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Effect of Hyaluronic Acid on Morphological Changes to Dentin Surfaces and Subsequent Effect on Periodontal Ligament Cell Survival, Attachment and Spreading

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Abstract:

Aim: Hyaluronic acid (HA) is a natural constituent of both soft and connective tissues and plays an important role in their development, maintenance and regeneration. Recently, HA has been shown to improve wound healing-However no basic *in vitro* study to date has investigated its mode of action. Therefore, the purpose of this pilot study was to examine morphological changes of dentin surfaces following HA coating and thereafter investigate the influence of periodontal ligament (PDL) cell survival, attachment and spreading to dentin discs.

Methods: HA was coated onto dentin discs utilizing either non-cross-linked (HA) or cross-linked (HA cl) delivery systems. Morphological changes to dentin discs were then assessed using scanning electron microscopy (SEM). Thereafter, human PDL cells were seeded under 3 in vitro conditions including 1) dilution of HA (1:100), 2) dilution of HA (1:10) and 3) HA coated directly to dentin discs. Samples were then investigated for PDL cell survival, attachment and spreading using a live/dead assay, cell adhesion assay and SEM imaging respectively.

Results: While control dentin discs demonstrated smooth surfaces both at low and high magnification, the coating of HA altered surface texture of dentin discs by increasing surface roughness. HA cl further revealed greater surface texture/roughness likely due to the cross-linking carrier system. Thereafter, PDL cells were seeded on control and HA coated dentin discs and demonstrated a near 100% survival rate for all samples demonstrating high biocompatibility of HA at dilutions of both 1:100 and 1:10. Interestingly, non-cross-linked HA significantly increased cell numbers at 8 hours whereas cross-linked HA improved cell spreading as qualitatively assessed by SEM.

Conclusions: The results from the present study demonstrate that both carrier systems for HA were extremely biocompatible and demonstrated either improved cell numbers or cell spreading onto dentin discs. Future in vitro and animal research is necessary to further characterize the optimal delivery system of HA for improved clinical use.

Key words: Hyaluronic acid, hyaluronan, periodontal regeneration, soft tissue regeneration, connective tissue regeneration

Introduction:

Chemotherapeutic agents are used in combination with mechanical standard treatment modalities involving non-surgical (1) and surgical methods (2, 3) in the therapy of periodontitis. These antimicrobial reagents topically administered into the periodontal pocket aim to reduce the bacterial load (4, 5). Their application is considered to support healing processes mainly by reducing inflammatory parameters e.g. bleeding on probing (BOP) and probing depth (PD) (6). More, recently, a variety of growth factors such as platelet derived growth factor and enamel matrix derivatives have gained interest for clinical applications as bioactive substances in regenerative procedures in periodontology (7).

Hyaluronic acid (HA) - also known as hyaluronan - was originally discovered in 1934 in the vitreous body of the eye and synthesized in 1964 (8). It is a hydrophilic, negatively charged, non-sulfated polymer (up to $10^5/10^7$ kDa) classified as a glycosaminoglycan (8). This macromolecule forms a viscous gel-like structure and can be found localized in high concentrations in the extracellular matrix (ECM) of the skin, cartilage, bone and more recently, has been identified in the periodontal ligament (9). In contrast to other glycosaminoglycans, HA is synthesized on the cell surface of fibroblasts, chondroblasts and osteoblasts thereby interacting with plasmamembrane bound receptor CD44, which is subsequently released into the ECM and degraded by the activated hyaluronidase enzymatic complex (10). HA is characterized by well-conserved structural properties and linked to several ECM proteins and collagenous fibres responsible for mediating cell adhesion, motility, migration and proliferation (11, 12). A variety of biological functions in wound healing processes including angiogenesis and re-epithelialization have been documented both in vitro and in vivo following topical application of HA (13, 14). Furthermore, HA has played a prominent role as a treatment agent for chronic osteoarthritis and is frequently used in aesthetic surgery, dermatology, ophthalmology, as well as for tissue engineering applications (8, 15).

In addition, the anti-inflammatory effect of HA applied as an adjunct to the conventional periodontitis therapy scheme in dentistry has been discussed (16). In vitro studies have demonstrated that HA induced reduction of periodontal pathogens including AAC (*A.actinomycetem-comitans*) and P.g. (*P. gingivalis*) (17). Moreover, HA has been linked with minimizing early bacterial re-colonialization after and in combination with mechanical debridement (18, 19). Therefore, a significant decline of PD (0.2-0.9 mm) and BOP (2.28–19.5%) compared to control groups has been observed in clinical studies using HA at different concentrations and dosage forms (20). Noteworthy, the application mode of HA and the appropriate carrier system are important parameters for periodontitis therapy. Therefore, the aim of the present study was to examine the biocompatibility of HA administered on dentin and the consequent biological outcome on periodontal ligament (PDL) cells in vitro. We hypothesize that HA stimulates the adhesion, proliferation and spreading of PDL cells seeded on dentin disks in a concentration dependent manner.

Materials and methods:

Dentin disc preparation, cell source and reagents

180 bovine roots of freshly extracted teeth were separated from their crowns and the approximate area was first ground flat and polished using water-cooled silicon carbide paper (Stuers, Erkrat, Germany) up to P4000 grit to a diameter of 6.0 mm and a thickness of 1.5 – 1.6 mm to fit directly into 96 well in vitro culture plates. Dentin discs were prepared using a diamond-coated trephine under constant water-cooling. The discs were then stored in the dark in tap water at a temperature of 4° Celsius until experimental seeding.

HA was kindly provided by Regedent (Switzerland) utilizing 2 carrier systems including non-cross-linked native HA (Hyadent, BioScience GmbH, Germany, Switzerland) as well as a cross-linked HA (Hyadent BG, BioScience GmbH, Germany, crosslinked to butanediol diglycidyl ether (BDDE). Both HA carrier systems were cultured at dilutions of 1:10 and 1:100 as well as by pre-coating discs slices with 10 μl of HA.

Primary human PDL cells were obtained from the middle third portion of 3 teeth extracted from healthy patients with no signs of periodontal disease extracted for orthodontic reasons as previously described (21, 22). Following ethical approval by the university, written informed consent was obtained from all patients. Primary human PDL cells were detached from the tissue culture plastic using trypsin solution. Cells used for experimental seeding were from passages 4-6. Cells were cultured in a humidified atmosphere at 37°C in growth medium consisting of DMEM (Gibco, Life technologies, Carlsbad, CA), 10% fetal Bovine serum (FBS; Gibco), and 1% antibiotics (Gibco). For *in vitro* experiments, cells were seeded with HA in 96 well culture plates at a density of

5,000 cells per well for all experiments including cell attachment, cell survival (live/dead assay) and morphological variation as qualitatively assessed via SEM.

Scanning electron microscopy

Dentin discs were fixed in 1% glutaraldehyde and 1% formaldehyde for 2 days for scanning electron microscopy (SEM). Following serial dehydration with ethanol, samples were critical point dried (Type M.9202 Critical Point Dryer, Roth & Co. Hatfield, PA, USA) and allowed to dry overnight as previously described (23, 24). The following day, samples were sputter coated using a Balzers Union Sputtering Device (DCM-010, Balzers, Liechtenstein) with 10 nm of gold and analyzed microscopically using a Philips XL30 FEG scanning electron microscope to determine surface variations between samples. Furthermore, primary human PDL cells seeded onto dentin discs with/without HA were also investigated for cell surface spreading in response to the various HA coating and dilution protocols.

Cell viability

Primary human PDL cells were seeded in 96-well plates at a density of 5,000 cells per well onto dentin slices either coated with non-crossed-linked HA (HA) at a dilution of 1:100, 1:10 or pre-coated HA, as well as cross-linked HA (HA cl) at a dilution of 1:100, 1:10 or pre-coated with HA cl. Cells were evaluated using a live-dead staining assay according to the manufacturer's protocol (Enzo Life Sciences AG; Lausen, Switzerland) as previously described (25). Experiments were performed in triplicate with three fluorescent images taken per experimental condition.

Adhesion assay

Primary human PDL cells were seeded in 96-well plates at a density of 5,000 cells per well onto dentin slices either coated on control, non-crossed-linked HA at a dilution of 1:100, 1:10 or pre-coated HA as well as cross-linked HA at a dilution of 1:100, 1:10 or pre-coated with HA cl. Cells were quantified using fluorescent imaging (from live/dead assay) at 8 hours for cell numbers as previously described (26). At desired time point of 8 hours**,** cells were washed with phosphate buffered solution (PBS), fixed with 4% formaldehyde solution (Grogg-Chemie AG, Stettlen, Switzerland) for 5 min, and mounted with VECTASHILD (Vector, Burlingame, CA). Fluorescent images were quantified with a fluorescent microscope (OLYMPUS BX51, Tokyo, Japan). Experiments were performed in triplicate with five images captured per group. Data were analyzed for statistical significance using one-way analysis of variance with Tukey's test (*, p values < 0.05 was considered significant).

Results:

Surfaces characteristics of dentin slices with/out HA coating

SEM was first utilized to visualize morphological variation in surface topography prior to HA coating (Fig. 1-2). Control dentin discs were characterized by the presence of very smooth surfaces at low magnification (100x). Higher resolution SEM images demonstrated surfaces that were still smooth, but with slight variations in surface topography likely due to a smear layer present on dentin discs during preparation (Fig. 1). Following non-cross-linked HA coating, surface characteristics of dentin slices demonstrated a more roughened surface with the presence of a surface layer of HA found on their surfaces (Fig. 2B). Interestingly, analysis at the same magnification revealed that cross-linked HA (HA cl) demonstrated surfaces with more roughened surface topography with the presence of an observable cross-linked pattern found coated on dentin surfaces (Fig. 2C).

PDL cell survival, attachment and spreading

Morphological differences in HA coating were then investigated for effects on cell survival, attachment and spreading of PDL cells (Fig. 3-5). It was first observed that cells seeded on dentin discs demonstrated close to a 100% survival rate irrespective of HA coating (Fig. 3). Therefore, the present in vitro conditions indicated that HA was an extremely biocompatible material that supported cell survival at either 1:10, 1:100 or surface coated dentin discs with HA (Fig. 3). Thereafter, cell numbers were quantified after 8 hours to determine the effects of HA coating on cell attachment (Fig. 4). It was found that non cross linked HA showed significantly higher levels of PDL cells on both HA 1:10 and HA pre-coated surfaces when compared to control and HA 1:100 dentin discs (Fig. 4). The effects of cross-linked HA did not seem to significantly affect cell numbers (Fig. 4). Thereafter, cell spreading was qualitatively assessed using SEM imaging (Fig. 5). It was found that cross-linked HA surfaces (HA cl) demonstrated more elongated cell shapes with more spreading observed on dentin discs when compared to control and regular HA surfaces (Fig. 5).

Discussion:

Successful periodontal regeneration following adequate infection control implies migration, adhesion and proliferation of periodontal progenitor and mesenchymal stem cells located in the intact part of the periodontal ligament towards the previously diseased root surface thus leading to formation of new cementum with inserting collagen fibers and of new alveolar bone (27-29). In this context, the generation of synthetic and/or xenogenic scaffold systems combined with bioactive substances capable of supporting periodontal regenerative procedures is a major clinical interest. The focus of the present study was to evaluate the biological effect of native HA and cross-linked HA (HA cl) coated on dentin surfaces and to study the viability, adhesion and spreading of PDL cells upon HA treatment. We demonstrated that i) changes to the dentin surface topography following application of either HA or HA cl (Fig. 1-2); ii) congruent biocompatibility of both carrier systems on the survival rate of PDL cells seeded on dentin discs (Fig. 3) and iii) attachment of PDL cells compared to native HA and HA cl (Fig.4-5) in a concentration dependent manner as well as a increased PDL cell spreading on HA cl in contrast to non cross-linked HA and control uncoated dentin surfaces.

Structured surfaces either naturally occurring on the tooth root or artificially developed in carrier systems (30) are necessary for cell adherence and spreading thereby forming elongated cell shapes (31). Dangaria et al. (2011) showed a direct influence of root surface topography triggering PDL cell elongation thereby influencing periodontal fiber re-attachment (32). Here, the application of HA modified the dentin surfaces resulting in more roughness in both HA and HA cl when compared to non-coated control dentin surfaces (Fig. 1, 2). In the presence of HA cl, the surface developed an even more distinctive surface roughness with cross-linked fibrils found more evenly distributed throughout the dentin surfaces. The observed effect of either high molecular mass HA (HMW: ~ 4000 kDa) and HA cl may be due to their physiochemical properties. HMW HA has previously been characterized by an optimal viscoelasticity, prolonged dwell time and extended biocompatibility (33). Previous studies performed in pulps treated with HMW HA resulted in the production of calcification nodules inducing reparative dentin formation (34). Therefore, accumulation of HMW HA and its hydration effect on the coated dentin surface structure exposing tubules upon chemical treatment could establish an ideal network for cells to settle. The formation of precipitates due to degradation of HMW HA into smaller fragments may evocate the up-regulation of genes involved in the proliferation and migration of cells (35). Based on these observations, we conclude that either HA or HA cl applied to dentin surfaces provide an optimal surface topography resulting in advanced cell attachment.

Biodegradation, mechanical stress resistance and biocompatibility are required features of medical devices maintaining optimal deposition of bioactive molecules thereby accelerating healing processes. The favorable effect of HA on the viability of various cell types e.g. odontoblasts and fibroblasts (36) and PDL cells in vitro (37) has been reported previously. Combination of HA cross-linked to BDDE (38, 39) commercially used in dermal fillers (e.g. Restylane), adhesion barriers and drug delivery result in prolonged residence time and accumulation of HA products (40). Cell viability and survival are not affected using BDDE (41). Previous studies comparing HA and HA cl concerning biological decay reported more stress resistance of HA cl. Moreover, both pure HA solutions (0.3-10%) and HA cl resulted in an anti-inflammatory response down regulating NO production by macrophages in a concentration dependent manner in the presence of BDDE (0.1-1.0 ppm) (40). Here, we observed cells seeded on HA and HA cl dentin discs surviving up to 100% at concentrations between 1:10 and 1:100 (Fig. 3, 4). Moreover, Fujioka-Kobayashi et al. reported unaffected PDL cell viability under similar treatment conditions on plastic dish (in preparation). Hence, both systems are considered exceptionally biocompatible with the ability to further stimulate PDL cell attachment and spreading in vitro.

The concentration dependent exertion of externally added HA on cell behaviour has been discussed controversially. Former investigators found induction of signal transduction and activation of intracellular cell cycle related pathways depending on the cell type used upon HA treatment at high concentrations (42, 43). The biological role of HA on cell migration and proliferation is yet not fully understood. David-Raoudi et al. reported increasing proliferation of fibroblasts in the presence of HA ranging in concentrations from 1mg/ml up to 5 mg/ml compared to HA oligosaccharid fragments (44). In wound repair and early phases of inflammation, HMW HA has been shown to suppress collagen and protein synthesis in human skin fibroblasts (42). Moreover, HMW HA inhibited the expression of cytokines e.g. TNF-α, IL-1β and IL-6 involved in inflammatory processes (45). Further, HMW HA interaction with the ubiquitously transmembranous located CD44 adhesion receptor affects cell adhesion, proliferation and HA metabolism (13). We therefore speculate, that HA and likely more so HA cl exert their biological function on

PDL cells spreading due to the dentin surface topography and the interaction with membrane located adhesion molecules. Taken together, our results confirm the exceptionally high biocompatibility of HA and HA cl on PDL cell survival. Moreover, the beneficial effect on cell adhesion and spreading demonstrate further suggest that HA may be a viable treatment option for periodontal wound healing applications. Future animal as well as further in vitro studies are necessary to investigate the regeneration potential and cell-to-cell interaction level including their participating molecules.

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Figure Legends

Figure 1: SEM images of control dentin slices at low (100x), medium (400x) and high (1600x) magnification. Smooth surfaces were observed at low magnifications with slight variations observed at high magnification (1600x).

Figure 2: SEM images of control, non-cross-linked (HA) surfaces, and HA cross-linked surfaces at 1600x magnification. Control surfaces demonstrated smooth surfaces, whereas surfaces roughness increased on non-cross-linked HA surfaces and cross-linked HA surfaces.

Figure 3: Live/dead staining of primary human primary PDL cells on control, HA and cross-linked HA surfaces. For cell viability, Live-Dead staining was done with viable cell appearing in green and dead cells in red. The results from these experiments demonstrated that HA is highly biocompatible at dilutions of 1:100 and 1:10 as well as pre-coated on dentin slices either utilizing regular or cross-linked HA carrier systems.

Figure 4: Cell number of primary human PDL cells seeded on control dentin slices and HA and HA cl dentin slices at dilutions 1:100, 1:10 and coated. (* denotes significant difference when compared to control samples, $p<0.05$)

Figure 5: SEM images of primary human PDL cells seeded on control dentin, HA and HA cl dentin slices. PDL cells seeded on cross-linked HA demonstrated qualitatively more elongated cell morphology when compared to control and HA surfaces.

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