

Three-Dimensional Loading Model for Periodontal Ligament Regeneration *In Vitro*

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In this study we present a new three-dimensional (3D) model to study effects of mechanical loading on tendon/ligament formation *in vitro*. The model mimics a functional periodontal ligament (PDL), which anchors dental roots to the jaw bone and transfers the axial load of mastication to the jaw bone. A collagen gel containing human PDL fibroblasts was seeded in a PDL space between an artificial root and bone surface. The effects of 3-day loading on the fibroblasts were studied *in vitro* by axial and intermittent displacement of the root to which the gel was attached. Cell responses were recorded by measuring expression of three sets of genes: (i) cyclooxygenase 1 and 2 (COX-1, COX-2) producing prostaglandins (signaling molecules); (ii) Runx2, a transcription factor for the osteogenic lineage; and (iii) the extracellular matrix proteins osteopontin, dentin matrix protein 1, and collagen type I (COL1). Loading for 3 days resulted in magnitude-dependent changes in the expression of COX-2 and COL1. A low loading magnitude significantly decreased COX-2 expression, an intermediate magnitude increased its expression, while a high magnitude increased COL1 expression. We concluded that the 3D loading model provides a useful, well-controlled method to examine ligament fibroblast responses to mechanical loading. The model may serve to explore the application of mechanical loading as an anabolic factor for ligament reconstruction.

Introduction

TENDON AND LIGAMENT INJURIES are widely distributed clinical problems. Such injuries may heal, but the original complex structure and mechanical properties of the tissue often do not return to normal.^{1,2} Tendon/ligament tissues can also be lost by trauma, tissue resection, or infection. Tissue engineering is now being explored as a way to build new tissues to replace lost ones and to restore the original architecture and function of original tissues. Three components are required for tissue engineering: (i) (stem) cells, (ii) a scaffold or extracellular matrix (ECM) to provide physical support to the cells, and (iii) proper signals to trigger a cellular response. The effects of biological signals provided by growth factors and cytokines have been extensively studied for tissue regeneration purposes. Mechanical loading as an anabolic signal is less familiar, though it is well recognized that loading can induce changes in the structure, composition, and mechanical properties of tendon/ligament tissues, mediated by the fibroblasts occupying these tissues.³⁻⁵ In addition, the type (compression, tension, hydrostatic pres-

sure) and magnitude of loading are thought to control cell differentiation.^{6,7}

Several types of tendons and ligaments, for example, anterior cruciate ligament (ACL), medial collateral ligament (MCL), and periodontal ligament (PDL), harbor load-responsive fibroblasts.⁸ As mechanical signals are known to affect the activity of fibroblasts, including cell morphology, cytoskeletal organization, cell survival, cell differentiation, and gene expression, loading may be a useful tool to stimulate regeneration of the connective tissues. A number of genes in these fibroblasts were previously reported to be mechanosensitive, including those coding for ECM proteins (e.g., COL1, COL3, and COL12), tenascin-C, fibronectin, connectin/titin, enzymes involved in ECM turnover (e.g., matrix metalloproteinase (MMP)-1, 3, 13), proteins controlling activity of different enzymes (e.g., TIMP-2, cystatin), growth factors (transforming growth factor-beta1 [TGF-β1]), signaling molecules such as prostaglandins (produced by cyclooxygenase 2 [COX-2] and mPGES-1, an inducible enzyme functionally linked to COX-2), and intracellular calcium-binding proteins like calmodulin that regulate activity of other proteins.⁹⁻¹⁷

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To study the relationship between loading and cell response, cultures of tendon and ligament fibroblasts are mechanically loaded in different models, such as substrate stretching or movement of fluid over the cells.^{18,19} The tension resulting from a defined strain (deformation) is transmitted from the ECM to the cells through their membrane-anchored adhesion sites connected to the ECM.^{20,21} However, cultures of fibroblasts on two-dimensional (2D) substrates do not sufficiently represent the more complex three-dimensional (3D) network of ECM macromolecules with which these cells interact *in vivo*. Therefore, 3D collagen lattices are more suitable culture models for tissue engineering purposes. Fibroblasts can be cultured in such matrices under both stretched and relaxed conditions.²²⁻²⁴ Compared with relaxed lattices, overall protein synthesis by fibroblasts can be increased 4–6 fold in internally stressed collagen gels.²⁵ This suggests that tension exerted on the ECM by fibroblasts may be required to maintain tissue structure and function. In addition to internally generated tensile stresses, external mechanical loading can be applied to the fibroblast-populated 3D collagen gels.²⁶

In this study we present a new 3D *in vitro* loading model to examine cell responses to loading. This model is useful in exploring mechanical loading as a contributing factor to regenerate tendons and ligaments. The model mimics the PDL, a dense, fibrous tissue connecting the dental roots to the alveolar bone across a narrow gap of approximately 200 μm .²⁷ The PDL is small in width and has one of the highest turnover rates among the connective tissues in the body.²⁸ Both the cells and the ECM components respond to applied mechanical forces resulting from tooth movement during, for example, mastication, which ultimately results in remodelling of the fibers.²⁹ In terms of ECM synthesis and degradation, PDL fibroblasts respond differently to tensile and compressive strains as well as to different stretching magnitudes.^{13,14}

We describe here the 3D *in vitro* model and its use in exploring the responsiveness of PDL cells to intermittent axial loading. We hypothesized that mechanical loading induces a mechanobiological response of the fibroblasts resulting in increased synthesis of ECM molecules.

Materials and Methods

Materials

Dulbecco's minimal essential medium (DMEM) and minimal essential medium (MEM) (10 \times) were purchased from Gibco BRL (Paisley, Scotland). Fetal calf serum (FCS) was obtained from HyClone (Logan, UT) and trypsin from Difco Laboratories (Detroit, MI). Antibiotic-antimycotic solution (penicillin, streptomycin, amphotericin B), bovine serum albumin (BSA, fraction V), β -glycerophosphate (β -GP), cycloheximide, N-ethylmaleimide (NEM), and type VII bacterial collagenase (specific activity 1389 U/mg) were purchased from Sigma Chemical (St. Louis, MO). Alkaline phosphatase (ALP, calf intestinal; specific activity >2500 U/mg) was obtained from Roche Diagnostics Nederland (Almere, The Netherlands), 2-phospho-L-ascorbic acid trisodium salt from Fluka Chemie (Buchs, Germany), rat tail COL1 from BD Biosciences (Bedford, MA), Trizol[®] from Invitrogen (Carlsbad, CA), and L-[2,3-³H]proline from GE Healthcare (Amersham, Buckinghamshire, UK). Culture flasks were

from Costar (Cambridge, MA), and polyetheretherketone (PEEK) was from Vink Holding (Zeist, The Netherlands).

Fibroblasts

PDL fibroblasts were obtained from male individuals (age 22–38 years) who underwent extraction of a third molar (wisdom tooth). Informed consent was obtained from each individual. PDL was taken only from teeth without overt signs of gingival inflammation and periodontitis (no plaque, periodontal probing ≤ 3 mm, no bleeding on probing, and no sign of loss of attachment).

Fragments of PDL were obtained from the middle third of the roots by means of a scalpel knife. These were cut into small pieces and distributed in six-well dishes with 1.5 mL DMEM + 10% FCS + antibiotics (100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 250 ng/mL amphotericin B) per well. The dishes were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. PDL fibroblasts were expanded for three passages and frozen in 10% dimethylsulfoxide + 90% FCS and stored in liquid nitrogen. Prior to the experiments, the cells were rapidly thawed in a water bath at 37°C, mixed with 4 mL culture medium, and incubated in a 25 cm² culture flask. The medium was changed after 1 day, and subconfluent cultures from two different individuals were used for the experiments between passages 4 and 8.

Subconfluent cultures were trypsinized using 0.25% trypsin and 0.1% EDTA in phosphate-buffered saline (PBS), suspended in culture medium, centrifuged for 10 min, and resuspended in culture medium. The number of cells in a sample of the cell suspension was counted in a Bürker-Türk chamber.

Culture chambers and actuator

Culture chambers were designed to mimic a moving tooth with respect to the bone socket at a distance of 200 μm . The chambers were manufactured from Vinplast[®] PEEK, a high-strength material resistant to high temperatures and chemicals. Figure 1 presents a schematic drawing of the model. Figure 2 shows the actual model manufactured from PEEK (A is the top view, B is a longitudinal section of a chamber embedded in plastic). The heart of the chamber is a tapered cylinder (\emptyset 6 mm) suspended between two spiral springs, which assure an exact vertical motion of the cylinder upon axial loading. The radial distance between the cylinder and the housing is 200 μm , which mimics the average width of the PDL.²⁷ The effective height of the artificial PDL space is 3 mm. The culture chambers were used for an experiment only once (disposable).

Six tapered cylinders are moved in parallel by a computer-driven voice coil linear microactuator (type NCM04-25-250-2LVE; H2W Technologies, Valencia, CA; Fig. 3). Specifications of the actuator can be obtained from the authors. A hinge system limits the maximum axial motion of the voice coil actuator to 1000 μm , with an accuracy of less than 0.2 μm (0.02%). The maximum load for each of the six chambers is 67 N (static), with a peak load of 200 N (dynamic). The whole system (except the computer) is placed in an incubator at 37°C and 5% CO₂ in air to allow for physiological culture conditions.

Coating of chambers

Calcium phosphate (CaP). Parts of the chambers representing the artificial root and the artificial bone (Figs. 1, 2)

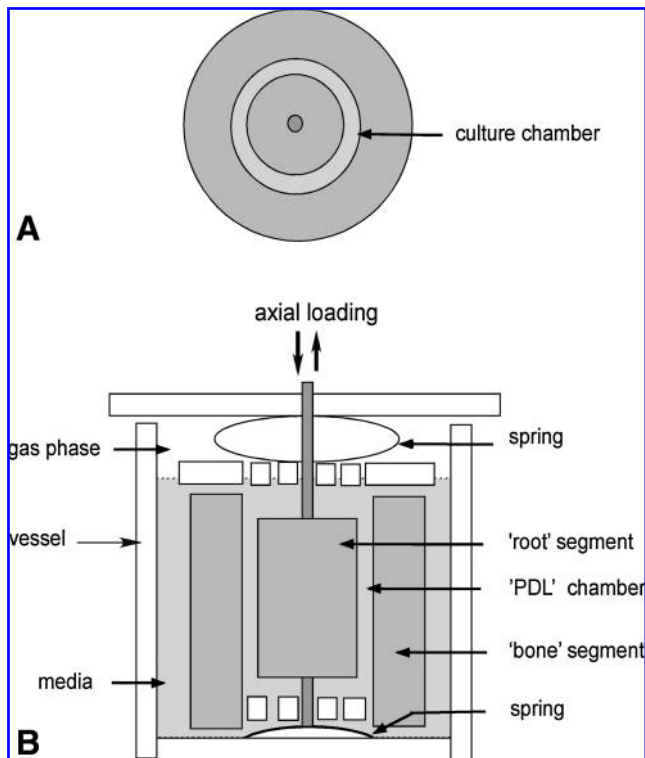


FIG. 1. Schematic drawing of the periodontal ligament (PDL) chamber, showing top (A) view and side (B) view. The model is placed in a culture vessel containing culture medium and in contact with gas phase.

were first coated with CaP using a radiofrequency magnetron sputter coating system (Edwards ESM 100, Crawly, UK). The final coating thickness was measured with a universal surface tester (UST; Innowep, Würzburg, Germany) and approximated to $0.8 \mu\text{m}$ (for more details, see Berendsen *et al.*³⁰).

Alkaline phosphatase (ALP). The CaP-coated parts of the chambers were treated with a radio frequency glow discharge for 10 min at a pressure of 2.0×10^{-2} mbar (Harrick Scientific, Ossining, NY). To prevent detachment of collagen gels from the PEEK surfaces, the material was incubated with ALP solution (250 U/mL sterilized Tris buffered saline [TBS]) for 60 min at room temperature.³⁰ The solution was removed and the chambers were allowed to dry under sterile conditions.

Preparation of collagen gels. Collagen gels were composed of 100% COL1 (rat tail), with a final collagen concentration of 2 mg/mL (0.2%). Preliminary studies revealed that at this concentration the fibroblasts did not sink downward in the gels, while the viscosity of the nonpolymerized gel solution was appropriate for applying into the PDL spaces of the culture chambers.

The purity of COL1 was determined, and collagen gels containing fibroblasts were prepared as described previously.³¹ After coating the culture chambers with CaP and ALP, the PDL spaces of the chambers (Fig. 1) were filled with COL1 gel containing PDL fibroblasts. For initial gene expression studies, low cell-seeding densities were used (1.0×10^4 cells/20 μL gel). Later, cell density was increased to

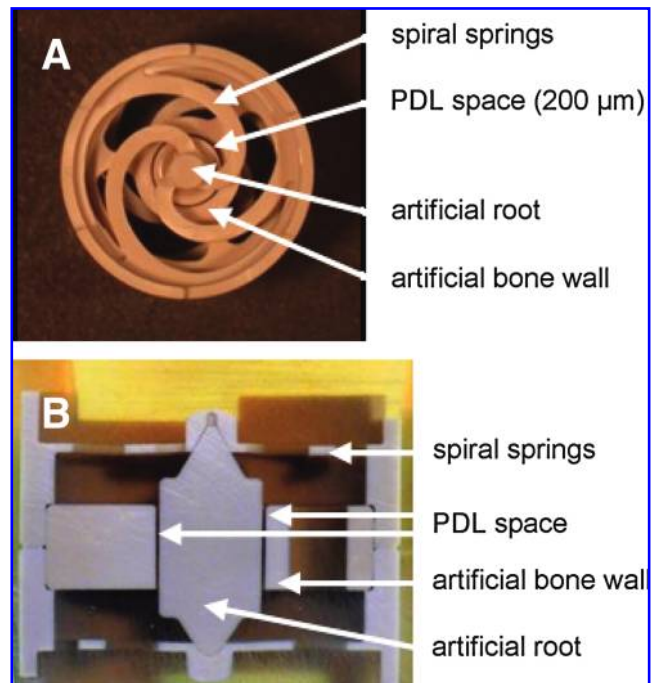


FIG. 2. Polyetherether ketone (PEEK) culture chambers for reconstruction of PDL by tissue engineering. (A) Top view of the culture chamber, showing the spiral springs holding the central, tapered cylinder representing the artificial tooth root. The circular space of $200 \mu\text{m}$ (PDL space) between the cylinder and the housing representing the artificial bone wall is clearly visible. (B) Longitudinal section of the same culture chamber embedded in plastic, showing the PDL space and the available volume for the culture medium. Color images available online at www.liebertonline.com/ten.

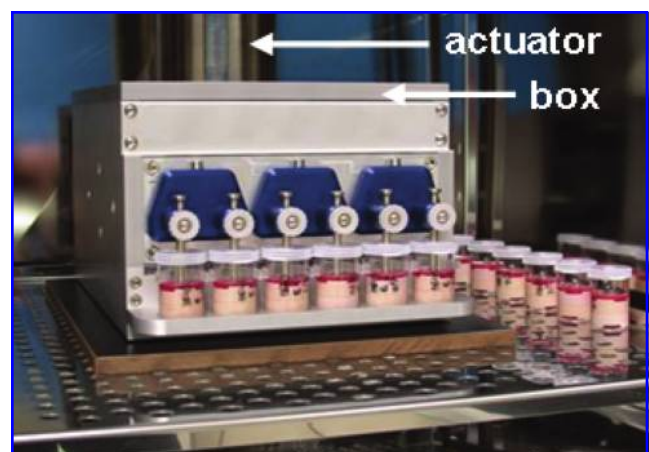


FIG. 3. PDL culture chambers ($n = 6$) mounted in the microactuator. The actuator is computer driven and is placed in an incubator at a temperature of 37°C . The cylinder on top of the box is the voice coil linear actuator. The box itself contains a hinge system, which increases the axial motion accuracy of the actuator by a factor of five and decreases the maximum amplitude of the axial motion by the same factor. Color images available online at www.liebertonline.com/ten.

1.0×10^5 cells/20 μ L). After gel polymerization the chambers were put into sterile culture vessels; 5 mL DMEM supplemented with 10% FCS, antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin, and 250 ng/mL amphotericin B), 50 μ g/mL 2-phospho-L-ascorbic acid trisodium salt, and 10 mM β -GP was added; and the vessels were covered with a breathseal (gas permeable).

Loading regime

The culture chambers (sets of $n = 12$) were preincubated for 1 day under static conditions to allow mineral deposition by ALP-mediated hydrolysis of β -GP, which released phosphate near the wall of the chamber that precipitated with calcium and attached the gels to the solid surfaces of the chambers.³⁰ Intermittent axial loading was then applied onto the artificial root (Fig. 1) of six culture chambers simultaneously at days 2, 3, and 4 of culture. Loading consisted of the following regime: four loading periods per 24 h; 4 h recovery time between separate loading periods; loading frequency of 1 Hz; 90 cycles per loading period (90 sec); sinus displacement of the artificial root varying from 0–20 μ m, 0–100 μ m, or 0–200 μ m.^{32–37} This resulted in shear deformations (and corresponding strains) of the cell-seeded collagen matrix of 5.7° ($\pm 0.5\%$), 27° ($\pm 12\%$), or 45° ($\pm 40\%$), respectively. The other set of six culture chambers was used as static controls. The experiment was stopped at day 5 of culture, and the chambers were further analyzed.

Histological analysis

To analyze the vitality and distribution of the fibroblasts and to ensure that gels had not detached during culture, chambers (static and loaded by 200 μ m displacement) were embedded *en bloc* in low-viscosity Spurr's resin at the end of the experiments (after 4 days of culture). Briefly, the medium was removed and the chambers rinsed with PBS and fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 120 min. Then chambers were rinsed twice in sodium cacodylate buffer (pH 7.4) for 60 min, dehydrated, and embedded in Spurr's resin following routine procedures. Longitudinal serial sections 1 μ m thick were cut, containing the gel in the entire length of the PDL space. The sections were

stained with Richardson's toluidin blue (a mixture of 0.5% methylene blue and 0.5% azure II in 0.5% sodium borate) to view cells and matrix, or with alizarin red to stain for minerals, and examined by light microscopy.

Using an ocular grid, cells were counted under a $\times 400$ magnification along the entire length of the culture chamber. Based on the morphology of the nucleus, cells were scored as vital (intact nucleus with prominent nucleolus) or nonvital (fragmented nucleus or cell) at a $\times 1000$ magnification. Shape of the cells was scored as elongated or round. Cells within 14 μ m of the wall surface were considered to be in contact with the surface, whereas the rest of the cells were scored as cells attached to the collagen matrix.

RNA analysis and quantitative real time polymerase chain reaction

To study gene expression by the fibroblasts, the total RNA from cultured cells was isolated from the culture chambers using TRIzol reagent according to the manufacturer's instructions. The RNA concentration was measured with the NanoDrop (Nanodrop Technologies, Wilmington, DE). The reverse transcriptase reaction was performed according to the MBI Fermentas cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers. Primers were designed such as to avoid amplification of genomic DNA, and therefore each amplicon spans at least one intron (Table 1).

Real time polymerase chain reaction (PCR) was performed on the ABI PRISM 7000 (Applied Biosystems, Foster City, CA). The reactions were performed in a total volume of 25 μ L containing SYBR Green PCR Master Mix, consisting of SYBR Green I dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP instead of dTTP, and Rox as passive reference (Applied Biosystems), and 300 nM of each primer.

After an initial activation step of the AmpliTaq Gold DNA polymerase for 10 min at 94°C, 40 cycles were run of a two-step PCR, consisting of a denaturation step at 95°C for 30 sec and an annealing and extension step at 60°C for 1 min. Subsequently, the PCR products were subjected to melting curve analysis to test if any unspecific PCR products were generated.

TABLE 1. PRIMER SEQUENCES USED FOR REAL TIME POLYMERASE CHAIN REACTION

Target gene	Oligonucleotide sequence	Accession number product length
GAPDH forward	5'-atggggaaggtgaaggtcg-3'	Human, ENSG00000149397
Reverse	5'-taaaagcagccctggtagc-3'	68 bp
COX-2 forward	5'-gcattctttgccagcactt-3'	Human, NM_000963
Reverse	5'-agaccaggcaccagaccaaaga-3'	299 bp
COX-1 forward	5'-agtacagctacgagcagtt-3'	Human, NM_000962
Reverse	5'-gagactccctgatgacatcc-3'	165 bp
COL1A1 forward	5'-tccaacgagatcgagatcc-3'	Human, NM_000088
Reverse	5'-aagccgaattcctggctt-3'	195 bp
Runx2 forward	5'-atgcttcattgcctcac-3'	Human, NM_001024630
Reverse	5'-actgcttgacgcttaaat-3'	156 bp
OPN forward	5'-ttcaagtaagtccaacgaaag-3'	Human, AF052124
Reverse	5'-gtgaccagttcatcagattcat-3'	181 bp
DMP1 β forward	5'-cctctttgagaacatcaacctgattt-3'	Human, ENSG00000152592
Reverse	5'-gagcagatgctgatcttcatagtt-3'	106 bp

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COX-2, cyclooxygenase-2; COX-1, cyclooxygenase-1; COL1A1, $\alpha 1(I)$ procollagen; Runx2, runt-related factor-2; OPN, osteopontin; DMP1 β , dentin matrix protein 1.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Expression of this gene was not affected by loading. Samples were normalized for GAPDH expression by calculating ΔCt ($Ct_{\text{gene of interest}} - Ct_{\text{GAPDH}}$), and expression of the different genes was represented as $2^{-\Delta Ct}$.

$[^3\text{H}]$ proline incorporation

To analyze matrix protein production by the fibroblasts, $[^3\text{H}]$ proline (5 $\mu\text{Ci}/\text{mL}$) was added to 5 mL of the culture medium at the start of culture. Culture media and gels with PDL fibroblasts were collected after 4 days of culture, and the amount of $[^3\text{H}]$ proline incorporated into newly synthesized collagens and noncollagenous proteins was determined by collagenase digestion (according to the method by Peterkofsky and Diegelmann³⁸). Briefly, culture media were removed, of which 1 mL was used for analysis. The chambers were washed two times with PBS, opened, and the collagen gels harboring fibroblasts collected by addition of 0.5 mL 5% trichloroacetic acid (TCA; icecold) solution and by scraping cell fragments from the surface of the chambers. This solution was transferred into 1.5 mL Eppendorf tubes, and the chambers were washed again with 0.5 mL of 5% TCA added to the tubes. Ten μL of 1% BSA was added as carrier, and the tubes were centrifuged for 5 min at maximum speed. The supernatant was removed and the pellet washed again three times with 1 mL of 5% TCA. To remove TCA, the pellet was washed twice with 1 mL of 100% ethanol. Pellets were allowed to dry and then dissolved in 50 μL 0.4 M TBS (pH 7.4). This solution was mixed with 50 μL 40 mM calcium chloride and 25 mM NEM in 0.2 M TBS (1:1), and then 100 μL bacterial collagenase (100 U/mL distilled water) was added. The tubes were incubated for 60 min at 37°C. To stop digestion, 200 μL of a mixture of 20% TCA and 0.5% tannic acid was added and samples centrifuged for 5 min at maximum speed. The supernatant (containing collagenase-digested material) was added to scintillation fluid, and the pellet (containing material not digested by collagenase) was dissolved in 50 μL 0.2 M NaOH for 30 min followed by addition of 200 μL 0.2 M HCl. This was added to scintillation fluid, and the tubes were washed with 200 μL 0.2 M HCl, which was added to the samples. The amount of radioactivity was assessed using a scintillation counter. Samples of the culture media were precipitated and repeatedly washed with 1:1 volumes of 5% TCA and treated similar to the extracts from gels and cells.

Preliminary studies in which 1 mM cycloheximide (a protein synthesis inhibitor) was added to cultures blocked $[^3\text{H}]$ proline incorporation into proteins, indicating that the incorporated $[^3\text{H}]$ proline represents ECM proteins newly synthesized by the fibroblasts.³⁹

Statistical analysis

Data were statistically analyzed using an unpaired *t*-test. Effects were considered statistically significant at $p < 0.05$ (one-tailed).

Results

Culture of cell-gel constructs in PDL model

To evaluate the behavior and vitality of the cells in culture, cell-gel constructs cultured for 4 days were fixed *in situ* and

the entire model processed for histology. Preliminary experiments indicated that sectioning of Methyl Methacrylate (MMA)-embedded PEEK was not possible. Low-viscosity Spurr's resin was harder and had better sectioning characteristics, but folding artifacts in the sections were unavoidable. A sample that received maximal displacement (200 μm) is presented in Figure 4.

Histology showed that cells were equally distributed over the entire length of the culture chamber but cell density was low. After seeding 1.0×10^4 cells/20 μL one to three cells were found per section including the entire PDL space. Part of the PDL space is presented in Figure 4A, B. After high-magnitude (200 μm) loading, high-density gels contained 32 ± 4 cells per section, corresponding to a density of 48 cells/ mm^2 . There were no indications that the gels after high-magnitude loading had detached from the walls during culture.

About 10% of the cells ($n = 411$) were located within 14 μm of the wall surface, and the remaining 90% were present completely within the collagenous network that spanned the width of the chamber. Twenty percent of the cells (6 ± 2 per section) appeared to be nonvital, with degraded nuclei or fragmented cytoplasm. These were located randomly between vital cells, which were very active due to the presence of a prominent nucleolus indicating transcriptional activity, suggesting high protein synthesis (Fig. 4A, J, K). Forty percent of the total number of cells were elongated with two long processes at opposite ends (Fig. 4C, E, H), and 60% were more or less round with multiple short processes (Fig. 4D, F, G, J). There was no histological indication that matrix had accumulated around the cells or in the extracellular space. External fibers of the original scaffold could barely be resolved at the light microscopical level, suggesting they were very thin.

Small deposits of alizarin red staining (calcium staining) within the collagen matrix indicated that mineral was hardly deposited within the matrix. A thin layer of alizarin red stained material was noticed at the surfaces of the artificial root and bone (not shown), which likely represents the CaP coating that had been applied prior to culture.

Loading induces magnitude-dependent expression of COX-2 and COL1 genes

We then analyzed whether intermittent axial loading influenced expression of the mechanosensitive genes COX-2 and COL1.

Loading of fibroblasts (density 1.0×10^4 cells/20 μL) differentially affected COX-2 and COL1 gene expression, which was magnitude dependent. COX-2 gene expression was decreased ($\pm 50\%$) by a loading regime consisting of 20 μm displacement of the artificial root (Fig. 5A), whereas expression was increased ($\pm 125\%$) for 100 μm (Fig. 5B) and not significantly affected for 200 μm (Fig. 5C). COL1 gene expression was not significantly affected by displacements of the artificial root by 20 μm (Fig. 5D) or 100 μm (Fig. 5E), whereas a displacement of 200 μm resulted in an increase ($\pm 30\%$) of COL1 gene expression (Fig. 5F). Expression of the noninducible COX isoform (COX-1), the osteoblast-specific transcription factor runt-related factor-2 (Runx2, early osteogenic marker), and the bone matrix proteins osteopontin (OPN, a bone differentiation marker) and dentin matrix protein 1 (DMP1, an osteocyte/cementocyte marker) was not affected by either loading regime (data not shown).

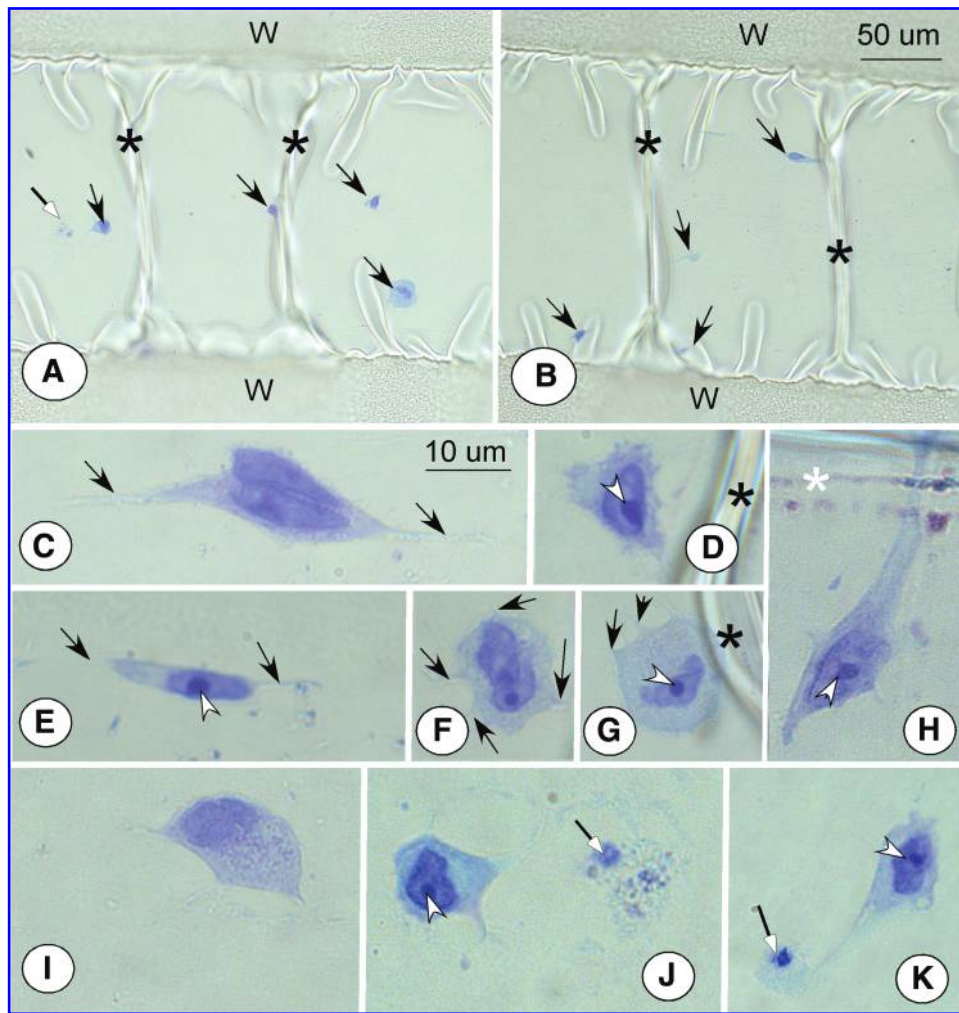


FIG. 4. Histological evaluation of a (maximum) loaded construct after 4 days of culture. (A) and (B) show low-power images of part of the culture chamber containing cells (black arrows) embedded in the gel. Note that there is some staining of the matrix molecules of the gels and few cells are in contact with or near the walls of the chamber. Asterisks represent histological artifacts (unavoidable folding of the plastic embedding). W denotes the walls of the chamber made of PEEK. (C-K): Examples of the shape and vitality of the cells in the chamber. Cells are elongated and with two long processes parallel to their long axis (C, E, H, K), triangular (D), or more round (F, G, J) with multiple short cell processes (black arrows). Most of them have a prominent nucleolus (white arrow heads). White arrows in (A), (J), and (K) point at non-vital cells with degraded nuclei located between vital cells. White asterisk represents the staining artifact. Richardson's stain. (A, B): original magnification, $200\times$, bar = $50\ \mu\text{m}$; (C-K): original magnification, $1000\times$, bar = $10\ \mu\text{m}$. Color images available online at www.liebertonline.com/ten.

High-magnitude loading has no effect on protein production

Since only the highest displacement ($200\ \mu\text{m}$) resulted in an increased COL1 gene expression, this loading regime was used to analyze the effect of intermittent axial loading on production of collagens by measuring [^3H]proline incorporation into (total) proteins and determining the collagenase digestible fraction. Low cell density studies gave very low ^3H counts without differences between loaded and nonloaded cultures. A high cell density (1.0×10^5 cells/ $20\ \mu\text{L}$ gel) resulted in the formation of a higher level of [^3H]proline-labeled proteins (Fig. 6A), of which 27% was collagenase digestible (Fig. 6B). However, loading had no measurable effect on synthesis of these ECM proteins as both the total amount of incorporated [^3H]proline (Fig. 6A) and its distribution into

collagenase digestible (collagens) and nondigestible (other proteins) material (Fig. 6B) had not changed.

The media contained insignificant amounts of TCA-precipitable [^3H]activity and were not further examined.

Discussion

Characterization of the PDL model

Mechanical loading can stimulate cells to preserve tissue structure or tissue formation and thus is a potential factor contributing to reconstruction of tissues. Loading modalities such as type of loading, frequency, or amplitude to stimulate regenerative processes are still far from known and likely differ from cell type to cell type. Because of the complex relation between different modalities of mechanical loading

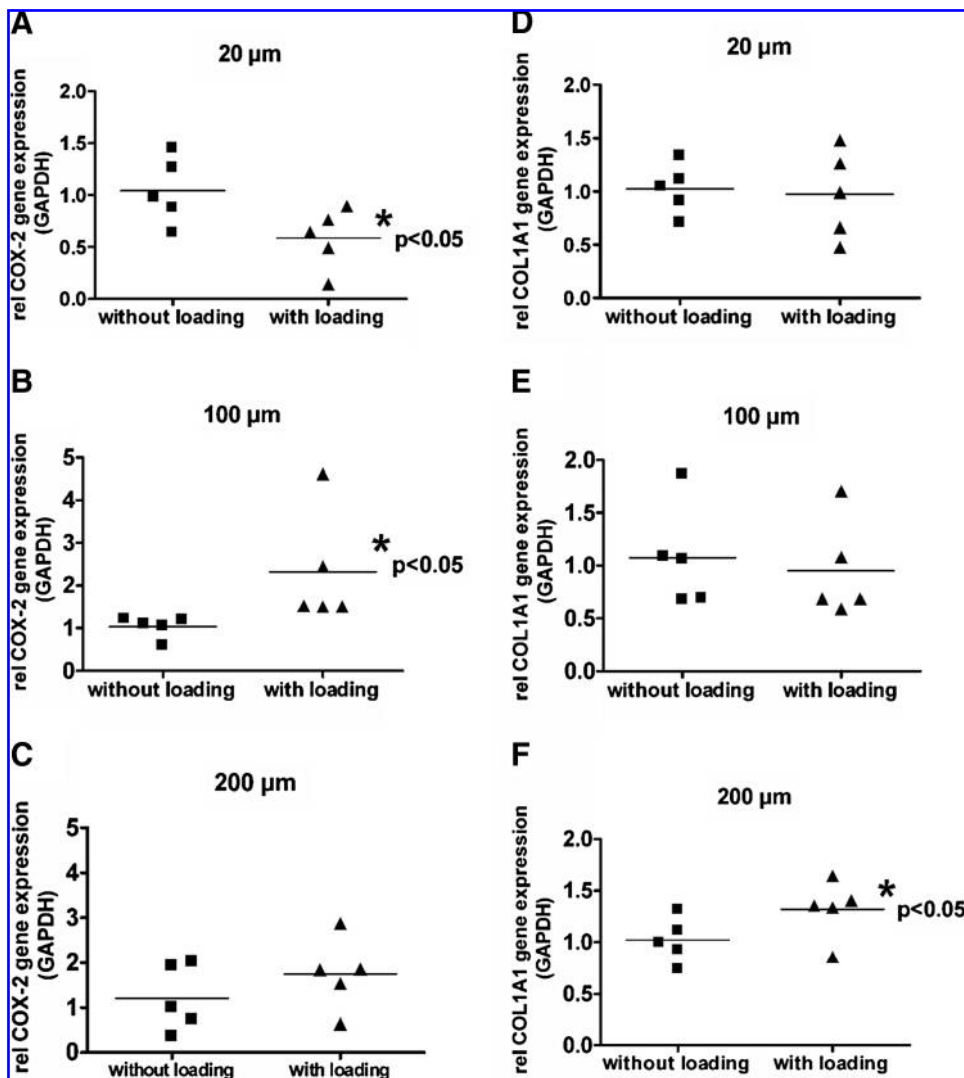


FIG. 5. Effect of intermittent axial loading of different magnitudes on relative gene expression of COX-2 (A-C) and COL1A1 (D-F) by PDL fibroblasts after 4 days of culture. Displacements of the tooth root by 20 μm (A, D), 100 μm (B, E), or 200 μm (C, F) were applied starting at day 2 of culture for 3 days with 360 loading cycles/day separated into four loading periods with recovery periods of 4 hours in between. Data are normalized to GAPDH expression ($n=5$). Loading induced a magnitude-dependent response in COX-2 and COL1 gene expression compared to static controls.

and the biological responses *in vivo*, *in vitro* models are very useful. The objective of this study was to design a model in which human PDL cells embedded in a 3D scaffold can respond to mechanical loading similar to an authentic PDL, in order to explore the use of mechanoloading in the construction of a new ligament.

We hypothesized that loading increases expression of signaling factors and ECM proteins as part of the adaptation to increased mechanical demands. The results show that human PDL cells in a 3D construct respond to even a small strain of daily loading cycles by changing expression patterns in a magnitude-dependent way. The effects of loading under the given conditions are relatively small, but the fact that responses were in opposite ways (reduction of COX-2 expression at low loading but stimulation at intermediate loading) and only some genes were responding is interesting and promising. Additional studies are required to determine whether responses can be enhanced.

The PDL model was chosen to study ligament tissue engineering for several reasons. First, the small size of the ligament is beneficial. Small tissue constructs require relatively small number of cells. This is an advantage as primary cells can be used without extensive subculturing, which

may change the phenotype of the cells. Second, small tissue constructs provide easy access of diffusible nutrients and oxygen needed for cell survival from surrounding culture media in the absence of a vasculature. We chose a tissue construct height as small as possible and made the construct freely accessible to culture media at both the top and the bottom. The majority of the cells in the cultured constructs proved healthy and vital. Third, small tissue constructs enable proper attachment of the construct to the walls of the chamber to build internal tension and enable axial displacement. Internal contraction of the gels by the cells result in detachment of the gels from the walls of PEEK culture wells.^{30,31} The width of the ligament tissue construct was therefore kept low to provide a high surface area/volume ratio, which implies a relatively large area for gel attachment but a low number of cells that can contract the gel. The PDL width in humans is 150–380 μm , and hence we considered a space of 200 μm physiologically meaningful.²⁷ The total volume of the gel was 20 μL . To further improve gel attachment, the surfaces of the chambers were precoated with apatite and the gel after setting was anchored to the surface by ALP-mediated mineral deposition.³⁰

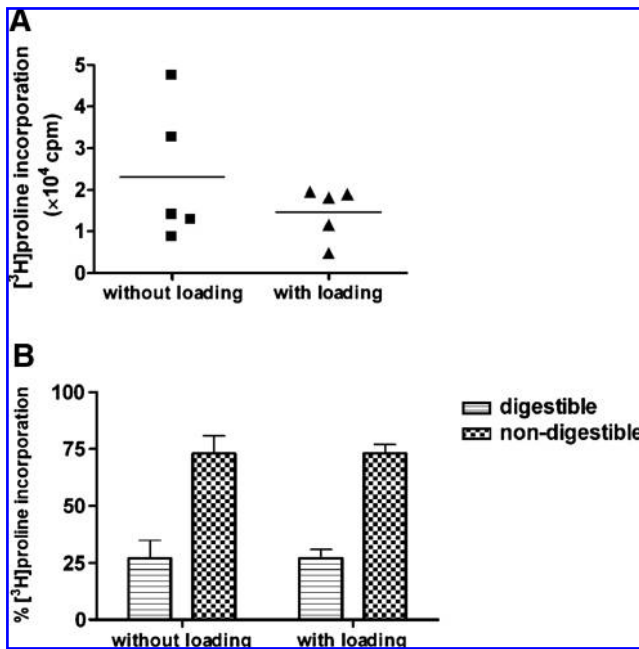


FIG. 6. (A) Effect of intermittent axial loading of high magnitude (200 μ m) on [³H]proline incorporation by PDL fibroblasts after 4 days of culture. Displacements of the tooth root by 200 μ m was applied starting at day 2 of culture for 3 days with 360 loading cycles/day separated into four loading periods with recovery periods of 4 hours in between. (B) Effect of loading on relative [³H]proline incorporation into either collagenase digestible (collagens) or nondigestible material (other proteins). Values represent the mean % [³H]proline incorporation \pm SD ($n = 5$).

The design of the model fulfilled its requirements: the gel did not detach from the wall surfaces during culture or following loading, and the cells remained viable and active. Only a minor fraction of the cells appeared nonvital and exhibited nuclear fragmentation. The random distribution of these nonvital cells located between vital cells suggests that culture conditions are not responsible for cell death but other factors possibly programmed cell death. Programmed cell death is a normal physiological process found in most adult and developing tissues including developing⁴⁰ and mature⁴¹ PDL, apoptosis being an integral part of the development process. The percentage of dead cells in our gels may seem high, but *in vivo* the percentage of apoptotic cells in tissue sections may be as high as 25% when measured by dynamic labeling studies.⁴² Due to the low-density seeding of cells in our 3D gels, apoptotic cells cannot be phagocytosed by neighboring cells and remain trapped in the gel. *In vivo*, apoptotic cells are rapidly cleared from the tissue by phagocytosing neighboring cells or scavenger cells, leaving only a fraction of the apoptotic cells for detection. In 2D cultures dead cells detach from the surface of the wells and are removed from the dishes during medium change or wash procedures and thus escape detection.

Response of PDL fibroblasts to loading

A small displacement (20 μ m) within the physiological range of tooth displacement by mastication resulted in decreased COX-2 gene expression, but an intermediate dis-

placement (100 μ m) resulted in an increased expression of the gene. Such opposite responses at different magnitudes were also found for COX-2 and COL1 gene expression after cyclic stretching of monolayers of human tendon fibroblasts.¹⁰ Stretching tendon and ligament fibroblasts from different sources with different magnitudes enhances gene expression of COL1.^{9,12,14} Based on the hypothesis of Pauwels we expected that strains higher than 15% would result in increased production of fibrous connective tissue.^{6,7} In our model, COL1 gene expression was enhanced but to a moderate extent and only after the largest displacement (200 μ m). The reason for this relatively low response is unclear. Frequency of loading in our model may have been too low or strain perception by cells suspended in a thin network and experiences only a low degree of stiffness may be lower than *in vivo* and lower than cells grown as monolayers on a solid support. Stiffness of the substrate has recently been reported to greatly influence cell behavior, activity, and commitment into a particular lineage.⁴³

The number of mRNA transcripts for COL1 was increased but without increase in collagen protein synthesis. This indicates that changes in mRNA levels do not necessarily imply changes in protein synthesis. Poor translation of mRNA into protein has been reported for a number of different protein species; translation can be restricted by several recently discovered mechanisms.^{44,45}

Strain perception by PDL fibroblasts

The relatively low response in our model may be associated with low density of matrix proteins and cells compared to high cell and matrix densities in PDL. Collagen fibers occupy 42% of the space of ECM⁴⁶ and make up almost 35% of the dry weight of the PDL,⁴⁷ which is 175 times higher than the 0.2% collagen used in the cell-gel constructs in our study. Also, the composition of both ECMs differ substantially; the matrix constituting the gel is extremely simple compared to the complex and rich nature of the ECM of genuine PDL tissue. PDL tissues contain at least three different types of collagen and approximately 3.5% non-collagenous proteins^{47,48} including proteoglycans, different species of glycoproteins such as OPN and osteonectin, and other minor proteins and growth factors. These organic molecules are involved in tissue structure; provide strength, elasticity, tension, stiffness, and anchoring sites for cells; and act as reservoirs for soluble factors⁴⁹ that influence cell behavior. The dense ECM network *in vivo* is thought to transfer even small deformations resulting from loading by mastication to the cells.⁵⁰ Due to the paucity of ECM proteins in our model and as a result of a lower number of contacts between matrix and cells, the load applied to the collagen matrix may not have been experienced by the fibroblasts at its full potential as *in situ*. Authentic PDL tissue is also much more densely populated; human fibroblasts constitute almost 25% of the periodontal space,⁴⁸ which is at least 50 times more cell dense than our cell-gel constructs (estimated 0.5% cell density). The high cell densities *in vivo* also highly enable cell-cell signaling transmitted by short-distance acting paracrine factors that stimulate cells to proliferate, differentiate, and suppress programmed cell death. Several authors have suggested that it is the increased production of paracrine growth factors, such as TGF- β 1, that links mechanical

loading to enhanced collagen expression in tendons *in vitro* and *in vivo*.^{9,51-54} In our model the distance between the cells may be too great to signal each other by such locally produced factors. More work has to be done to examine whether the perception of the actual strain on the fibroblasts in the model can be increased, for example, by providing a more dense collagen network, enriching the matrix with non-collagenous proteins and growth factors, and enhancing cell density. In short, the *in vitro* PDL model supports cell viability and matrix synthesis and allows the cells to respond to mechanical loading without overtly disrupting the gel matrix. Although still far from a clinical application, the *en bloc* implantation of a (partial) bioresorbable version of the model containing an *in vitro* engineered periodontal construct may be an option to explore. Implanted in the edentate jaw it could be a more attractive approach to generate a flexible connection around artificial roots than transplanting PDL cells or unsupported soft tissue constructs that collapse or transform into bone.

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Disclosure Statement

No competing financial interests exist.

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