Histological and immunohistochemical studies of tissue engineered odontogenesis*

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Summary. The successful regeneration of complex tooth structures based on tissue-engineering principles was recently reported. The process of this regeneration, however, remains poorly characterized. In this study, we have used histochemistry to examine the regeneration process of tissue engineered teeth in order to determine the cell types that give rise to these engineered tooth structures. Porcine third molar tooth buds were dissociated into single-cell suspensions and seeded onto a biodegradable polyglycolic acid polymer scaffold. Following varying periods of growth in rat hosts, the specimens were evaluated by histology and immunohistochemistry. Aggregates of epithelial cells were first observed 4–6 weeks after implantation. These aggregates assumed three different shapes: a natural tooth germ-like shape, a circular shape, or a bilayer-bundle. Based on the structure of the stellate reticulum in the dental epithelium, the circular and bilayer-bundle aggregates could be clearly classified into two types: one with extensively developed stellate reticulum, and the other with negligible stellate reticulum. The epithelial cells in the circular aggregates differentiated into ameloblasts. The continuous bilayer bundles eventually formed the epithelial

sheath, and dentin tissue was evident at the apex of these bundles. Finally, enamel-covered dentin and cementum-covered dentin formed, a process most likely mediated by epithelial-mesenchymal interaction. These results suggest that the development of these engineered teeth closely parallels that of natural odontogenesis derived from the immature epithelial and mesenchymal cells.

Introduction

One important goal of dental research is the efficient regeneration of lost teeth (Earthman *et al.*, 2003; Chai and Slavkin, 2003). Tooth formation, or odontogenesis, is a complex process that has been characterized as a series of reciprocal epithelial-mesenchymal interactions, culminating in the differentiation of the interacting tissues (Thesleff, 1989; Thesleff *et al.*, 1995; Jernvall and Thesleff, 2000; McCollum and Sharpe, 2000). Tissue engineering of tooth structures on biodegradable polymer scaffolds has been recently achieved (Young *et al.*, 2002). The method involves dissociating porcine third molar tooth buds into single-cell suspensions and seeding them onto biodegradable polymers, but this regeneration process is not yet fully understood. To characterize the process in greater detail, we followed the regeneration of tissue-engineered teeth by histology and immunohistochemistry with specific markers of epithelial and mesenchymal differentiation as well as of ameloblasts, odontoblasts, and cementoblasts. In the present study, we show that the development of these engineered teeth closely parallels that of natural odontogenesis.

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Materials and Methods

Preparation of biodegradable polymer scaffolds

Three-dimensional scaffolds were prepared as described previously (Mikos *et al.*, 1993), with the following modifications: polyglycolic acid (PGA) fiber mesh (fiber diameter=13 μm ; density=60 mg/ml; Albany International Research Co., Mansfield, MA, USA) was packed into 96-well plates and sterilized in 75% ethanol. The scaffold dimensions were approximately 1 cm³. Before seeding the cells, the scaffolds were collagen-coated overnight at 4°C (1 mg/ml type I collagen in 10 mM HCl), followed by three washings in phosphate-buffered saline (PBS) and three times in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA).

Isolation and dissociation of porcine third molar tissue

Tooth bud cells were harvested and prepared as described previously (Young *et al.*, 2002). The tooth buds were removed early in development—at early stage of crown formation—from the mandibles of 6-month-old pigs, which were purchased from a local slaughterhouse and transported to the laboratory on ice. Briefly, third molar tooth buds were removed from the fresh mandibles with their inner and outer epithelial layers as well as the dental papilla and dental follicle intact. After the calcified tissue was removed, all tooth bud tissue, including the dental follicles, was minced into <1 mm³ pieces in Hanks Balanced Salt Solution (HBSS, Gibco)(Fig.1) and dissociated with 2 mg/ml collagenase (Wako, Tokyo) and dispase (Roche, Indianapolis, IN, USA) for 50 min at 37°C, followed by

Fig. 1. Schematic diagram of the strategy used to produce the tissue-engineered tooth. Tooth-germ was derived from isolated cells seeded on a PGA fiber mesh scaffold.

Fig. 2. **a:** A third molar tooth bud used in experiments stained with toluidine blue. The cusps of the molar are already calcified (black arrow). **b:** Single cells dissociated by straining through a nylon filter (70- μ m pores) before seeding onto the PGA fiber mesh scaffold (black arrow).

gentle trituration. Digested tooth bud tissues were then strained through a nylon filter (70- μ m pores), and the isolated single cells (1.0×10^7 cells) were seeded onto a polyglycolic acid (PGA) fiber mesh scaffold (Albany International Research Co.)—that had been precoated with type I collagen (1 mg/ml type I collagen in 10 mM HCl)—for 3 h at 4°C before placing them into the omentum in the host rats (Fig. 1).

In vivo implantation and histology

Four- to five-week-old male athymic rats (F344/N Jcl-rnu; Chubu Kagaku Shizai Corporation, Nagoya) were used, and all experiments were conducted in accordance with the Guidelines for Animal Experimentation of Nagoya University.

The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (15 mg/kg), and the scaffolds seeded with cells were implanted into the omenta of the athymic rats ($n=30$) (Choi and Vacanti, 1997). Samples developed for 2, 4, 6, 8, 10, 15, 20, and 25 weeks were dissected and immediately fixed in freshly prepared 4% v/v paraformaldehyde in PBS at 4°C for 6–8 h. After fixation, the tissues were demineralized for 4 h in 0.2 N HCl and, after extensive washing in PBS, were dehydrated in an ethanol gradient, cleared in xylene, and embedded in paraffin. Tissue sections 6- μ m thick were mounted on

glass slides and stained with hematoxylin and eosin (H-E) (Stevens *et al.*, 1990).

Immunohistochemical analysis

Immunohistochemical analysis was performed with a Vectastain ABC kit (Vector Laboratories Inc, Burlingame, CA, USA). The antibodies used were: anti-cytokeratin-14 (CK14) mouse monoclonal IgG (1:500 dilution; No. MBA3232; CHEMICON International, Inc., Temecula, USA), mouse anti-pig vimentin (1:500 dilution; MS129P; Lab Vision UK, LTD, Suffolk, UK), affinity-purified rabbit anti-pig amelogenin polyclonal antibody (1:2,000 dilution; a gift from Dr. J. P. Simmer, University of Texas, San Antonio, USA); and rabbit anti-pig bone sialoprotein (BSP) polyclonal antibody (1:500 dilution; kindly supplied by Dr. J. Sodek, University of Toronto, Canada). The immunohistochemical procedure by Hsu *et al.* (1981) was followed, with modifications as described in detail by Chen *et al.* (1991). Briefly, paraffin-embedded sections were de-waxed and rehydrated in TBS-Tween (0.05% Tween-20 in 50 mM Tris and 150 mM NaCl). To inactivate endogenous peroxidase, the sections were incubated with 1% v/v hydrogen peroxide in methanol, and the non-specific binding of immunoglobulins was blocked by incubation with 3% v/v goat serum for cytokeratin 14 and Vimentin antibodies or 3% v/v normal mouse

Fig. 3. a–b: At 6–8 weeks after implantation, the cells have formed either circular aggregates (black arrow) (a) or sheets of a bilayer of cells (black arrow) which separate two very different densities of surrounding cells (b). At higher magnification, the cells in circular aggregates (c) are low columnar in shape with nuclei in the center of the cytoplasm (black arrow), while the cells in the sheets clearly form a two-cell thick layer (d), which separates a low density of cells (black arrow) from a denser region of cells (white arrowhead), which is densest just adjacent to the bilayer (white arrow).

serum for amelogenin and BSP antibodies. The sections were subsequently washed in TBS-Tween and incubated for 30 min with biotinylated horse anti-mouse IgG for cytokeratin 14 and vimentin diluted 1:100 in PBS, and goat anti-rabbit IgG for amelogenin and BSP, followed by 30 min with a diluted ABC reagent from an avidin-biotin-peroxidase complex kit (Vectastain ABC kit, Vector Laboratories), and finally reacted with 0.02% w/v diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St Louis, MO, USA) in a 0.1 M Tris-HCl buffer (pH 7.2) containing 0.005% v/v hydrogen peroxide. The slides were then washed in cold running water before being mounted

in Permount and examined by light microscopy. Controls involved replacing the first antibody with pre-immune serum eluted from the corresponding affinity column.

Results

Normal pig tooth germ

Figure 2a shows the gross morphology of a third molar tooth bud from a 6-month-old pig. The enamel organ is in

Fig. 3. e–h: Immunohistochemistry shows that an inner layer of the circular aggregates (e; black arrow) and one layer of the bilayer bundles (f; black arrow) express the epithelial marker CK14, while cells surrounding the epithelial aggregates stain positive for the mesenchymal marker vimentin (g; black arrow and h; black arrow).

the early stage of crown formation, and the pattern of the cusps and the outline of the future dentine-enamel junction have formed. The tooth buds were minced, dissociated by enzyme treatment, and strained through a nylon filter (70-μm pores). The isolated single cells were rounded (Fig. 2b).

Weeks 2–8 post-implantation

Single cells were seeded onto collagen-coated PGA fiber scaffolds and incubated into the omentum in the rats. At 4 weeks, there was no evidence of tooth formation, but thin layers of fibrous tissue had encapsulated the PGA based constructs (data not shown). Between 4 to 8 weeks, some

cellular aggregates or sheets had formed in the presumptive regenerated tooth tissue (Fig. 3a, b). Higher magnification revealed that the cellular aggregates were composed of low columnar cells (Fig. 3c), while the sheets were a long bilayer of cells (Fig. 3d). By immunohistochemistry, an inner ring of cells in the circular aggregates stained positive for cytokeratin 14 (CK14), an epithelial marker, while the surrounding cells stained positive for Vimentin, a marker of mesenchymes (Fig. 3e, g). In the cellular sheets, the outer layer cells facing the loose connective tissues stained with CK14 (Fig. 3f), and the surrounding cells stained with Vimentin (Fig. 3h). These results suggest that the first step in tissue-engineered odontogenesis in the PGA scaffolds is the aggregation of epithelial cells.

Fig. 4. **a:** At 10 weeks after implantation, tissue organization typical of dental lamina is evident (black arrow). **b:** At higher magnification, there is an apparent border between an area of dense cells and more sparsely distributed cells (black arrow). **c:** Most of the cells in the cell-dense region stain positive for CK14 (white arrow), but the tall columnar cells in the adjacent region are negative (black arrow). **d:** The circular region increases in size (black arrow) and contains a central stellate reticulum-like structure (black arrowhead). In box region, the epithelial cells are low columnar. The detail is in **g** of the Figure4. **e:** A layer consisting of epithelial cells (black arrow) runs adjacent to the mesenchymal tissue, which stains with H-E. On the opposite side of the mesenchyme tissue, there are sparse cells of the stellate reticulum (black arrowhead).

Fig. 4. **f:** The layer of epithelial cells stained with CK 14 (black arrow). The stellate reticulum encircled by epithelial cells shows weak staining for CK14 (black arrowhead). **g:** A higher magnification of the box in **d** shows that the epithelial cells are low columnar (black arrow). The nucleus is located distal to the epithelial cells in the long cytoplasm (black arrowhead). The density of the cells in stellate reticulum tissue is higher than that in **f**. **h:** Positive staining for vimentin is seen adjacent to the epithelial cells in the mesenchymal tissues (black arrow). **i:** Epithelial cells did not stain positive for amelogenin at this timepoint (black arrow).

Weeks 8–12 post-implantation

Tissue organization typical of dental lamina was seen at 10 weeks post-implantation (Fig. 4a). At high magnification, the dental lamina-like tissue was composed of numerous low columnar cells (Fig. 4b). Most of the low columnar cells inside the dental lamina-like tissues were stained positive for CK14; however, the tall columnar cells facing the connective tissue did not express CK14 (Fig. 4c).

These three types of structures—cellular aggregates (Fig. 3a), cellular bilayer-sheets (Fig. 3b) or dental laminal structures (Fig. 4a)—were often found in the same sample and occurred at approximate ratios of 40%, 40%, and 20% respectively.

Where epithelial cells that stained with CK14 formed open circular structures, they appeared to encircle

stellate reticulum-like tissue (Fig. 4d–i). At this stage, the epithelial cells continued to proliferate in the PGA fiber mesh scaffold, the circular epithelial structures gradually increased in size, and the region of stellate reticulum-like tissue (Fig. 4e)—which stained positive for CK14—expanded (Fig. 4f). At higher magnification, the nuclei of the tall columnar epithelial cells were observed to be in the middle of cells, whereas the nuclei of the mesenchymal cells were localized to edge of the cells most distal to the epithelial cells (Fig. 4g). Many of the low columnar epithelial cells appeared to be developing into tall columnar cells. Positive staining for vimentin was seen in the mesenchymal tissues adjacent to epithelial cells (Fig. 4h). At this time, none of the epithelial cells stained positive for amelogenin polypeptides (Fig. 4i). No hard tissue had formed at this time.

Fig. 5. **a:** At 15 weeks after implantation, there is the formation of hard tissue that stains with H-E (black arrow). The onset of mineralization was at about 12 weeks after implantation. **b:** The cells within the circular structures stain strongly for CK14 (black arrow). Stellate reticulum-like tissue is also weakly stained for CK 14 (black arrowhead). **c:** Mesenchymal tissue, but not epithelial cells, stain strongly for vimentin (white arrow). **d:** The epithelial cells facing the hard tissues are now stained positive for amelogenin (black arrow), however, epithelial cells that face the mesenchymal tissue that has not yet mineralized do not express amelogenin (black arrowhead).

Weeks 12–18 post-implantation

From week 12 post-implantation there was evidence of hard tissue forming between the CK14-positive epithelial and vimentin-positive mesenchymal cell layers (Fig. 5a–c). Epithelial cells had become tall columnar in shape, made contact with the regenerated hard tissues, and stained strongly with the amelogenin antibody, strongly suggesting they were ameloblasts (Fig. 5d). The low columnar epithelial cells that were not in contact with the hard tissue did not stain with the amelogenin antibody. Surrounding these ameloblasts was the stellate reticulum-like tissue that stained weakly for amelogenin (Fig. 5e).

Epithelial cells also formed continuous bilayer sheets to become the epithelial sheath. Dentin tissue was evident adjacent to these sheets, which had the appearance of Hertwig's epithelial root sheath. The Hertwig's epithelial root sheath-like tissue was deflected away from the regenerated dentin-surface at a right angle (Fig. 5f). The inner epithelial cells that abutted mesenchymal cells were cuboidal, whereas the outer epithelial cells were flattened; both cell types stained with the CK14 antibody (Fig. 5g) but not the amelogenin antibody (data not shown). Mesenchymal tissues that stained positive for vimentin lay underneath the epithelium (Fig. 5h).

Fig. 5. **e:** At higher magnification, amelogenin expression is specifically localized to columnar epithelial cells (black arrow), and at the border between these columnar cells and the hard tissues (black arrowhead). Stellate reticulum tissue adjacent to the columnar cells also has a weak expression of amelogenin (white arrow). **f:** At 12 weeks after implantation, hard tissue has formed at the apex of the continuous bilayer sheets (black arrow). The nuclei in the adjacent columnar epithelial cells are localized to the edge of the cytoplasm against the hard tissue and bilayer sheets (black arrowhead). **g:** A higher magnification of the apex of the continuous bilayer sheets showing positive staining for CK14 (black arrow). **h:** A higher magnification of the apex of the continuous bilayer sheets showing positive staining for vimentin in the cells facing the bilayer-bundles (black arrow).

Weeks 18–25 post- implantation

At this stage, the tissue-engineered tooth consisted of two major regions: dentin with overlying enamel, and dentin with overlying cementum which resembled a root (Fig. 6a, b). Enamel tissues were readily identified as a deep-stained layer in demineralized sections stained with H-E (Ten Cate, 1998) (Fig. 6a). Hertwig's epithelial root sheath-like tissue was also recognized (Fig. 6b). There was positive staining for amelogenin in the ameloblasts and in sites that were exclusively enamel (Fig. 6c). Strong BSP expression was observed in the cementum-dentin border (Fig. 6d). There was no evidence of any stratum intermedium in the

regenerated tissue between the ameloblasts and stellate reticulum. The ameloblasts were oriented with their long axes almost perpendicular to the surface of the hard tissue and the nuclei at the edge of the cytoplasm, suggesting that these ameloblasts were at the secretory stage (Fig. 6e).

At higher magnification, dentin tubular was identified in the dentin tissues. Moreover, the cells were incorporated into the cementum tissue, and there were a few cell layers covering the cementum tissues (Fig. 6f). Cementum tissues stained weakly for BSP, but dentin, the dentinal tubule, and cementum-covered cell layers did not stain for this marker (Fig. 6h). These results suggest that, by this time, the root-analogue structure had matured and was nearly in its final

Fig. 6. **a:** At 25 weeks after implantation enamel has formed (black arrow) between the ameloblasts and dentin tissues, which stain with H-E. **b:** The dentin tissue has formed a dental root-like tissue including dentinal tissues and Hertwig's epithelial root sheath-like tissues (black arrow), which stain with BSP. **c:** Amelogenin expression (brown) is restricted to the ameloblasts (black arrow) and enamel (black arrowhead). **d:** At higher magnification, strong staining for BSP (brown) is seen along the peripheral dentin surface (black arrow). Pre-dentin tissue and the tissue that covers the dentin are weakly stained for BSP (black arrowhead). White arrowhead: cementum-like tissue

form. Control sections, which were subjected to treatment with a blocking agent before being incubated with any antibodies, showed no reactivity, indicating that all staining was specific (data not shown).

Beyond 25 weeks after implantation, the tall columnar epithelial cell had disappeared and the enamel was covered with the flattened cells (data not shown). Thus root formation was not yet complete, although we were unable to follow this process to its conclusion beyond 28 weeks because the rats used in the study died around this time.

Discussion

In this study, we employed histochemistry to examine the regeneration process of tissue-engineered teeth in order to determine the cell types that give rise to these engineered tooth structures. Two possible mechanisms may underlie this *in vivo* regeneration process in the PGA scaffold: tissue-engineered odontogenesis may involve the reorganization of fully differentiated ameloblasts, odontoblasts and cementoblasts that are already present

Fig. 6. **e:** More tall columnar cells are aligned with enamel tissue (black arrow). The nuclei of the tall columnar cells are localized at the end of the cytoplasm adjacent to the enamel tissue. **f:** At higher magnification, dentinal tubules can be recognized inside the dentin tissue (black arrow). Cementum-like tissues cover the dentin tissues (black arrowhead). **g:** Amelogenin is strongly expressed at the border between the enamel and dentin tissue (black arrow), the border between the enamel and ameloblasts (black arrowhead), and in ameloblasts. **h:** BSP is strongly expressed at the border of the cementum and dentine tissues (black arrow), and weakly expressed in the cementum-like tissue (black arrowhead).

in the original tooth bud; or, alternatively, it may involve a series of epithelial-mesenchymal interactions between progenitor cells that are derived from the epithelium and mesenchyme of the original tooth bud. The first morphological signs of tissue engineered odontogenesis were seen at 4–6 weeks and involved the aggregation of CK14-reactive epithelial cells inside the vimentin-reactive mesenchymal tissue. None of these cells expressed amelogenin or BSP at this stage. We speculate that the tissue-engineered odontogenesis occurs *via* cooperation between these epithelial and mesenchymal progenitor cells

although it remains unclear from our investigation which cell is responsible for triggering the development of tissue-engineered odontogenesis. Further studies are needed to clarify this point.

Tissue-engineered odontogenesis appears to involve the independent and concurrent formation of two types of tissue: enamel-covered dentin and cementum-covered dentin. These are distinguished by the absence or presence of a stellate reticulum-like tissue inside the epithelial bilayer sheets. In general, in the mouse molar, the cervical loop epithelium loses its capacity for crown formation; that

is, it loses the stellate reticulum and switches from crown to root formation (Thomas, 1995; Zeichner-David, 2003). However, in tissue-engineered odontogenesis, enamel and cementum tissue regeneration appeared to occur at the same time. It therefore appears that the development of tissue-engineered teeth does not coincide with that of the natural tooth in this respect. These results suggest that the mechanisms for the regulation of cell differentiation present in the natural tooth are lost after dissociation of the tooth bud. We presume that this is due to residue of the independent progenitor cells to develop the enamel or cementum tissue in the dental epithelium at this time, the early stage of crown formation. We are currently clarifying this phenomenon using a mouse model.

Cementum matrix deposition began when the Hertwig's epithelial root sheath-like tissue emerged in the continuous epithelial bilayer bundle. The dental follicle is believed to contain progenitors of cementoblasts, periodontal ligament cells, and osteoblasts (Bosshardt, 1996, 1998; Kagayama, 1998; Handa *et al.*, 2002). In our study, tooth bud cells containing the dental follicle cells were transplanted into the rat omentum. Based on our observations, we propose that tissue-containing enamel is formed when an interaction is triggered between the dental epithelial cells and the dental papilla cells. Conversely, if the dental epithelial cells come in contact with dental follicle cells, cementum-covered dentin tissue is formed. This is in contrast with the finding in the normal developing tooth where the formation of cementum-covered dentin proceeds as a result of an interaction between the dental epithelium and dental papilla cells as was the case for the enamel-covered crown dentin. In future experiments, we aim to identify the relationship not only between the papilla cells derived from the ectomesenchyme and the epithelial cells, but also between the dental follicle cells derived from the ectomesenchyme and the dental epithelial cells.

In conclusion, we have shown that the pattern of tissue-engineered odontogenesis is similar to that of natural tooth development. Our results suggest that the entire tooth structure—including the enamel, dentin, and cementum—is regenerated in this technique. However, we have identified several potential problems with this technique: the regenerated teeth remain quite small, it is not possible to regulate the shape of regenerated tissue, or the timing of development including the formation of enamel and cementum is difficult. We are now investigating new approaches to tooth regeneration that involve careful seeding of the cells to regenerate more complete teeth.

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