ExoView Platform on an anti-CD63 spot as described previously (25). GCF was eluted with 100 μ l PBS. Twenty μ l of the sample was diluted 1:1 with PBS then introduced into the ExoView apparatus. Samples were incubated for 12 H, washed and labeled for 2 H with antibody, washed again and nanoparticles were detected by either light scatter or fluorescence. Round circles indicate particles detected by light scatter. Bright particles were those labeled with anti-CD63. Most of the anti-CD63 labeled EVs were not detected by the interferometric method indicating the CD63 labeled particles are mostly smaller than about 60 nm in diameter, the detection limit of the interferometric technique.

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Figure 2. Schematic of microfluidic device using acoustic wave to isolate EVs.

(a) A local $\lambda/2$ acoustic standing wave in a fluidic channel using piezoelectric transducer generates. Seeding particles are aspirated after trapping in the acoustic field. The excess seeding particles are washed away. (b) Polystyrene seeding particles are retained by the acoustic standing wave. (c) Sample containing EVs is aspirated and the EVs are attracted and trapped with seeding cluster by the secondary acoustic forces as a result of particle–particle interactions. (d) Seeding cluster and trapped vesicles are washed and released after the acoustic wave is turned off. (e) Photograph of the automated trapping device, AcouTrap. This figure is used with permission (30).

Figure 3. MicroRNAs can be detected from single GCF samples, but accuracy of measurement is reduced.

MiR-146a-5p and miR-103-3p were analyzed from GCF from two Periopaper dips into two separate sites, the buccal sides of the left and the right upper central incisor following a procedure approved by the University of Florida Internal Review Board (IRB201600476). These methods were as described previously (2). The method for performing microRNA analysis from GCF was as described previously except using only two pooled dips rather than six (3). The ΔCq is the difference between miR-146a-3p and miR-103-3p. GCF indicates the result from two pooled samples. GCF/2 indicates dilution of the pooled sample with PBS by two prior to analysis. The ratio (which should be the same) changes as the sample is diluted, although the two values are not significantly different by T-test, and the standard error is much higher in the analysis of the diluted sample. This suggests that the limit for quantitative detection for these microRNAs is from GCF is approached