The application of this technology has enabled the retrieval of confluently cultured cells, such as keratinocytes (Yamato et al., 2001), corneal epithelial cell sheets (Nishida et al., 2004a), and oral mucosal epithelial cells (Ohki et al., 2006) in the form of a "cell sheet". The epithelial cell sheets are multi-layered and preserve the integrity of proteins such as E-cadherin and laminin 5 that are typically destroyed in the process of enzymatic treatments (Yamato et al., 2001). In addition, recent studies revealed that epithelial cell sheets can be fabricated using temperature responsive culture inserts without feeder layers (Murakami et al., 2006a; b), thereby eliminating exclude xenogeneic factors for animal-free cell transplantation (Takagi et al., 2011).

To fabricate thick tissues, cell sheets can be stacked in layers because they can connect to one another very quickly. A study demonstrated that bilayer cardiomyocyte sheets were completely coupled 46 ± 3 min (mean ± SEM) after the initial layering (Haraguchi et al., 2006), suggesting that multi-layered cell sheets can communicate and become synchronized as functional tissues. Based on this study, multi-layered transplantation was performed (Shimizu et al., 2006b). When more than three cardiomyocyte sheets were layered and transplanted into the subcutaneous space in rats, the appearance of fibrosis and disordered vasculature indicated the presence of fibrotic areas within the transplanted laminar structures. Although the rapid establishment of microvascular networks occurred within the engineered tissues, this formation of new vessels did not rescue the tissues when the thickness was above 80 μm. Using a multiple-step transplantation protocol at 1 or 2 day intervals resulted in rapid neovascularization of the engineered myocardial tissues with a thickness of more than 1 mm (Shimizu et al., 2006b), and these results led us to fabricate prevascularized cell sheets (Sekine et al., 2011). Recent studies demonstrate that the combination of different types of cells, for example an endothelial cell sheet sandwiched with other types of cell sheets, can lead to pre-vascularization *in vitro*, which may allow the graft to survive and function (Haraguchi et al., 2012; Pirraco et al., 2011). Furthermore, the three-dimensional manipulation of fibroblast cell sheets and micro-patterned endothelial cells with a gelatin-coated stacking manipulator produced microvascular-like networks within a 5-day *in vitro* culture (Tsuda et al., 2007). Non-patterned endothelial cell sheets and other types of cell sheets with a fibrin gel manipulator can also produce pre-vascular networks both *in vitro* (Asakawa et al., 2010) and *in vivo* (Sasagawa et al., 2009).

2.2. Cell sheet transplantation in animal models

From the beginning of the 21st century, various types of cells have been extracted, cultured in temperature responsive dishes, and fabricated as cell sheets. Transplantation has been performed, and the efficacy of these cell sheets was evaluated in most of the studies.

2.2.1. Corneal regeneration

Limbal stem-cell deficiency by ocular trauma or diseases causes corneal opacification and loss of vision. To recruit limbal stem cells, a novel cell-sheet manipulation technology that takes advantage of temperature responsive culture surfaces was developed (Nishida et al., 2004a). The results reveal that multi-layered corneal epithelial cell sheets were successfully

fabricated and that their characteristics were similar to those of native tissues. Transplantation of these cell sheets induced corneal surface reconstruction in rabbits. For patients who suffer from unilateral limbal stem deficiency, corneal epithelial cell sheets can be cultured from autologous limbal stem cells. When the objective is to repair the bilateral corneal stem cell deficiency, autologous oral mucosal epithelial cells are utilized to create oral mucosal epithelial cell sheets. The cell sheets contain both cell-to-cell junctions and extracellular matrix proteins, and can be transplanted without the use of any carrier substrates or sutures. Therefore, oral mucosal epithelial sheets were examined as an alternative cell source to expand the possibilities of autologous transplantation. Autologous transplantation to rabbit corneal surfaces successfully reconstructed the corneal surface and restored transparency. Four weeks after the transplantation, epithelial stratification was similar to that of normal corneal epithelia, although the keratin expression profile retained characteristics of the oral mucosal epithelium.

2.2.2. Cardiac regeneration

To enhance the function of cardiac tissue, neonatal rat cardiomyocyte sheets were fabricated and examined (Shimizu et al., 2002). When 4 sheets were layered, spontaneous beating of the engineered constructs was observed. When they were transplanted subcutaneously, heart tissue-like structures and neovascularization within the contractile tissues were observed. The long-term survival of pulsatile cardiac grafts was confirmed for more than one year in rats (Shimizu et al., 2006a). Another study was performed to create thick tissue in rats (Shimizu et al., 2006b). However, the thickness limit for the layered cell sheets of subcutaneous tissue was ~80 μm (3 layers). To overcome this limitation, several transplantations of triple-layer grafts were performed, resulting in an approximately 1 mm-thick myocardium with a well-organized microvascular network. Other types of cell sheets were also examined to improve cardiac function. Adipose-derived mesenchymal stem cells in mice (Miyahara et al., 2006) and skeletal myoblasts in dogs, rats, and hamsters (Hata et al., 2006; Hoashi et al., 2009; Kondoh et al., 2006) were transplanted as cell sheets, demonstrating the efficacy of the method for cardiac repair.

2.2.3. Cartilage regeneration

Chondrocyte sheets applicable to cartilage regeneration were prepared using cell sheet manufacturing technique that takes advantage of temperature responsive culture dishes. The layered chondrocyte sheets were able to maintain the phenotype of cartilage and could be attached to sites that exhibited cartilage damage. The cell sheets act as a barrier for preventing the loss of proteoglycan from these sites and for protection against catabolic factors in the joints of rabbits (Kaneshiro et al., 2006).

2.2.4. Esophageal regeneration

With the recent development of endoscopic submucosal dissection (ESD), large esophageal cancers can be removed using a single procedure. However, complications, such as postoperative inflammation and stenosis, frequently occur after an aggressive ESD procedure,

which can considerably affect the quality of life of the patient. Therefore, a novel treatment combining ESD and the endoscopic transplantation of tissue-engineered cell sheets created using autologous oral mucosal epithelial cells, was examined in a canine model (Ohki et al., 2006). The results confirm the efficacy of the novel combination of the endoscopic approach with the potential treatment of esophageal cancers that can effectively enhance wound healing and possibly prevent postoperative esophageal stenosis.

2.2.5. Hepatocyte regeneration

To address the demand for therapeutic benefits for patients suffering from liver disease, the development of new therapeutic applications is crucial. Therefore, hepatic tissue sheets transplanted into the subcutaneous space of mice have been investigated, resulting in the efficient engraftment of the surrounding cells, as well as the formation of a two-dimensional hepatic tissues network, which was stable for more than 200 days (Ohashi et al., 2007). The engineered hepatic cell sheets also showed several characteristics of liver-specific functionality, and the use of bilayered sheets enhanced these characteristics.

2.2.6. Fibroblast sheet transplantation for sealing air leaks

In thoracic surgery, the development of postoperative air leaks is the most common cause of prolonged hospitalization. To seal the lung leakage, use of autologous fibroblast sheets on the defects was demonstrated to be an effective treatment for permanently sealing air leaks in a dynamic fashion in rats (Kanzaki et al., 2007). Using roughly the same procedures, pleural defects were also closed by fibroblast sheets in pigs (Kanzaki et al., 2008).

2.2.7. Mesothelial cells for the prevention of post-operative adhesions

Post-operative adhesions often cause severe complications such as bowel obstruction and abdominopelvic pain. The use of mesothelial cell sheets was investigated to prevent post-operative adhesions in a canine model (Asano et al., 2006). Mesothelial cells were harvested from tunica vaginalis (Asano et al., 2005) and cell sheets were fabricated on a fibrin gel. The results demonstrated that mesothelial cell sheets are effective for preventing post-operative adhesion formation.

2.2.8. Retinal Pigment Epithelial (RPE) cell regeneration

The retinal pigment epithelium (RPE) plays an important role in maintaining the health of the neural retina. RPE cell sheets were fabricated as a monolayer structure with intact cell-to-cell junctions, similar to that of native RPE (Kubota et al., 2006). In the transplantation study, RPE cell sheets attached to the host tissues in the subretinal space were more effective than the use of injected isolated cell suspensions in rabbits (Yaji et al., 2009).

2.2.9. Urothelial regeneration

Augmentation cystoplasty using gastrointestinal flaps may induce severe complications such as lithiasis, urinary tract infection, and electrolyte imbalance. The use of viable, contig-

uous urothelial cell sheets cultured *in vitro* should eliminate these complications. Canine urothelial cell sheets were grown and their structures were shown to be appropriate (Shiroyanagi et al., 2003). Urothelial cell sheets were autografted onto dog demucosalized gastric flaps successfully, with no suturing or fixation, and generated a multi-layered urothelium *in vivo* (Shiroyanagi et al., 2004). The novel intact cell-sheet grafting method rapidly produced native-like epithelium *in vivo*.

2.2.10. Islet regeneration

To establish a novel approach for diabetes mellitus, pancreatic islet cell sheets were fabricated and transplanted in rats (Shimizu et al., 2009). Laminin-5 was coated on temperature responsive dishes to enhance the initial cell attachment, and the presence of specific molecules, such as insulin and glucagon, was also observed in the recipient site.

2.2.11. Thyroid regeneration

For hormonal deficiencies caused by endocrine organ diseases, continuous oral hormone administration is indispensable to supplement the shortage of hormones. To verify the cytotherapeutic approach, cells from rat thyroid were spread on temperature responsive culture dishes, and cell sheets were created (Arauchi et al., 2009). Rats were exposed to total thyroidectomy as hypothyroidism models and received the thyroid cell sheet transplantation 1 week after the total thyroidectomy. The transplantation of the thyroid cell sheets was able to restore the thyroid function 1 week after the cell sheet transplantation and the improvement was observed long after the surgery.

2.3. Cell sheet transplantation in human clinical trials

In Japan, 6 clinical trials using cell sheet engineering technology have been started or have already been completed.

2.3.1. Corneal reconstruction

The first clinical trial of the cell sheet engineering technology involved a corneal reconstruction using autologous mucosal epithelial cells, and the results were published in 2004 (Nishida et al., 2004b). Oral mucosal tissue was harvested from 4 patients with bilateral total corneal stem-cell deficiencies. Subsequently, cells were cultured for two weeks using a mitomycin C-treated 3T3 feeder layer and transplanted directly into the denuded corneal surfaces without sutures. The results demonstrated that complete re-epithelialization of the corneal surfaces occurred, and the vision of all patients was restored. Recently, autologous oral mucosal epithelial cell sheets cultured with UpCell-Insert technology (CellSeed, Tokyo, Japan) without the feeder layer were transplanted into 25 patients for the treatment of corneal limbal epithelial deficiency in France The safety of the products was established during the 360-day follow-up, and the results confirmed its efficacy for reconstructing the ocular surface. (Burillon et al., 2012).

2.3.2. Endoscopic treatment of esophageal ulceration

Using a canine model (Ohki et al., 2006), autologous oral mucosal epithelial cell sheets were fabricated using the UpCell-Insert technology. After performing the esophageal endoscopic submucosal dissection to remove superficial esophageal neoplasms, cell sheets were transplanted, resulting in the complete prevention of stricture formation in patients with partial circumferential resection (Ohki et al., 2009; Ohki et al., 2012).

2.3.3. Improvements in ischemic cardiomyopathy

Autologous myoblast cells from a patient's thigh were fabricated as cell sheets, and these cell sheets were transplanted into end-stage dilated cardiomyopathy patients in need of left ventricular assist systems (Sawa et al., 2012). The myoblastic cell sheets were transplanted into the affected part of the heart in the patients. The first patient was successfully treated and discharged from the hospital without requiring a ventricular assisting device.

2.3.4. Cartilage regeneration

A clinical trial for cartilage regeneration began in 2011 at Tokai University, Japan. In this study, autologous chondrocytes and synoviocytes were co-cultured with the UpCell-Insert technology. After a period of cultivation, co-cultured cell sheets were combined into three layers and transplanted into the cartilage defects of patients.

2.3.5. Nasal mucosa epithelial cell sheet transplantation to the middle ear bone for preventing hearing loss

A clinical trial for preventing hearing loss began in 2014 at The Jikei University, Japan. Autologous nasal mucosa epithelial cell sheets were transplanted to the surface of bone of the middle ear, and inhibit such as the hyperplasy of granulation tissue and bone, and the progression of fibroblast within middle ear cavity, which induce hearing loss after the surgery of otitis media.

3. Periodontal regeneration

Our laboratory started to introduce cell sheet engineering for periodontal regeneration since sometime after 2000. A key event in periodontal regeneration involves the formation of periodontal ligament and cementum complex (MacNeil and Somerman, 1999), which is a thin surface structure that anchors the tooth to the alveolar socket. Several studies have demonstrated that the cell sheet engineering approach can deliver functional cells in the form of a thin layered sheet, wherein the extracellular matrices, cell-cell junctions, and cell-matrix interactions are well-preserved (Kumashiro et al., 2010). Thus, we have attempted to regenerate this periodontal attachment apparatus based on the technology of "cell sheet engineering" (Yang et al., 2007).

3.1. Small animal studies

Human PDL (hPDL) cell sheets were successfully created using temperature responsive dishes, and the characteristics of hPDL cell sheets were investigated (Hasegawa et al., 2005). In this study, explant culture methods were utilized for the primary culture of hPDL cells. The hPDL cell sheets cultured with ascorbic acid were recovered from the culture dishes as a contiguous sheet accompanied by abundant extracellular matrix components, including type I collagen, integrin β1 and fibronectin. Then, hPDL cell sheets were transplanted as cell pellets into a mesial dehiscence model in athymic rats. Four weeks after surgery, newly formed immature fibers with obliquely anchored dentin surfaces were observed in all the experimental sites, whereas no such findings were observed in any control sites (Figure 2).These results suggest that this procedure based upon the principles of cell sheet engineering can be applied to periodontal regeneration.

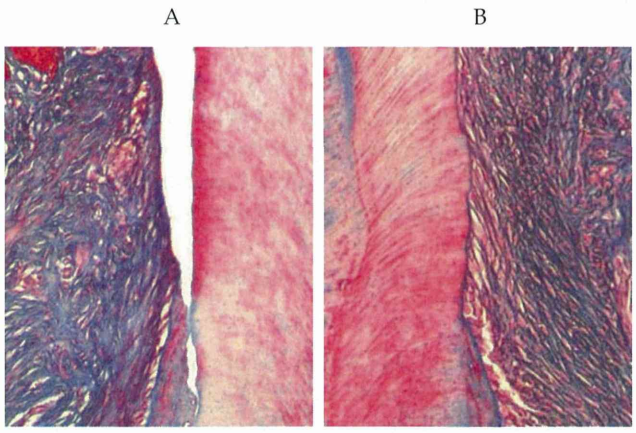

Figure 2. PDL regeneration at 4 weeks postsurgery.

A: Nontransplanted control site. B: hPDL transplanted experimental site. Regeneration of periodontal ligament-like structure was observed only in the experimental site. Azan staining. Modified and reprint from Hasegawa et al., 2005.

Next, the optimal culture condition was examined. Because the osteoinductive medium, which contains 50 μg/ml of ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone, enhanced both osteoblastic/cementoblastic and the periodontal differentiation of PDL cells *in vitro*, we compared hPDL cell sheets cultured in the absence and presence of these osteoinductive supplements in a xenogeneic transplantation model (Flores et al., 2008a). Three layered hPDL cell sheets were constructed with fibrin gel and transplanted with a human dentin block into the back of a subcutaneous athymic rat. The constructs were excised for histological investigation 6 weeks after the transplantation. The three-layered hPDL cell sheets-dentin block constructs induced a new cementum-like hard tissue on the surface of the dentin in more than 60% of the samples. Collagen fibers were inserted perpen-

dicularly into the newly formed cementum-like tissue, and this orientation resembled the native Sharpey's fibers. In addition, the regenerative potential of hPDL cell sheets cultured with the osteoinductive medium the was confirmed, when hPDL cell sheets were transplanted onto the root surface of periodontal defects in athymic rat mandibles (Flores et al., 2008b). The results indicate that most of the specimens in the experimental group exhibited a newly-formed cementum and a new attachment of collagen fibers to the cementum layer. No clear cementum layer was observed in the control group (in the absence of osteoinductive supplements). As shown in these experiments, hPDL cells cultured with osteoinductive medium could contribute to the simultaneous regeneration of cementum and PDL.

3.2. Large animal studies

Based on the successful results from small animal studies, we next utilized canine periodontal defect models. Dog PDL (dPDL) cells were extracted using collagenase/dispase digestion. Four individual dPDL cells were successfully isolated and expanded *ex vivo*. Cells were cultured in a standard medium with osteoinductive supplements for 5 days, because longer cultivation induced spontaneous detachment of cell sheets from the UpCell Surfaces. Three-layered dPDL cell sheets were fabiricated with woven polyglycolic acid (PGA) for cell sheet transfer. This PGA product has a number of advantages, including: 1) cell sheets can be easily peeled from temperature responsive dishes, because cell sheets can be attached to the fibers of the woven PGA, 2) the shrinkage of cell sheets can be prevented, 3) easy stacking of multi-layered cell sheets can be achived in a short period of time (see the video attached to the manuscript (Iwata et al., 2009)), 4) easy adjustment of different sizes of cell sheets can be used to cover any defect shape by simply trimming the cell sheets, 5) the ability to make contact on hard tissues and curved surfaces, and 6) the transplant is visible to the operators. dPDL cell sheets were transplanted into the surface of dental roots containing three-wall periodontal defects in an autologous manner, and bone defects were filled with porous beta-tricalcium phosphate (β-TCP). Cell sheet transplantation regenerated both new bone and cementum connecting with the well-oriented collagen fibers, while only limited bone regeneration was observed in the control group where cell sheet transplantation was not performed. These results suggest that PDL cells have multiple differentiation properties that allow for the regeneration of periodontal tissues composed of hard and soft tissues.

Next, we evaluated the safety and efficacy of PDL cell sheets in a one-wall infrabony defect model (Tsumanuma et al., 2011), which is considered to be a severe defect model (Kim et al., 2004). In this study, we also compared the differences in the periodontal healing of various cell sources. PDL cells, bone marrow derived mesenchymal stem cells, and alveolar periosteal cells were obtained from each animal, three-layered canine cell sheets were transplanted in an autologous manner, and bone defects were filled with porous β-TCP with 3% type I collagen gelto stabilize the graft shape. Eight weeks after transplantation, significantly more periodontal regeneration was observed in the newly formed cementum and well-oriented PDL fibers more in the PDL cell sheets group than in the other groups. These results indicate that PDL cell sheets combined with β-TCP/collagen scaffold serve as a promising tool for periodontal regeneration.

3.3. Optimization of human PDL cells

To protect human rights as subjects in clinical trials, the protocol of cytotherapy should be designed based on Good Clinical Practice (GCP) and Good Manufacturing Practice (GMP). Culturing hPDL cells from a single tooth is essential in performing our clinical trial. However, appropriate method for the extraction and expansion of hPDL cells are still not well understood. Thus, we determined the optimal method of isolation and expansion of hPDL cells and then examined their gene expression levels and differentiation potentials, and eventually validated the common characteristics of hPDL cells from 41 samples (Iwata et al., 2010). The hPDL cells were successfully extracted with collagenase/dispase, and then clonal proliferation was performed. Typically, 10 to 100 colonies were observed for a few days after the initial spreading. hPDL cells exhibit the ability to be highly proliferative when cultured at a low cell density. The cells were subcultured for 3 to 4 days, reaching one million cells in 2 weeks. Then, cells were spread on temperature responsive dishes to create a cell sheet in the presence of the osteoinductive medium. Cell sheets were harvested 2 weeks after spreading because the mRNA expression of osteogenic marker genes was strong after that period of time. Quality assurance tests were performed on at least 7 samples, and then the standard phenotypes of hPDL cell sheets were determined.

According to the GCP and GMP guidelines, hPDL cell sheets were created from three healthy volunteer donors at the GMP-grade Cell Processing Center (CPC) in our university (Washio et al., 2010). GMP-grade reagents and certified materials were used for culturing the hPDL cells. The safety and efficacy of "the product (hPDL cell sheets in this case)" was validated for a clinical trials. Prior to performing the cell culture, autologous serum was prepared from the donors. The hPDL cells were cultured under xeno-free conditions, and cell sheets were fabricated using the temperature responsive dishes. Culture sterility was confirmed using conventional tests. Safety was evaluated using the following tests: 1) the soft-agar colony-formation assay, 2) transplantation into nude mice, and 3) the karyotype test (Yoshida et al., 2012). The efficacy of the cell sheets was verified by transplantation with a dentin block into SCID mice. All of these tests revealed that hPDL cell sheets created at the CPC were safe and exhibited the ability to regenerate periodontal tissues. Another set of three hPDL cell sheets from healthy volunteer donors were created at the CPC to optimize the procedures.

3.4. The clinical trial

After approval on the 5th of January 2011, our clinical trial called "Periodontal regeneration with autologous periodontal ligament cell sheets" was initiated to treat patients presenting with the following ailments: 1) infrabony defects with a probing depth of more than 4 mm after the initial therapy, 2) radiographic evidence of infrabony defects, and 3) a redundant tooth that contains healthy periodontal tissue as a cell source. All patients provided written informed consent according to the GCP. Exclusion criteria included the following: 1) relevant medical conditions contraindicating surgical interventions (e.g., diabetes mellitus, cardiovascular, kidney, liver, or lung disease, or compromised immune system), 2) pregnancy or lactation, and 3) heavy tobacco smoking (more than 11 cigarettes a day). The primary out-

come of this trial is to evaluate the safety and efficacy of autologous transplantation of periodontal ligament cell sheets. As of the end of May in 2014, 10 cases of autologous PDL cell sheets were transplanted, and the healing process took place uneventfully.

4. Conclusion

The applications of cell sheet engineering for regenerative medicine are mentioned. Various types of cells have been examined and most of them improved the functions of recipients, suggesting that cell sheet engineering can be an alternative strategy for the therapy of tissue engineering. The implementation of robotic systems that allow the safe mass production of sterile cell sheets automatically, as well as further collaboration between researchers and medical professionals will make "cell sheet engineering" the leading edge solution for regenerative medicine (Elloumi-Hannachi et al., 2010).

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●セルシートエンジニアリング

歯周組織再生

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要旨

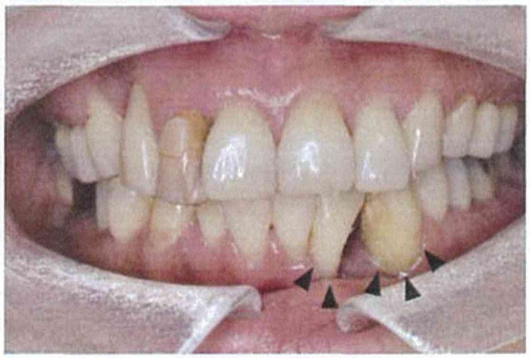
歯周病は多くの日本人が罹患している細菌感染による炎症性骨吸収であり，進行すると抜歯に至る．さまざまな再生療法が試されてきた本領域ではあるが，今世紀以降，大学を中心として細胞を用いた治療法が研究されてきた．筆者らは細胞シート工学を用いて効率良くかつ高い機能を保持した細胞移植法を開発し，自己歯根膜幹細胞を歯周病罹患部位へ移植する臨床研究を実施しており，次世代型の再生医療製品の開発を目指している．

はじめに

“歯周炎”とは，口腔内細菌による炎症性骨吸収を伴う疾患である．歯周ポケット周囲のプラーク（細菌叢）を放置すると炎症を引き起し“歯肉炎”になるが，“歯肉炎”は可逆的病態であり，適切な対応により健康状態に戻ることができるものの，炎症を放置し周囲の歯槽骨の炎症性吸収が起ると，通常は元に戻らない．よって，患者自身が適切なプラークコントロールを実施することと医療者による定期的なメインテナンスが協働することにより，予防することは十分可能であると考えられている[1]が，40歳以上の日本国民の40％以上が，歯周炎に

キーワード：細胞シート工学，歯根膜細胞，歯周組織再生

図1　歯周病の臨床所見



45歳女性．主訴は数ヵ月ごとの疼痛．左下犬歯周囲に歯肉の退縮と歯間空隙が見受けられた．X線写真より骨吸収が根尖に達していたため，抜歯を選択せざるをえなかった．

罹患していることが，厚生労働省の歯科疾患実態調査より明らかになっている[2]．歯周炎初期の段階では自覚症状に乏しいため，明らかな症状を患者自身が自覚した際には歯周炎はかなり進行していることが多く，抜歯に至るケースも少なくない（図1）．

歯周組織の再生

歯周炎で歯槽骨が破壊されると歯肉の退縮が起り，それにより咀嚼障害・発音障害などの機能障害のみならず，審美障害・知覚過敏と根面カリエスなどの，さまざまな問題が起る．さらにはプラークコントロールが困難化し，歯周炎再発を防止するには，患者・医療者双方へ多大な負担がかかることが分かっている．そこで，歯周組織の再生療法が，古くより実践されてきた．歯周組織再生は歯と歯槽骨をつなぐ付着器官を再生することを目的とし，単なる歯槽骨の再生を指すわけではなく，“硬組織（歯根表面のセメント質）-軟組織（歯周靱帯）-硬組織（歯槽骨）”という付着ユニットの再生を同時に促さなければいけないために，困難を伴う．歯周組織再生療法としては，① 自家・他家・合成物などの骨補填剤，② 遮断膜，③ 成長因子などの生物製剤，などがすでに臨床で応用されているが，適応症が限られていたり，予知性が不安定であるなどの問題点を包含している[3]．また，これらのバイオマテリアルを用いた再生療法はそもそも直接的ではなく，欠損部に適用したバイオマテリアルが患者自身の細胞に働きかけることで

再生を引き起すという，間接的な再生療法である．具体的にはマテリアルが患部の細胞に働きかけることで歯周組織の各コンポーネント（歯根膜，セメント質，歯槽骨）を形成していく．そこで，マテリアルには，① 周囲の幹細胞を引き寄せる，② 幹細胞を目的の細胞に分化させる，③ 新生組織の形成を促進させる，などの性能が求められる[4]．再生における主役はあくまでも細胞なのである．

近年の幹細胞生物学と組織工学の発展により，患者から微量な組織を採取し，その組織の中から幹細胞を抽出し，生体外で増幅させる技術が確立されてきた．このような幹細胞生物学と組織工学を背景とした細胞治療の研究が，歯周領域においても2000年以降，大学を中心に進められている．歯周病を歯周組織幹細胞疲弊症としてとらえ，生体に存在する幹細胞をバイオマテリアルとコンビネーションで移植する細胞治療である．本邦ではすでに4つの大学でヒト臨床試験が完了ならびに開始しており，本稿では，歯根膜幹細胞と細胞シート工学を用いた歯周組織の再生に関して解説する．

細胞ソースとしての歯根膜組織由来幹細胞

歯牙とそれを支える歯槽骨の間には歯根膜という靭帯様軟組織が介在し，咀嚼時のクッション機能や噛み心地を脳に伝える神経伝達機能，ならびに口腔内細菌に対する防御機能などを保持することが分かってきた．また近年では，歯根膜組織内には多分化能を有する幹細胞集団が存在し，歯周組織再生の担当細胞であることが示唆されている[5]．よって我々の臨床研究では，患者自身の抜去歯牙から健全な歯根膜組織を採取し，歯根膜幹細胞を単離・培養し，患部への移植を実施している[6]．

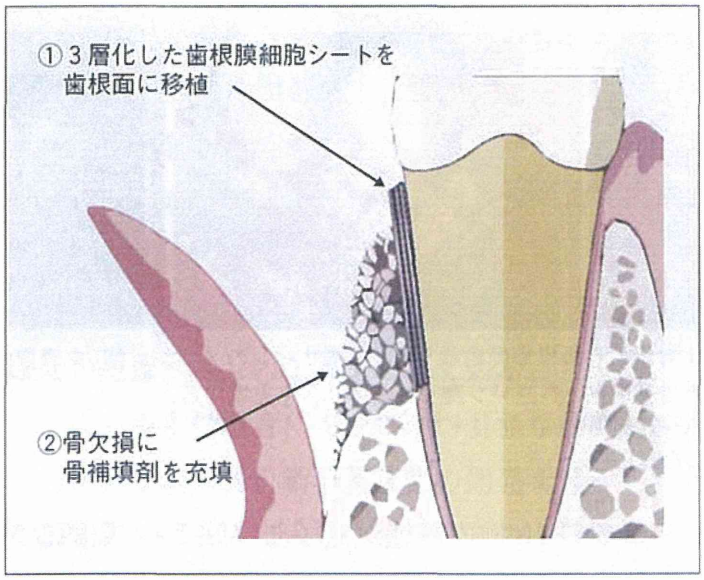
ヒト歯根膜幹細胞は発生学的には神経堤由来の細胞と考えられているが，ヒト間葉系幹細胞（hMSC）と性質が非常によく似ていると考えられている[7]．我々の研究では，ヒト歯根膜幹細胞はhMSCの必要最小用件である ① プラスチック（培養基材）に接着する，② 95％以上の細胞がフローサイトメーターでCD105，CD73ならびにCD90陽性であり，かつ，CD45（白血球共通抗原），CD34（造血前駆細胞のマーカー），CD14もしくはCD11b（単球やマクロファージのマー

カー），CD79α もしくは CD19（B細胞のマーカー）などの血球系マーカー，ならびに HLA-DR 陰性（2％以下）である，③ *in vitro* での分化環境下で骨芽細胞，脂肪細胞，軟骨細胞に分化しうる，の3点の性質[8]を保持しているが，軟骨細胞への分化能は低いことが分かった[7]．体幹の骨のように軟骨内骨化によって形成されず，顎骨の発生様式が膜内骨化であることが原因なのかも知れない．ヒト歯根膜幹細胞は，アルカリホスファターゼ活性ならびに骨芽細胞への分化能が比較的高く，*in vitro* で培養すると石灰化結節の形成が観察される．

温度応答性培養皿と細胞シート工学

温度応答性培養皿は，温度応答性高分子であるポリ-*N*-イソプロピルアクリルアミド（PIPAAm）を，電子線を用い培養皿に表面グラフトしたものである．この培養皿の特徴は通常の細胞培養で用いられる37℃（相転移温度以上）では細胞は良好に接着・増殖するが，培養温度を相転移温度以下（32℃以下）にするだけで，表面の親水化に伴って細胞が自発的に脱着してくることである．さらに，細胞を密な状態に培養した場合は，細胞と細胞外マトリックス（ECM）から成る細胞シートを脱着，回収できる．この技術は，トリプシンなどのタンパク分解酵素を用いた既存の細胞回収法では不可能であったことを可能としている．すなわち，細胞やECM，細胞間の接着が切断されることなく維持されるため，細胞をシート状に回収できる．さらには，シートがフィブロネクチンなどの細胞接着分子を健常な状態で保持できることから，それらの接着分子があたかも“糊”の役割を演じ，細胞と移植される組織が接着し縫合が不要となる．この細胞シートを用いることにより，眼科領域では自己口腔上皮細胞シートを用いた角膜の再生がヒト臨床応用され[9]，先進医療に認定された．消化器領域においては，内視鏡下での表層がん切除後に，自己口腔上皮細胞を用いることにより術後の狭窄を予防できることが確認され[10]，現在国内外での共同研究が進められている．呼吸器系においては，気胸の修復に細胞シートが有用であることが示された[11]．上記のような単層細胞による組織再生のみならず，この細胞シートを重層化し3次元構築することにより，心筋や肝臓の再生も行われている[12~14]．

図2　細胞シート移植の模式図



自己歯根膜由来細胞シートを用いた歯周組織再生の臨床研究の開始

東京女子医科大学では，大型動物や実験室レベルでの安全性有効性を確認し，「ヒト幹細胞を用いる臨床研究に関する指針」に合致した臨床研究として，2011年1月に厚生労働大臣より臨床研究実施の承認を得た．具体的には，患者自身の抜去歯から歯根膜幹細胞を抽出し，“細胞シート工学”を用いてシート状に回収された“自己培養歯根膜幹細胞シート”を歯周欠損の根面に移植する臨床研究を進めている．この臨床研究は“細胞シート工学”を用いることで，細胞を非破壊的にECMと共に移植することが可能である．細胞間相互作用を保持したまま移植することが可能であるため，移植した細胞が拡散することなく，移植したい場所（ここでは歯根面）に高次機能を保ったまま移植できるのが，大きな利点であると考えられる（インジェクションによる細胞治療では，酵素処理による単一細胞化の過程で細胞外基質や細胞膜上のタンパクなどの有効成分が分解されてしまう）．無菌的に細胞を培養できる“細胞プロセッシングセンター”（CPC）と呼ばれる特別な施設で作製された細胞シートは3層に重ね合わされ，郭清術の行われた歯周欠損の歯根面に設置され，骨欠損にはβ-三リン酸カルシウムを充填することで，付着器官の再生を促す（図2）．2014年