Introduction

The human dental follicle (DF) is an ectomesenchymal tissue surrounding the developing tooth germ. In recent years, progenitor cells have been identified in the DF, demonstrated to differentiate along osteogenic pathways. Additionally, the DF attached to impacted tooth has been used to analyze the differentiation into various cell types including cementoblasts, adipocytes, chondrocytes, fibroblasts or osteoblasts. Homogenized DF attached to impacted tooth surgically removed from the jawbone has been used to elucidate the differentiation capability in animal studies.

However, there are only few studies regarding the histological and immunohistochemical analysis in surgically removed DF. Hence, the purpose of this study was to examine the histopathological and immunohistochemical characteristics of DF attached to impacted tooth.

Materials and Methods

Specimen

The study was comprised of 38 cases of DF diagnosed histopathologically between 2000 and 2011 by 2 oral pathologists at the Department of Oral Pathology, Nihon University School of Dentistry, Matsudo City, Japan. The DFs used were attached to impacted third molars after surgical removal. However, DFs clinically diagnosed that developed cystic structure were excluded. A total of 38 cases of DFs were gathered from 9 male and 29 female patients whose average age was 22.8 ± 10.7.

Study was conducted with patients’ informed consent considering sufficient privacy, diagnostic outcome and management before retrieving the pathological specimens (Ethical Committee: AP11MD034).

For immunohistochemical analysis, 10 cases which did not undergo decalcification were selected among the 38 cases to avoid...
Table 1 Histopathological findings of dental follicle (ratio*)

<table>
<thead>
<tr>
<th>Myxoid appearance</th>
<th>Capillary enlargement</th>
<th>Vessels density†</th>
<th>Vessels area††(¼−m²)</th>
<th>Lymph vessels density†</th>
<th>Lymph vessels area††(¼−m²)</th>
<th>Odontogenic epithelial area††(¼−m²)</th>
<th>Enamel epithelium</th>
<th>Calcification</th>
<th>Peripheral nervous system</th>
<th>Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner layer</td>
<td>38(100.0)</td>
<td>30(83.3)</td>
<td>37.4±10.7</td>
<td>107.8±124.0</td>
<td>2.0±2.9</td>
<td>43.4±4.1</td>
<td>21(55.3)</td>
<td>7(18.4)</td>
<td>12(31.6)</td>
<td>10(26.3)</td>
</tr>
<tr>
<td>Outer layer</td>
<td>0(0.0)</td>
<td>0(0.0)</td>
<td>3.4±2.4</td>
<td>12.5±7.5</td>
<td>0.1±0.3</td>
<td>5.4±0.6</td>
<td>2(2.9)</td>
<td>0(0.0)</td>
<td>1(2.6)</td>
<td>0(0.0)</td>
</tr>
<tr>
<td>Epithelial island</td>
<td>38(100.0)</td>
<td>8(38.1†)</td>
<td>15.7±8.1</td>
<td>58.6±80.2</td>
<td>0.2±0.2</td>
<td>11.5±12.4</td>
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<td>-</td>
<td>1(8.3‡)</td>
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</table>

ratio*: number of cases / all cases
§: ratio / 30 cases with capillary enlargement in the inner layer
Vessels density†: Vessels density (average number / all cases)
Vessels area††: Vessels area (average area / all cases)
Lymph vessels density†: Lymph vessels density (average number / all cases)
Lymph vessels area††: Lymph vessels area (average area / all cases)
‡: 8 cases were shown in the inner layer with inflammatory reaction
‡‡: ratio / 12 cases with calcification in the inner layer

Table 2 Immunohistochemical findings of ectomesenchymal cells and odontogenic epithelial islands in the dental follicle

<table>
<thead>
<tr>
<th>S-100</th>
<th>GFAP</th>
<th>Vimentin</th>
<th>α-SMA</th>
<th>CD34</th>
<th>D2-40*</th>
<th>VEGF</th>
<th>Runx2</th>
<th>FGF-2*</th>
<th>Fibronogen</th>
<th>Ki-67</th>
<th>p63*</th>
<th>CD68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner layer</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>[ ]</td>
<td>[ ]</td>
<td>-</td>
<td>[ ]</td>
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<td>-</td>
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<tr>
<td>Inflammatory lesion</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Outer layer</td>
<td>-</td>
<td>-</td>
<td>[ ]</td>
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<td>+</td>
</tr>
<tr>
<td>Odontogenic epithelial island</td>
<td>-</td>
<td>-</td>
<td>[ ]</td>
<td>[ ]</td>
<td>[ ]</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

* Positive rates (%): positive cells / total cells of odontogenic epithelial islands
Figure 1. The inner layer (*) with abundant vessels and outer layer with fibrous connective tissue (**) (HE, x10)

Figure 2. (A) Myxomatous inner layer (HE, x20); (B) eosinophilic fibrous outer layer (HE, x20)

Figure 3. Scattered odontogenic epithelial islands in the inner layer (HE, x40)

Figure 4. Inflammatory cell infiltration around the ovoid calcification (x60)

Figure 5. Peripheral nerve positive to S100 in the inner layer (x10)

Figure 6. Abundant vessels with CD34 positive cells in the inner layer (*) (x10) and positive ectomesenchymal cells scattered in the inner layer (inset, x20)
Figure 7. Almost all mesenchymal cells strongly expressed αSMA in the inner layer but no reaction in the outer layer (x40)

Figure 8. Cells positive to CD68 are scattered in the inner layer (x40)

Figure 9. Scattered positive reaction to VEGF in the inner layer (x40)

Figure 10. Positive reaction in the inflammatory lesion in the inner layer (x40)

Figure 11. (A) Positive reaction to FGF-2 in odontogenic epithelial island and weakly positive for mesenchymal cells in the inner layer (x60); (B) weakly positive reaction in inflammatory lesion (x40)

antigenic inactivation. From the 10 cases, 4 cases of DFs with inflammatory cell infiltration were included to compare the immunohistochemical reaction. EnVision+ Polymer System (Dako Glostrup, Denmark), also used in secondary antibody, was utilized for antigen detection. Primary antibodies used were directed against the following antigens: S100 (S100, 1:1000; Dako), Glial Fibrillary Acidic Protein (GFAP, 1:1000; Dako), Vimentin (Vim3B4, 1:200; Dako), α-SMA (1A4, 1:100; Dako), VEGF (ab46154, 1:100; Abcam), Runx2 (QBEnd10, 1:100; Dako), FGF-2 (ab106245, 1:50; Abcam), CD34 (QBEnd10, 1:100; Dako), CD68 (PG-M1, 1:100; Dako), D2-40 (D2-40, 1:50; Dako), Ki-67 (MIB-1, 1:50, Dako), p63 (ab53039, 1:100; Abcam) and Fibrinogen (Fibrinogen, 1:500; Dako). Antigen retrieval was performed with citrate buffer solution (pH 6.0 for Fibrinogen, GFAP, S100, Vimentin, Ki-67, CD34 and α-SMA, Runx2, CD68, D2-40 for pH 9.0, respectively) in a pressure cooker. Antigenic reactions were revealed using 3, 3'-dianisobenzidine tetrahydrochloride (DAB) and finally counterstained with Mayer's hematoxylin. For positive controls, oral fibroma was used for FGF-
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Table 3 Result of review of literatures

<table>
<thead>
<tr>
<th>Year</th>
<th>Reference number</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>6)</td>
<td>S-100</td>
</tr>
<tr>
<td>2003</td>
<td>7)</td>
<td>CD68</td>
</tr>
<tr>
<td>2006</td>
<td>8)</td>
<td>p53</td>
</tr>
<tr>
<td>2007</td>
<td>9)</td>
<td>α3β1 integrin</td>
</tr>
<tr>
<td>2008</td>
<td>10)</td>
<td>Ki-67</td>
</tr>
<tr>
<td>2009</td>
<td>11)</td>
<td>beta-catenin</td>
</tr>
<tr>
<td>2010</td>
<td>12)</td>
<td>Ki-67</td>
</tr>
<tr>
<td>2010</td>
<td>13)</td>
<td>ATF-2</td>
</tr>
<tr>
<td>2010</td>
<td>14)</td>
<td>p63</td>
</tr>
</tbody>
</table>

2 and Vimentin; inflammatory granulation tissue for Fibrinogen CD68, CD34, α-SMA, Ki-67; pleomorphic adenoma for S100, Runx2 and GFAP. For evaluation of immunohistochemical staining technique, mouse and rabbit universal negative controls (Dako Glostrup, Denmark) were used during the staining procedure instead of primary antibodies.

Review of literatures

Articles were searched from the website of National Institute of Health – PubMed electronic literature database. Articles listed in the database until April 2012 were included. Article selection was limited to studies in humans and those published in English. Data related to human dental follicle tissue and immunohistchemistry were extracted and summarized for this review.

Results

Histopathological analysis

Histopathological findings of DFs are shown in Figs.1 to 4 and the features are summarized in Table 1. Microscopically, no inflammatory reaction was noted in 28 cases. Specifically, the inner layer is filled with dense capillaries and the outer layer consists of fibrous tissue (Figs.1, 2a and 2b). The stroma of the inner layer is composed of loose collagenous or myxoid connective tissue with scattered ectomesenchymal cells which are spindle or oval in shape. Peripheral nerves (Fig. 5), differentiated enamel epithelium and calcification were more predominant in the inner layer than in the outer layer (Fig. 4). Myxoid tissue in the inner layer was noted in all specimens. In DFs without inflammatory reaction, vessel density marked by CD34 in the inner layer is 11 times more than in the outer layer with 37.4% and 3.4% respectively (Fig. 6). Capillary dilatation in the inner layer was observed in 83.3% with 107.8 ±124.0 μm² but was not observed in the outer layer with 3.4±2.4 μm² in average, either. Lymph vessel density was almost the same as the outer layer, but the dilatation of them was observed with 11.5±12.4 μm² in average. There was also a case with many clusters of odontogenic epithelial islands. Enamel epithelium was frequent in the inner layer with 18.4%, though aorta was present mainly in the outer layer with 36.8%. Fibrosis was seen in the outer layer (Fig. 2b).

Lymphocytic infiltration and scattered ectomesenchymal cells with edematous background were noticed. Mild inflammatory reaction was observed in 10 cases (26.3%). Calcification was detected in 12 cases (31.6%) wherein 8 cases had inflammatory reaction. Lymphocytes were observed around an ovoid calcification (Fig. 4).

Immunohistochemical findings

Immunohistochemical findings are shown in Figs. 5 to 12 and the features of ectomesenchymal cells and odontogenic epithelial islands in the DFs are summarized in Table 2.

In the inner layer, ectomesenchymal cells positive to CD34 were scattered in the inner layer (Fig. 6, inset). Ectomesenchymal cells were positive to Vimentin and α-SMA (Fig.7) and were weakly positive to VEGF (Fig.9) and FGF-2 (Fig.11a). Moreover, CD68-positive cells were scattered in the inner layer (Fig.8) and no reaction to Fibrinogen, p63, S100 and GFAP was observed. In the outer layer, Fibrinogen was diffusely positive but CD68 was rarely observed. α-SMA (Fig. 7), VEGF, Runx2 and p63 showed negative reaction. Concerning about Runx2, positive reactivity to ectomesenchymal cells were observed in the area of inflammation in the inner layer (Fig.10).

In odontogenic epithelial island, no reactivity to Ki-67 was detected but D2-40 (Fig. 12a), FGF-2 (Fig. 11a) and p63 (Fig. 12b) expressed positive reaction. There positive rates were 38.4%, 86.2% and 70.8%, respectively. Positive distributions of FGF-2 and p63 were diffusely, though D2-40 was only the peripheral layer which is in contact with stroma of odontogenic epithelial islands.
Review of literatures

The results were summarized in Table 3. The immunohistochemical studies using human dental follicle extracted from jaw bone was only 9 papers 6-14.

Discussion

The DF or dental sac is an ectomesenchymal tissue containing epithelial islands (epithelial rests of Malassez) that surrounds the crown of an unerupted tooth responsible for the orientation of tooth eruption11. Recent studies had shown that DF contained stem cells or progenitor cells that could differentiate into various cell types including cementoblasts, adipocytes, chondrocytes, fibroblasts or osteoblasts in in vitro conditions 15).

To assess the osteogenic differentiation capacity in vivo, stem cells in DF derived from impacted third molars were inserted in bone16,17). Thus, homogenized DF adhering to impacted tooth surgically removed from the jawbone has been used in order to know the differentiation capability. However, histopathological and immunohistochemical assessments of the DF were not enough, and the characteristics of spindle cells in DF are still unknown.

Microscopically, the stroma of the DF consisted of loose collagenous or myxoid connective tissue with ectomesenchymal cells which are spindle or oval in shape with abundant blood vessels referred as the inner layer 1). Eosinophilic fibrous tissue in the coronal part of the DF was regarded as the outer layer 1). In the present study, myxoid appearance was observed in all specimens in the inner layer. Perlecan core protein was localized in fibroblastic cells and odontoblasts in DF, but its function is still unknown 19). Accumulation of perlecan in myxoid connective tissue was considered a factor in odontogenesis. Although the case was asymptomatic, mild inflammatory reaction was observed microscopically in 10% of the cases. A total of 80% of inflammatory cases showed variously-sized calcification.

Localization of α-SMA in stem cells and precursor cells in DF was examined during tooth development and its presence was assumed to have a role in alveolar bone formation19). Our result showed remarkable α-SMA distribution in the inner layer. However, since they were negative to Runx2, the ability of the stem cells in DF to participate in bone formation was not accepted.

Blood and lymph vessel density as well as dilatation were assessed with CD34 and D2-40 immunoreactions respectively. CD34 is a single-chain transmembrane protein of approximately 116 kDa expressed by immature hematopoietic stem/progenitor cells, capillary endothelial cells and embryonic fibroblasts. In this study, capillary dilatation in the inner layer was observed in 83.3%, although it was not observed in the outer layer in any case. Vessel density in the inner layer was 11 times more than in the outer layer. Hence, it was assumed that most ectomesenchymal cells in the inner layer consisted of hematopoietic stem cells or embryonic fibroblasts expressing CD34. Moreover, the vessels might have developed in the inner layer since chemokines and growth factors were needed for the differentiation of ectomesenchymal cells. Anti-D2-40 has been demonstrated to label lymphatic endothelium but not vascular endothelium. There were very few lymph vessels compared to capillary vessels in the DF with densities of 2.0% and 0.1% in the inner and outer layers respectively. It was believed that the DF might be a tissue with an excretory function which did not develop in the present study. Articles were searched from the website of National Institute of Health – PubMed electronic literature database. Articles listed in the database until April 2012 were included. Article selection was limited to studies in humans and those published in English. As a result, papers about lymph vessels of dental follicle were not detected.

Vascular endothelial growth factor (VEGF) is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth and reproductive functions. Ectomesenchymal cells surrounding the vessels slightly showed VEGF expression and CD68-positive cells were scattered in the inner layer although they were negative to S100 and GFAP. Human monocytes, macrophages and myeloid cells are labeled by CD68. Monocytes were concerned with dental growth and their influx was controlled by the secretion of monocyte chemotactic protein (MCP-1), which is an inflammatory chemokine for controlling infiltration10) by the dental follicular cells 12).

FGF-2 protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members bind heparin and possess broad mitogenic, angiogenic activities and nerve development. In the present study, FGF-2-positive ectomesenchymal cells were scattered in the inner layer. It was suggested that FGF-2 operated independently or dependently by the signals of MCP-1 and/or VEGF using ectomesenchymal cells with blood vessel derivative 20). FGF-2 has been reported not only in neovascularization but also in lymphangiogenesis 21). It was considered that FGF-2 was concerned in the development of blood and lymph vessel and/or peripheral nerve tissue in the inner layer of DF. However, the characteristics of nerve cells were lost in ectomesenchymal cells.

In the present study, odontogenic epithelial islands in DF were detected in the inner and outer layers in 55.3% and 2.9% respectively with FGF-2 and p63 diffusely positive expressions. p63 protein was expressed by the cells with stem cell characteristics such as basal cell of various epithelial tissues. Stronger p63 gene immunorexpression in the DFs in completely impacted teeth represented more number of stem cells than the partially impacted teeth 22). In addition, odontogenic epithelial islands in DFs of unerupted teeth were negative to Ki-67 as shown in previous report 23). Moreover, the positive D2-40 distribution with 33.4% in the peripheral layer which is in contact with stroma of an unerupted tooth responsible for the orientation of tooth eruption11). Recent studies had shown that DF contained stem cells or progenitor cells that could differentiate into various cell types including cementoblasts, adipocytes, chondrocytes, fibroblasts or osteoblasts in in vitro conditions 15).
Although the biologic function of D2-40 were not fully understood, this positive reaction was described as the character of cyst formation. Recently, it was reported that apoptosis should be induced in odontogenic epithelial islands in DF during tooth development. From the above-mentioned result, it was suggested that odontogenic epithelial islands in completely embedded DF implied a characteristic of stationary stem cell, but the slight possibility of cyst formation or proliferation was latent. Inflammation observed in ectomesenchymal components of the DF up-regulated the cell turnover of odontogenic epithelium and led to proliferation.

Both calcification and ossification were predominant in the inner layer with inflammatory reaction. Runx2 was observed in ectomesenchymal cells only when there was inflammatory reaction in the inner layer. Osteogenic reaction was not observed histologically in ectomesenchymal cells in the inner layer and this result coincided with the previous in vitro study. The DF showed an initial phase of osteogenic differentiation only when cultured with ectomesenchymal stem cell in osteogenic induction medium. Consequently, the results indicate that inflammatory irritation to the inner layer might have caused osteoblastic differentiation.

References

20. Baird A, Ling N. Fibroblast growth factors are present in the


