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Tissue-engineered ligament: implant constructs for tooth replacement

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Abstract

Aim: A tissue-engineered periodontal ligament (PDL) around implants would represent an important new therapeutic tool to replace lost teeth. The PDL is the key to tooth anchoring; it connects tooth root and alveolar bone, and it sustains bone formation.

Materials and Methods: Cells were isolated from PDL and cultured in a bioreactor on titanium pins. After the formation of multiple cellular layers, pins were implanted in enlarged dental alveolae.

Main Outcome Measures: Cell-covered implants integrated without adverse effects, and induced bone in their vicinity.

Results: A histological examination of a dog model revealed that cells were arranged in a typical ligament-like fashion. In human patients, product safety was ascertained for 6–60 months. Probing and motility assessments suggested that the implants were well integrated with mechanical properties similar to those of teeth. Radiographs demonstrated the regeneration of deficient alveolar bone, the development of a lamina dura adjacent to a mineral-devoid space around the implant and implant migration in an intact bone structure.

Conclusions: New tissue consistent with PDL developed on the surface of dental implants after implantation. This proof-of-principal investigation demonstrates the application of ligament-anchored implants, which have potential advantages over osseointegrated oral implants.

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Apart from its role in tooth anchoring, the periodontal ligament (PDL) provides progenitor cells for alveolar bone formation and remodelling (Devlin & Sloan 2002, Mizuno et al. 2006, Gay et al. 2007); at the bone side facing the tooth root, the PDL plays the role of the periosteum. Periodontal disease with tissue destruction by inflammation often leads to resorption and loss of alveolar bone (Wikesjö et al. 2004, Pinkerton et al. 2008), which may be followed by tooth loss (Cochran 2008). In contrast, a functional PDL induces bone, even at ectopic sites (Hamamoto et al. 2002). The PDL suppresses its own ossification, a biological function associated with the local expression of the homeobox gene *Msx2* (Yoshizawa et al. 2004).

Large, acute PDL and alveolar bone lesions may be repaired when they are induced experimentally; however, care must be taken to protect the repair site from invasion by competing tissues, especially gingival connective tissue (Polimeni et al. 2006). Likewise, tissue repair in periodontal disease is only possible by the protection of the repair site.

A possible approach to the replacement of lost teeth is tissue engineering of the PDL. In support of the feasibility of this concept, the PDL has been shown to possess a capacity for spontaneous regeneration, during which the biomechanical tissue strength is restored (Shionhara et al. 2004), and innervation is re-established (Yamada et al. 1999,

Conflict of interest and source of funding statement

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Maeda 2004). After clinical tooth transplantations, a new functional PDL can be regenerated, apparently from PDL tissue accompanying the transplanted tooth (Mandel & Viidik 1989, Andreasen et al. 1995, Gault & Warocquier-Clerout 2002). Even after transplantation at sites with deficient bone, restoration of alveolar bone has been observed, along with the recovery of functional tooth anchoring (Gault & Warocquier-Clerout 2002). Regeneration of the PDL likely emanates from PDL progenitor cells (Seo et al. 2004), which can assemble new PDL-like structures in vivo (Sonoyama et al. 2006). Regeneration proceeds with a new layer of cementum, attached to the original cementum of the tooth root, into which new transverse fibres are integrated (Ripamonti 2007). Importantly, if a new cementum layer were to be laid down on the surface of an engineered device, this would accommodate the integration of a properly attached PDL with the potential to stimulate the regeneration of adjacent alveolar bone.

Currently, to replace lost teeth without considering the PDL, implants of inert biomaterial are directly inserted into jawbones. In these procedures, local bone defects and generally poor bone quality necessitate bone reconstruction before implantation (Wikesjö et al. 2007), and localized bone loss around the implant fixture represents a clinical challenge (Sennerby et al. 2008). A further, commonly observed problem is gingival recession, possibly due to modified tissue architecture, which requires further surgical interventions (Ekfeldt et al. 2003). An implant system that would include a PDL with tissue-inducing properties might alleviate these problems. Technically, implants carrying a PDL may be installed in the extraction socket of the missing tooth, thereby facilitating the surgical procedure. Natural implant anchoring might also be compatible with further growth and development of the alveolar bone housing, and it may allow tooth movements during orthodontic therapy.

Here, we describe the technical development and the clinical application of so-called ''ligaplants'', the combination of PDL cells with implant biomaterial. After implantation, a new PDL-like tissue is produced. Ligaplants have the capacity to induce the formation of new bone, when placed in sites associated with large periodontal bone defects.

Materials and Methods

Implantation of human PDL cells in nude mice

Human teeth were extracted for non-study-related medical indications, and rinsed with PBS (Hyclone, Logan, UT, USA) containing 300 IU/ml penicillin, 3 μg/ml amphotericin (Bristol-Myers Squibb, Rueil-Malmaison, France) and 60 μg/ml gentamycin (Panpharma, Fougéres, France). The mid portion of the root was scraped to collect PDL tissue, which was treated with collagenase A (Roche, Neuilly, France) solution at 0.1 U/ml in DMEM/F12 culture medium containing gentamycin, fungizone, and penicillin for 16 h in an incubator at 37°C. Cells were cultured for 3 weeks in DMEM/F12 with HEPES, 20% newborn calf serum, 250 μg/ml ascorbic phosphate (Sigma-Aldrich, Saint Quentin, France), 10 μg/ml tetracycline, 50 IU/ml penicillin, 0.5 μg/ml amphotericin, and 10 μg/ml gentamycin, to obtain cell sheets (Black & Romette 2003). Sheets were rolled onto porous hydroxyapatite (HAP) implant discs [4 mm diameter, 1.5 mm high (Hugueny SA, Fleurieux, France); pore size of 300–600 μm with 15 μm interconnections, composed of 70% HA and 30% tricalcium phosphate], or onto NaOH-cleaned monkey tooth roots, then cultured for 2 more weeks, and implanted subcutaneously into the dorsa of 8-week-old male nu/nu mice. Mice were sacrificed after 12 weeks and the implants and corresponding associated tissues were harvested. Samples were fixed in 4% paraformaldehyde, demineralized in cacodylate buffer saturated with EDTA, embedded in paraffin and 6–8 μm sections were stained with Masson's trichrome, or subjected to immunostaining. All animal experiments (mice and dogs) were performed at the facility of Biomatech-Nasma (Chasse sur Rhone, France), under the authorization No. B3808710001.

Ligaplants in a canine model

Teeth were extracted from Beagle dogs, and cells were obtained as above. Cells were pre-cultivated for 4 days in plastic dishes to semi-confluence. Ti pins (cone-shaped cylinders, 15 mm long and 5–7 mm wide) were covered with HAP by treatment with simulated body fluid (Kim et al. 1996, see Fig. 7), followed by coating with commercial serum fibronectin. Those pins intended to be implanted are referred to as ''implants'', or as ligaplants, if they were covered with PDL-derived cells. Sixty thousand cells were seeded onto each implant by manually applying a cell suspension in a humified chamber, and the implant was placed in a bioreactor (Fig. 2). DMEM/F12 with HEPES, 20% newborn calf serum, 250 μg/ml ascorbic phosphate (Sigma), 10 μg/ml tetracycline, 50 IU/ml penicillin, 0.5 μg/ml amphotericin, and 10 μg/ml gentamycin was perfused at a constant rate of 1 ml/h at 37°C. Culture continued for 8 days (short culture) or 18 days (long culture), after which the ligaplant was inserted into an alveolus in the jaw of the cell donors that had been prepared 2 weeks in advance. The ligaplants, alternatively control implants without PDL cells, were maintained in the alveolus by the use of a flexible splint for 73 days (short-culture ligaplants) or 55 days (long-culture ligaplants). Dogs were sacrificed, the tissues containing the implants were dissected, fixed as above, dehydrated, embedded in resin and sectioned using a diamond-band saw. Sections were ground to 100 μm thickness and either stained with Masson's trichrome, or observed directly by phase-contrast microscopy.

Human clinical investigation

Initial safety assessments of ligaplants addressed the question of possible inflammatory reactions or other adverse effects of the presence of the ligaplants. Human patients were recruited with the main entry criteria of having one compromised tooth to be replaced, and a radicular area sufficient for PDL cell collection. Research participants were between 35 and 69 years of age, non-smokers, Caucasians, in good general health, and without acute or chronic oral inflammation except in the compromised tooth. Particular criteria for exclusion were seropositiveness for HBV, HCV, HIV, CMV, toxoplasmosis and syphilis; susceptibility to generate a local focus of inflammation (periodontitis was treated beforehand); necessity to place a removable prosthesis; allergy against penicillin; and pregnancy or breastfeeding. All patients signed an informed consent form. Ethical authorization for this study was obtained from the Institutional Review Board (IRB) of Centre Hospitalier Universitaire de Brest, and authorization to perform human clinical trials was obtained from the French Regulatory Agency (Agence Francaise de Securité Sanitaire des Produits de Santé, AFSSaPS). Cell culturing (as above) was

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performed in an AFSSaPS-certified facility under the control of components for human use at ''Etablissement Français du Sang'', Lyon, France. Ligaplants were prepared using the protocol used in the pre-clinical canine study, but without fibronectin coating. Only multi-layer cell cushions were used. After surgery, mechanical probing was performed and radiographs were obtained to study tissue healing. Periodontal pocket depths and ligaplant mobility were recorded by two independent evaluators, following standard periodontal evaluation routines. Nine ligaplants were inserted into the jaws of nine human research participants. One patient was dismissed from the study. This patient suffered a personal crisis, became depressed, developed allergic reactions, and started smoking. Ligaplants #1–4 were load-protected for a short period, up to 8 weeks, ligaplant #6 was kept load-protected for 3 months and ligaplants #5, 7 and 8 were kept under the protection of a composite fixed bridge to the neighbouring teeth before loading. Scanning electron microscopy for the analysis of failed ligaplants was performed at the service unit of the University of Marseille (France).

Results

Isolated PDL cells are able to form a new PDL on an apatite surface

PDL cells were isolated from avulsed human teeth, expanded in vitro, combined with porous HAP, or alternatively, with NaOH-cleaned monkey tooth root fragments and implanted subcutaneously into nude athymic mice. After 12 weeks, implants were examined histologically. In porous HAP samples, elongated cells had formed palisades resembling normal PDL, along a newly formed layer similar to cementum (Fig. 1a). Collagen fibre bundles were inserted into this layer and oriented perpendicularly to its surface. These collagen fibres were of human and not of mouse origin, because they were detected by anti-human collagen serum and not by anti-mouse collagen serum (Fig. 1b–d). The PDL-like cell arrangement was not observed around cleaned tooth root fragments (not shown). Specific experimental conditions might have played a role in this outcome, because PDL-like cell arrangements can also occur around dentin blocks too (Flores et al. 2008). Nevertheless, this experiment indicated that isolated PDL cells could recover their normal configuration, typical of bona fide PDL.

Culture of PDL cells in a bioreactor

A titanium pin was placed in a hollow plastic cylinder leaving a gap of 3 mm around the pin (Fig. 2a). Culture medium was continuously pumped through the gap. Single cell suspensions, obtained from human or dog PDL, were seeded first into plastic dishes, then onto the titanium pins. The pre-culture proved necessary to obtain enough cells for consistent results. To provide a surface layer

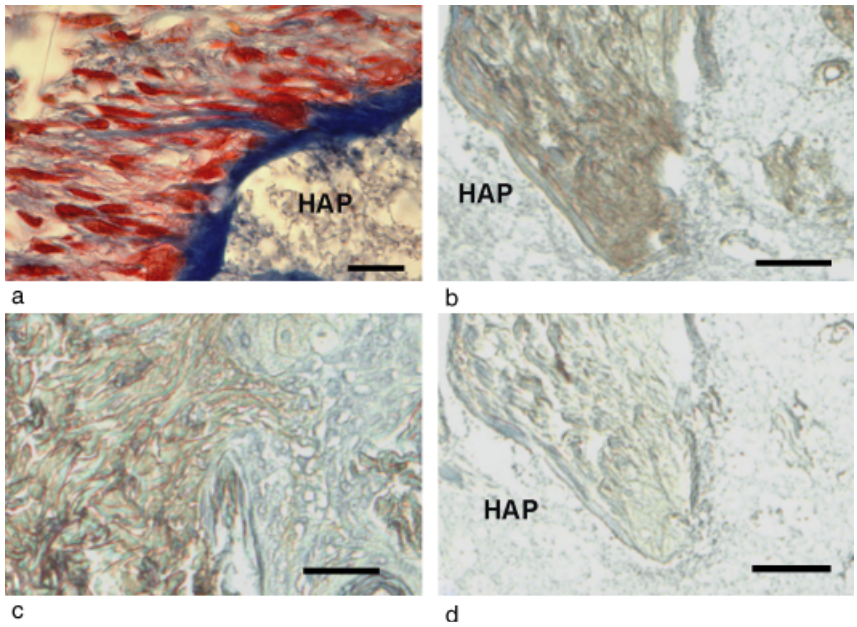

Fig. 1. Formation of periodontal ligament (PDL)-like structures by implantation of human PDL cells into nude athymic mice. Cells were harvested, grown in vitro, seeded on granular hydroxyapatite (HAP) and implanted subcutaneously into the neck region of mice. After 12 weeks, mice were sacrificed, and the implanted material was analysed histologically. (a) Cells and extracellular fibres arranged into PDL-like structures. (b) Immunostaining using anti-human collagen type I serum (brown) indicated the human origin of the HAP-associated fibres. (c) Staining with anti-mouse collagen type I serum revealed mouse collagen in scar tissue. (d) HAP-associated fibres are not stained with anti-mouse serum. Scale bar: 20 μm (a), 40 μm (b–d).

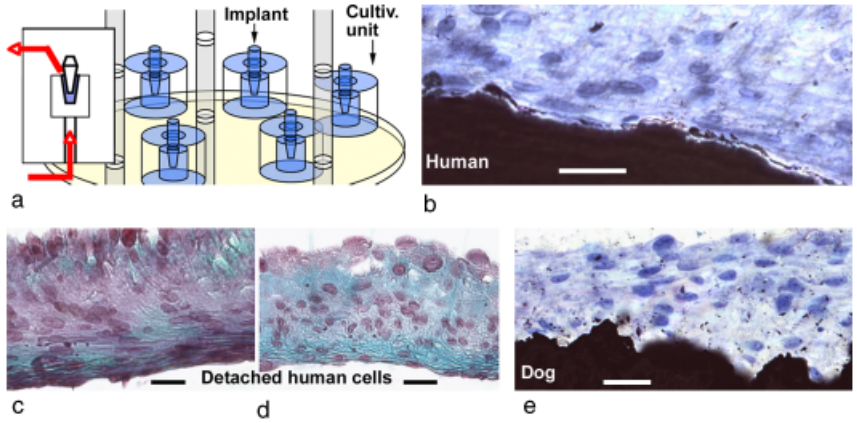

Fig. 2. Assembly of human and canine periodontal ligament (PDL) cells with Ti implants, resulting in ligaplants. (a) Bioreactor; cell suspensions were seeded on apatite-covered cone-shaped Ti cylinders (implant, vertical) and incubated in plastic vessels (cultivation unit) under a flow of growth medium (inset, red arrows) for 18 days. (c and d) Cell cushions were detached from the ligaplant to prepare paraffin sections (Richardson's stain). (b and e) Ligaplants were embedded in plastic and ground sections were prepared (methylene blue stain). Scale bars: 10 μm.

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that is similar to the granular HAP used for Fig. 1, the pin had been incubated in simulated body fluid (Kim et al. 1996). Ground sections from whole complexes were analysed (Fig. 2b and e), and paraffin sections of detached cell cushions were also examined. PDL cells had proliferated and formed stratified layers on the implant surface. Cell orientation and the arrangement of fibres were variable with respect to the support (Fig. 2c and d). PDL-typical tissue conformations were not observed.

Canine model for PDL reconstitution

HAP-coated Ti pins were combined with canine-derived PDL cells as above, to obtain ligaplants, and implanted into jaws of the respective cell donors. Additional fibronectin coating had proven favourable for the attachment of dog cells (data not shown). Eight-day cultures with cell monolayers and 18-day cultures with stratified cell cushions (Fig. 2) were tested. As an alternative to apatite coating, Ti pins were treated with H_2O_2 to enhance cell binding (Tavares et al. 2007), covered by PDL cells after both short- and long-term culture and implanted. Pins without cells served as controls. All pins were implanted into prepared alveoli. Each of the four dogs received one of each cell-covered implant type plus one control. The coronal regions of the implants were protected by a solid composite and fibre splint (Fig. 3a–c). After 20 weeks, nine implants had been lost due to apparent mechanical overloading of the implants exerted by the animals. All dogs were sacrificed after 20 weeks, and a comprehensive evaluation of ground sections of the remaining 11 implants was performed. Two control implants without cells did not firmly integrate; they were encapsulated in a tissue consisting mainly of cells. Collagen fibres at the bone side were mostly parallel to the implant surface; occasional perpendicular fibres were not in contact with the pin (Fig. 3f). Similarly, no PDL formation was observed with any of the four H_2O_2-treated pins, with either stratified cells (long-term culture) or as monolayer (short-term culture). Their surrounding tissue exhibited longitudinally oriented fibres and regions similar to the no-cell controls (not shown). PDL formation was obtained after apatite and fibronectin coating; two ligaplants containing stratified cells (long-term culture) exhibited mainly perpendicular fibres and properly oriented cells, one of them all along the implant surface (Fig. 3d,e,g). A new layer of tissue resembling repair cementum (Ripamonti 2007) was formed on the implant surface (arrows). After short-term culture (monolayer), one implant had adopted a PDL-like conformation, one had mostly longitudinally oriented fibres and the third one resembled the no-cell controls. The results indicated that the formation of PDL-like tissue arrangement can be attained in vivo, if a suitable implant surface and sufficient amounts of PDL cells are provided. However, tissue arrangement around the implants was in most cases not uniform, suggesting that the formation of PDL-like tissue from cultured cells is a slow process. Both dogs and macaque monkeys, used in an earlier trial, attempted continuously to shed their implants. This behaviour, in combination with the required time of PDL formation, limits the usefulness of these animal models.

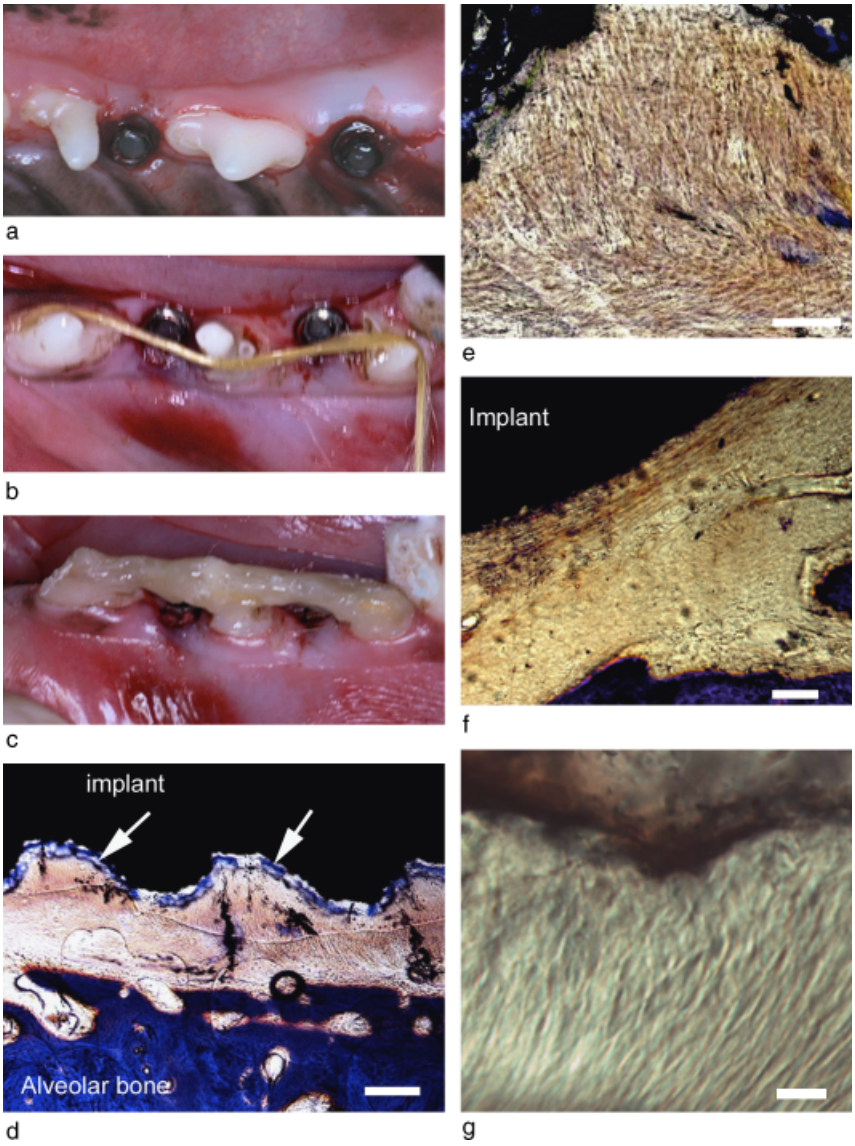

Fig. 3. Canine model to test the implant–periodontal ligament (PDL) cell complex in vivo. (a) Ligaplants (Fig. 2) were applied in the pre-molar region of four dogs. (b and c) The gingival lesions were closed by suturing, and the implant regions were covered by solid composite protections. (d–g) Dogs were sacrificed, and ground sections were prepared from the implant region. (d–f) Masson's trichrome staining; a new layer of dense collagen covered the ligaplant (d, arrows), and a PDL-like tissue had been formed with perpendicular fibres between implant and alveolar bone. (f) Implants without PDL cells, encapsulated without firm mechanical attachment. (g) Ligaplant, no staining, transverse fibres in phase contrast. Scale bars: 100 μm (c), 20 μm (d, e), 10 μm (f).

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Table 1. Human clinical evaluations of ligaplants

Ligaplants	Patient, age (years)	Anamnesis	Mobility of tooth to be replaced	Mobility of ligaplant after 6 months	Mobility of two control teeth at same occasion	Pocket depths (mm) before implantation mb/b/db/ml/l/dl/ **mean**	Pocket depths (mm) after 6 months mb/b/db/ ml/l/dl/**mean**
#1	Male, 51	Adult chronic periodontitis	3	3.3 (before removal)	1.3/0.7	10/4/6/9/12/10/**8.5**	7/6/6/8/8/8/**7.2** (before removal)
#2	Female, 69	Adult chronic periodontitis, root caries	1	1	1/2	3/3/5/4/4/6/**4.2**	4/4/2/4/2/2/**3.0**
#3	Male, 38	Endodontic infection	3	No data, covered by splint	1/1	4/1/2/4/1/4/**2.7**	No data
#4	Female, 35	Root caries, endodontic infection	0	3	0/1	3/1/2/3/2/2/**2.2**	1/3/1/2/1/3/**1.8**
#5	Female, 37	Root fracture	2	2.3	1/2	6/8/4/5/7/5/**5.8**	5/3/4/4/1/3/**3.3**
#6	Female, 52	Adult chronic periodontitis	3.3	No data, covered by splint	1/2.7	9/5/2/10/1/1/**4.7**	2/1/2/2/2/2/**1.8** (before removal)
#7	Female, 55	Root caries	0	1	0.7/1	1/1/1/1/1/1/**1.0**	*/*/*/2/1/3/**2.0**
#8	Male, 49	Root fracture	3	1.3	0.7/1	3/7/2/1/1/2/**2.7**	1/1/1/1/1/1/**1.0**

All patients were Caucasians. Criteria for inclusion in the study were (1) indication of a tooth to be replaced; (2) a root area allowing recovery of sufficient cell numbers; (3) an age of older than 18 years; (4) use of a reliable form of contraception (for women); (5) capability to efficiently communicate and to follow the protocol; (6) signature of the informed consent form. Criteria for exclusion were (1) medical conditions susceptible to interfere with ligaplant implantation; (2) seropositiveness for HBV, HCV, HIV, CMV, toxoplasmosis and syphilis; (3) susceptiblity to generate a local focus of inflammation (periodontitis was treated beforehand); (4) necessity to place a removable prosthesis; (5) allergy against penicillin; (6) pregnancy or breastfeeding; (7) abuse of drugs or alcohol; and (8) risk to lose track of the patient.

Ligaplants #1 and #6 were removed before the time limit of testing, 6 months. Mobility of ligaplants was tested by applying mechanical force; classification according to the Miller index. Periodontal pocket depths (in mm) were determined by mechanical probing around ligaplants.

*No measurement because of the presence of a splint.

mb, mesial–buccal; b, buccal; db, distal–buccal; ml, mesial–lingual; l, lingual; dl, distal–lingual.

Human clinical investigation of ligaplants

PDL cells from individual patients were allowed to attach to Ti pins, and implanted into the cell donors. With regard to safety assessment, no adverse tissue reactions were observed. In particular, there was no gingival inflammation or bone loss, among the eight ligaplants tested. In order to assess the reconstitution of solid tissue around the implant, two independent examiners performed mobility tests (Table 1) and mechanical probing at six sites (Table 1). Mobility tests indicated that ligaplant insertion was different from that of osseointegrated implants, the latter scoring zero in this test. Ligaplant scores were in the range of natural tooth scores (Table 1). Mechanical resistance around all ligaplants in a millimetre-range indicated firm, healthy tissue. In addition, in all cases except one, the probing depth was reduced around the implant, which indicated that the ligaplants had brought about reinforced tissue structure (Table 1). It should be noted that the tissue resistance developed after implantation because ligaplants were initially loosely fitted into their alveoli.

Ligaplant #8 is still in place after 5 years; it replaces a fractured root (Fig. 4a). The adjacent defect in the bone (Fig. 4b) exhibited substantial regeneration 2 years after implantation. During this time, the ligaplant became surrounded by spongy bone. Bone structure was similar to that around tooth roots; however, trabeculae were rather sparse and thin (Fig. 4d, f–h). A desmodontal gap, corresponding to a PDL space of normal width, was evident around the ligaplant, and the structure of the lamina dura resembled that around a natural tooth (Fig. 4e). Around an osseointegrated implant placed beside this ligaplant (Fig. 4e), there was no desmodontal gap. The two implants were furnished with crowns connected by a sliding device allowing vertical movements (Fig. 4e). Both types of implants are equally well tolerated with no indication of inflammatory reactions. Regarding ligaplant #2 (Fig. 5), radiographs indicated that it moved inside the apparently intact bone. It should be noted that the bone level remained constant, but that the implant moved coronally during the period of observation.

Two ligaplants lost anchoring during the first month and were removed, and five ligaplants had to be removed subsequently at 4, 15, 22, 51, and 52 months (Fig. 6). Failures were due to unintended occlusal disturbances and luxation in five cases, and to unknown reasons in two cases. We observed an egression of ligaplants of several millimetres that occurred during healing, which exposed them mechanically and made them more vulnerable than teeth. Shortly after implantation, ligaplants #2 and 4 loosened partially due to unintended mechanical impact. Re-attachment was observed after temporary protection by splinting (not shown). The subsequent ligaplants were therefore protected during the initial integration phase by a splint, which remained in place for 3 months (ligaplant #6), or for 6 months (#5, 7 and 8). In general, the bone level around failed ligaplants slightly recessed, and that around successful ligaplants was reinforced.

Failed implants were analysed by scanning electron microscopy with focus on the centre region that had been the main contact with PDL cells. After removal of these implants, very few surface-attached cells were observed. All implants contained a continuous layer of apatite deposit (Fig. 7c) with occasional small gaps (arrows in Fig. 7a and c). On some implants, we noted darker regions in a patchy appearance (asterisks in Fig. 7a and b). Higher magnification revealed that these patches were free of apatite; they were delineated by a transition zone, where apatite appeared to be partially removed. The patches were interpreted

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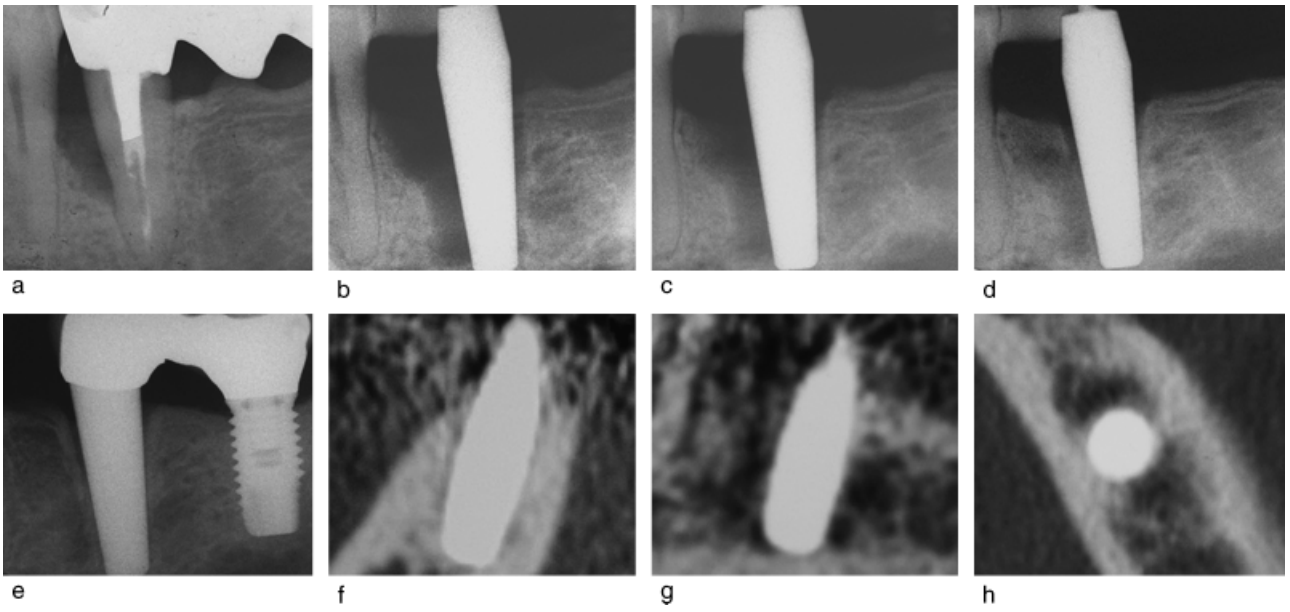

Fig. 4. Ligaplant #8, radiography. (a) Before surgery, root fracture in tooth #34 plus bone defect mesially (left) to the fractured root. (b) Same location immediately after ligaplant implantation. (c) Partial bone regeneration after 24 weeks. (d) After 24 months, note the almost complete replenishment of the major defect. (e) Forty-two months; the periodontium has partially restructured with a fairly regular desmodontal space and reinforced bony demarcation around this space. (f–h) Tomography analysis after 24 months; frontal view (f), side view (g), top view (h). The ligaplant is surrounded by bone trabeculae. Ligaplant dimensions: 15 mm in length, 5–7 mm in width.

to be due to regional resorption of apatite during the implantation period.

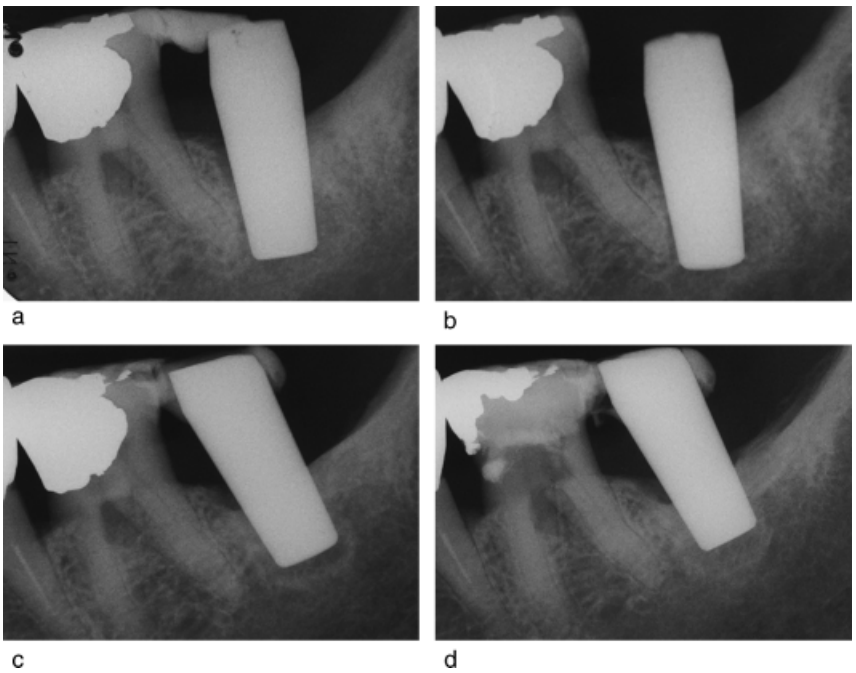

Fig. 5. Ligaplant #2, radiography. (a) After 2 weeks, (b) after 8 weeks, (c) after 2 years and (d) after 4 years. Note the displacement of the ligaplant with respect to the adjacent root and the bone surface. Trabeculation of the bone surrounding the ligaplant is intact, indicating that anchoring occurred via a functional PDL and not via osseointegration. The angle of projection was kept constant. Ligaplant dimensions: 15 mm in length, 5–7 mm in width.

Discussion

The ligaplant system mimics the natural insertion of tooth roots in alveolar bone. Tissue-engineered PDL has been obtained starting from a stratified cushion of PDL cells cultivated on HAP-coated Ti pins. Tissue-specific characteristics were acquired after implantation: a new cementum-like layer, typical for regenerated PDL (Ripamonti 2007), orientation of cells and fibres across the non-mineralized peri-implant space. PDL organization thus induced the cooperation of the tissues surrounding the ligaplant site. Bone formation was induced around ligaplants, suggesting an osteogenic potential of the new PDL. Therefore, functional inter-tissue communication occurred during the tissue-formation phase, as well as in response to the de novo-formed PDL. Physical contacts between implant and neighbouring tooth roots, whereby PDL cells could transgress (Jahangiri et al. 2005), were never observed.

Currently, our evidence concerning the nature of the cementum, PDL, and alveolar bone-like tissue that was formed around ligaplants depends on indirect observations. Our support for cementum (Fig. 3) resides in the positioning of this tissue along the HAP layer of the ligaplant, its limited thickness, a desmodontal-like space, and the absence of ankylosis. The support for bone induction in the clinical study is based on the radiography results: the appearance of a lamina dura, typical for the alveolar bone around tooth roots, and the trabecular bone around the lamina dura (Fig. 4).

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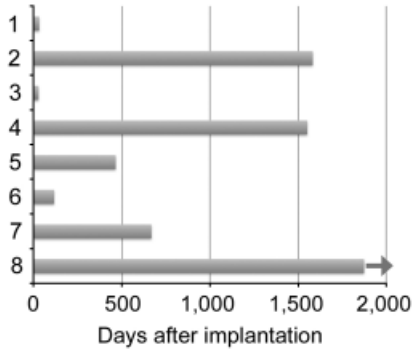

Fig. 6. Stability of ligaplants in their implantation sites. Implants #1–#4 were charged after a short protection period; implant #5–#8 after long-term protection. Ligaplant #8 is still in place.

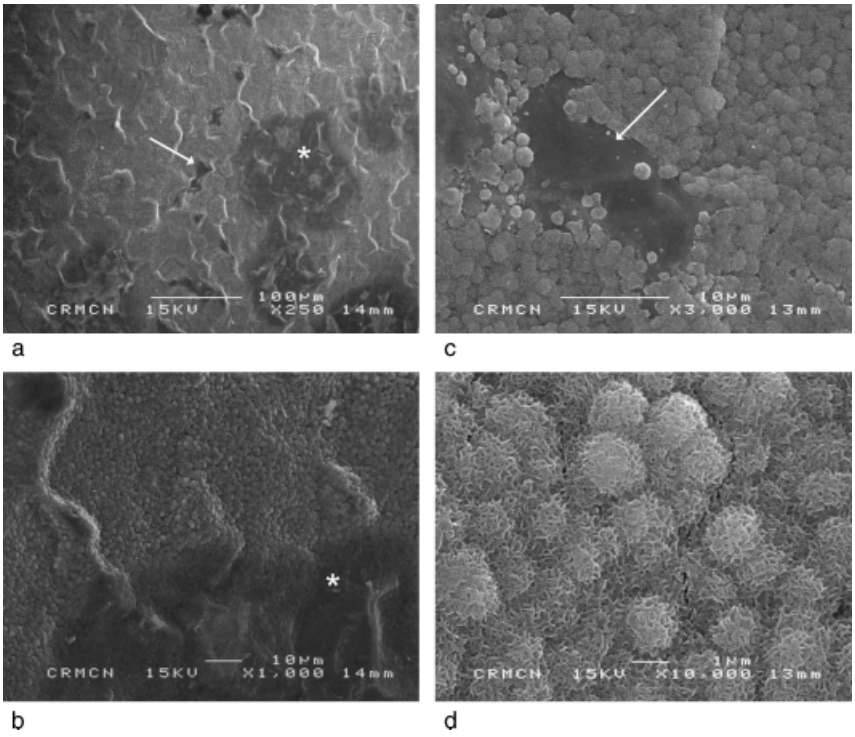

Fig. 7. Scanning electron microscopy of ligaplant #1 (a and b) and ligaplant #3, lost after 28 and 32 days, respectively. After removal, the ligaplant was covered by metal vapour without further cleaning, and analysed using a scanning electron microscope. (a) Overview, showing the structured ligaplant surface covered with apatite deposits (lighter) and apatite-deficient regions (darker, *), as well as minor gaps in the apatite layer (arrows); (b) transition between apatite-containing (up) and apatite-deficient (down) regions; (c) apatite surface showing a minor gap; (d) high magnification of intact apatite.

The implants of this first clinical test were applied in cases of periodontal bony defects, situations where conventional implants could not be installed. Ligaplants became firmly integrated without mechanical interlocking and without direct bone contacts, despite the initial fitting being loose in order to spare the PDL cell cushion. Bone formation was induced and movement of ligaplants inside the bone was often observed, suggesting an intact tissue communication between bone and the implant surface. Three ligaplants out of eight remained in place for >4 years, and two further ones for >1 year. Unknown factors contribute to the success of individual ligaplants, and therefore further technical development is required before routine clinical use.

Compared with the healing of the PDL after tooth transplantation (Mandel & Viidik 1989, Andreasen et al. 1995, Gault & Warocquier-Clerout 2002), the organization of the PDL from cultured cells was an inefficient and a slow process. The stability of implant #8 might suggest that a prolonged period of mechanical stress protection in the initial phase favours the establishment of a stress-resistant PDL. Furnishing ligaplants with cells is, at present, prone to two contradictory demands: on the one hand, a cushion of sufficient thickness favours the formation of a PDL (as the dog experiment suggests), and on the other, prolonged cell culturing may favour the appearance of non-PDL cell types.

In order to preserve the cell differentiation state and to obtain adequate cell stimulation, the bioreactor has been constructed with the aim to resemble the PDL situation during cell growth; cells are positioned in a narrow space between the ligaplant and surrounding hollow cylinder. It was thereby anticipated that the PDL phenotype would be favoured implicating a tight attachment of cells to the implant. However, two complications appeared. Firstly, if during the in vitro experimentation the space between Ti pin and outer wall was reduced to about 100–200 μm, the thickness of human PDL, the flow of growth medium was hampered and cell survival was compromised. In the present experiments, this space was at 3 mm, but it might also be possible to improve medium flow by minute mechanical movements. Secondly, the apatite-free patches on the surface of failed ligaplants (Fig. 7) suggested that the biomaterial used and the surface treatment might not be optimal.

The partial removal of HAP from the surface of failed ligaplants may also suggest that spurious non-PDL cell types are present; genuine PDL does not remove HAP. It is likely that cells differentiate during culture, because flow cytometry has indicated that the proportion of Stro-1-positive cells diminishes (not shown). The apatite-free regions may thus correspond to foci of cells arising during culture, in which the PDL phenotype was lost. For an explanation, we hypothesize that the development of a regenerative PDL depends on site-specific signalling, which in turn is mediated by an anatomic code, written in expression patterns of homeogene-coded transcription factors, as was earlier postulated for the skeleton (Wurtz & Berdal 2003). According to this concept, homeoproteins influence the synthesis of cell surface and signalling components, and signals from the cell surface feed back to modulate homeogene expression, whereby cell identities are established according to the anatomic site and the tissue type (Gómez-Skarmeta et al. 2001, Cheng et al. 2003). Homeogene *Msx2* has in fact been implicated in the segregation of mineralized bone *versus* non-mineralized PDL (Yoshizawa et al. 2004). For the inhibition of mineral formation in the PDL, a role of asporin has been proposed, an SLRP protein that is present in the extracellular matrix (Yamada et al. 2007). A possible connection between the transcription factor *Msx2* and extracellular asporin has, however, not been elucidated.

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According to our results, PDL cells are capable of conserving elements of original gene expression patterns during growth in vitro, which allow them to restore their proper tissue after implantation. However, local tissue-like microenvironments that produce non-PDL phenotypes can at present not be excluded. Signals emanating from dentin matrix and also from the ''rests of Malassez'', sparse agglomerations of epithelial cells at the root surface, may be absent in our culture system; however, their role for PDL regeneration is not clear (Wesselink & Beertsen 1993).

In conclusion, tissue engineering of the PDL has been achieved as a proof-of-concept. We observe two important inter-tissue interactions: firstly, the formation of a functional PDL is dependent on the implantation site, and secondly, ligaplants can induce the formation of bone in the vicinity. Ligaplants as tooth replacements have decisive advantages as compared with osseointegrated devices, due to their potential for periodontal tissue regeneration. It is proposed that therapeutic success requires the ability of a high proportion of the cultivated cells to organize into a new PDL. The ligaplant surgery is relatively easy, because the implant is not tightly fitted into its site. Future clinical use of ligaplants might avoid bone grafting with its expense, inconvenience, and discomfort for the patient.

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Clinical Relevance

Scientific rationale for the study: A tissue-engineered PDL mimics the anchorage system of natural teeth.
Principal findings: Implants covered with such a PDL are well tolerated and induce, in selected cases, the regeneration of large bone defects in their vicinity.
Practical implications: The principle of the surgical treatment is to make use of the original alveolus with limited adaptation. Implant fitting is loose, which facilitates the surgical procedure. The engineered tissue regenerates the anchoring apparatus between the implant and the residual alveolar bone. This system grants an extended range of implant application without preparative bone reconstruction.

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