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Regenerative capability of dental pulp cells after crown fracture

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Abstract – The purpose of this study was to evaluate the characteristics of dental pulp cells for tissue engineering derived from the fractured incisal portion of tooth crowns. Thirty Sprague-Dawley rats were used for histological and immunohistochemical analysis of nestin protein expression and to measure levels of mRNAs encoding osteocalcin, osteopontin, bone sialoprotein (BSP), dentin sialoprotein (DSP), heat shock protein (HSP) 27, vascular endothelial growth factor (VEGF), ATP-binding cassette transporter G2 (ABCG2), nestin, and $p57^{Kip2}$. Odontoblasts at the incisal portion in the control group were oriented in a regular pattern, but those in the experimental group were randomly stratified. Immunohistochemically, only a few odontoblasts were positive for nestin at the incisal portion in the experimental group at 2 days. Some cells in the inner area in the control group were positive for nestin, but nestin-positive cells in the experimental group at the incisal portion were not observed. The mRNA expression for osteogenic or odontogenic markers in the experimental group was higher than in the control group. HSP27 mRNA expression in the experimental group at 2 days was higher than in the control group and in the experimental group at 7 days. mRNA expression of stem cell markers, such as ABCG2 and nestin, in the experimental group tended to decrease compared with the control. In conclusion, this study demonstrates that dental pulp stem cells derived from fractured teeth differentiate to osteogenic or odontogenic cells.

In general dental therapy, the dental pulp of fractured teeth is usually removed. Thus, it would be an exciting advance if it was possible to use those dental pulp cells from fractured teeth for tissue engineering of hard tissues such as alveolar bone defects, bone loss for dental implants, and so on. A report about the isolation and characterization of dental pulp stem cells derived from crown-fractured teeth was published by Huang et al. (1). They reported that dental pulp cells derived from fractured teeth have a multilineage potential for adipogenic, chondrogenic, and osteogenic differentiation. However, they evaluated cultured dental pulp cells obtained from fractured teeth with a differentiation induction medium containing L-ascorbic acid-2-phosphate. Further, Mangano et al. (2) reported the osteoblastic differentiation of dental pulp stem cells on a titanium surface. Huang et al. (1) reported the isolation and characterization of human pulp stem/stromal cells derived from fractured teeth for root canal treatment.

Regenerative medicine is a strong focus of the medical and dental world (3). Recently, many reports have been published about the application of cells derived from all types of mature tissues and organs, including dental tissues (3–6). It has been reported that tissue stem cells have been detected in the dental pulp (7–9), and these pluripotent cells have been shown to act as tissue stem cells (7–10). Howard et al. (11) reported on the migration of dental pulp stem cells. Further, it has been suggested that dental pulp stem cells could differentiate into and be a source of odontoblasts. On the other hand, Kenmotsu et al. (12) investigated the characteristics of dental pulp stem cells derived from the dental pulp of young and aged rats using the method of isolating side population cells.

The purpose of this study was to evaluate the characteristics of dental pulp cells for tissue engineering derived from the fractured incisal portion of tooth crowns.

Materials and methods

All animal studies were conducted in compliance with the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College. Thirty 5-week-old male Sprague-Dawley rats, weighing 100–120 g each, were used in this study. Under general anesthesia with sodium thiopental, the incisors on the right side of the maxillae were fractured and were removed with forceps from the tooth crown. The dental pulp was exposed by tooth fracture. The incisors on the left side were used as controls without the fracture surgery (Fig. 1a); Figure 1b shows the evaluation area. Ten rats were used for the histological experiments, and 20 rats were used for the mRNA expression experiments. After the surgery, rats were fed only milk and water to guard against bacterial infection.

For histological observation, the observed area was divided into two portions, that is, the incisal portion and

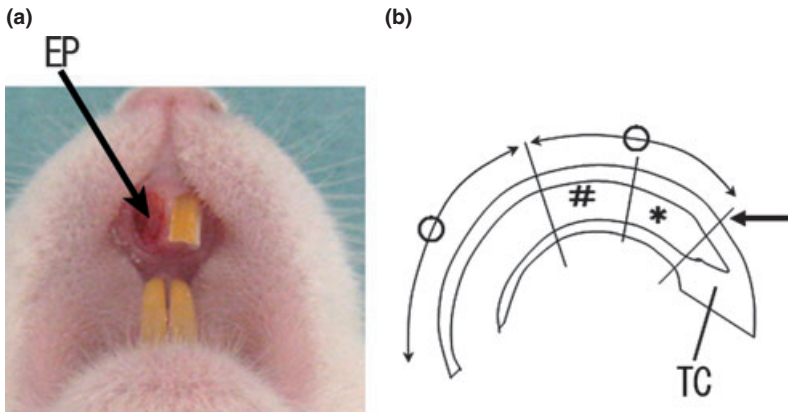

Fig. 1. Experimental design a: photograph of tooth fracture, b: scheme of evaluation area EP: exposed pulp, TC: tooth crown, arrow: fracture portion, #, *: evaluation area (*: incisal portion, #: middle portion) For histological observation, the observed area was divided into two portions, that is, the incisal portion and the middle portion.

the middle portion (Fig. 1b). At 2 and at 7 days, rats were sacrificed under general sodium thiopental anesthesia and were perfused intracardially with 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.3). The maxillae were then removed and were stored in the same fixative at 4°C for 2 days. Sections were cut in the mesiodistal sagittal plane at a thickness of approximately 5 μm and were collected on glass slides. The sections were stained with hematoxylin and eosin (HE) and were then observed by light microscopy. For immunohistochemical staining, paraffin sections were deparaffinized with xylene and were then incubated in 3% hydrogen peroxide with methanol for 13 min at room temperature to block endogenous peroxidase activity. For antigen retrieval, sections were treated with 3% bovine serum albumin (BSA) or with 10% goat serum for 30 min at room temperature. The sections were incubated at room temperature for 60 min with an anti-nestin antibody (diluted at 1:100; RAT-401: sc-33677, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then were incubated with a biotinylated secondary antibody: Histofine Simple-stain MAX-PO® (Nichirei Corporation, Tokyo, Japan) for 30 min at room temperature. Thereafter, the sections were rinsed with PBS and were stained with Histofine Simple-stain DAB® (Nichirei), counterstained with hematoxylin, and observed using light microscopy.

For mRNA expression studies, total RNAs were extracted using the acid guanidium thiocyanate/phenol-chloroform method as follows: at 2 and at 7 days after the surgery, dental pulp tissues from both the experimental and the control groups from the incisal part were sectioned at the center to allow removal of the dental germ tissues and lightly affected tissues were removed (Fig. 2b). Throughout the lifespan of rodents, the incisors continuously erupt at the apical end of the tooth, which comprises the dental germ tissue. Therefore, in this study, only the coronal pulp tissue from the incisor was used, thus excluding the dental germ tissue and half of the apical area. Each tooth was sectioned in half, and dental pulp tissue was removed mechanically. After the pulp tissues were homogenized in 1 ml TRIsol Reagent (Invitrogen Corp, Carlsbad, CA, USA), according to the standard method, quantitative RT-PCR was carried out using the TaqMan Gene Expression Assays (Applied Biosystems, Life Technologies Corp, Carlsbad, CA, USA) for the target genes: osteocalcin (OCN, Rn01455285 g1; Applied Biosystems), osteopontin (OPN, Rn00563571 m1; Applied Biosystems), bone sialoprotein (BSP, Rn01450118 m1; Applied Biosystems), dentin sialoprotein (DSP, Rn02132391 s1; Applied Biosystems), heat shock protein (HSP) 27 (HSP, 00583001 g1; Applied Biosystems), vascular endothelial growth factor (VEGF, Rn00582935 m1; Applied Biosystems), ATP-binding cassette transporter G2 (ABCG2, Rn00710585 m1; Applied Biosystems), nestin (nestin, Rn00564394 m1; Applied Biosystems), and p57 ($p57^{Kip2}$, Rn00711097 m1; Applied Biosystems). The TaqMan Endogenous Control for the target gene β-actin (RAT ACTB, Rn4352340; Applied Biosystems) was used as a control. All PCR reactions were performed using a real time PCR 7500 fast system, and quantitative RT-PCR analyses were reproduced three times. For statistical analysis, data were analyzed via one-way ANOVA and were compared by Scheffe's test.

Results

Histological observations

Odontoblasts at the incisal portion in the control group were oriented in a regular pattern, but those in the experimental group were observed to be randomly stratified. The fractured surface at 2 days was covered with a fibrin nest, necrotic cells, and inflammatory cells, and capillary dilation was observed. Further, calcified materials appeared at 7 days in the incisal portion. However, the wound surface was completely closed in with newly formed dentin at 7 days (data were not shown).

Immunohistochemically, nestin was strongly positive for odontoblasts in the control group at both the incisal and the middle portions, and in the experimental group at 2 days at the middle portion. However, only a few odontoblasts were positive for nestin at the incisal portion in the experimental group 2 days after the tooth

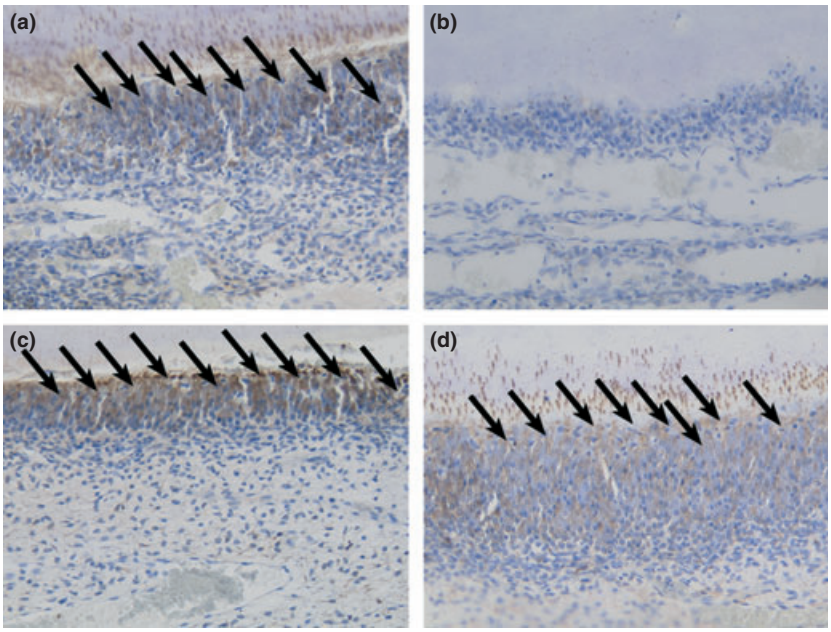

Fig. 2. Immunohistochemical staining with an anti-nestin antibody (a) control group at the incisal portion, (b) experimental group at the incisal portion 2 days after the tooth fracture, (c) control group at the middle portion, (d) experimental group at the middle portion 2 days after the tooth fracture. Arrow: positive cells Nestin was strongly positive for odontoblasts in the control group at both the incisal and the middle portions, and in the experimental group at 2 days at the middle portion. However, only a few odontoblasts were positive for nestin at the incisal portion in the experimental group 2 days after the tooth fracture. Some cells in the control group at the incisal and middle portions and in the experimental group at the middle portion were positive for nestin, but nestin-positive cells in the experimental group at the incisal portion were not observed.

fracture. Some cells in the control group at the incisal and middle portions and in the experimental group at the middle portion were positive for nestin, but nestin-positive cells in the experimental group at the incisal portion were not observed (Fig. 2).

mRNA expression

The expression of osteocalcin mRNA in the experimental group at 2 days (Fig. 3) and osteopontin mRNA expression in the experimental group at 7 days were significantly higher than in the control group (Fig. 4). BSP and DSP mRNA levels tended to be higher in the experimental group than in the control group (data were not shown). HSP27 mRNA expression in the experimental group at 2 days after tooth fracture was significantly higher than in the control group (Fig. 5). VEGF mRNA expression was not significantly different but tended to increase in the experimental group (data were not shown). ABCG2 mRNA expression in the experimental group tended to decrease compared with the control group (data were not shown). Nestin mRNA expression in the experimental group at 2 days was significantly lower than in the control group (Fig. 6). $p57^{Kip2}$ mRNA expression at 2 and at 7 days was significantly higher in the experimental group than in the control group (Fig. 7).

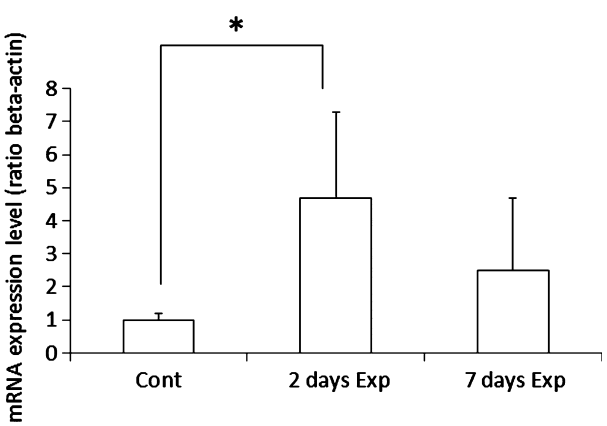

Fig. 3. Osteocalcin mRNA expression Osteocalcin mRNA in the experimental group at 2 days was significantly higher than in the control group. *: significantly difference ($P < 0.05$).

Discussion

There have been many reports about stem cells in dental pulp tissues (1, 11, 13). Clinically, fractured teeth are often extracted. To use cells from removed fractured teeth for tissue engineering is still a long-range vision and not a clinical possibility at this time. The possibility for pulp stem cells to differentiate to osteoblasts is well known since 1979 (14, 15). The purpose of this study was to evaluate the characteristics of dental pulp cells derived from fractured teeth. Generally, it is known that pulp cells differentiate to odontoblasts 2 days after injury in rat incisors (14). Therefore, this study evaluated cells at 2 and at 7 days after tooth fracture. Rat incisors are known to continuously erupt, and the osteogenetic or odontogenetic activity of rat incisor dental pulp is high.

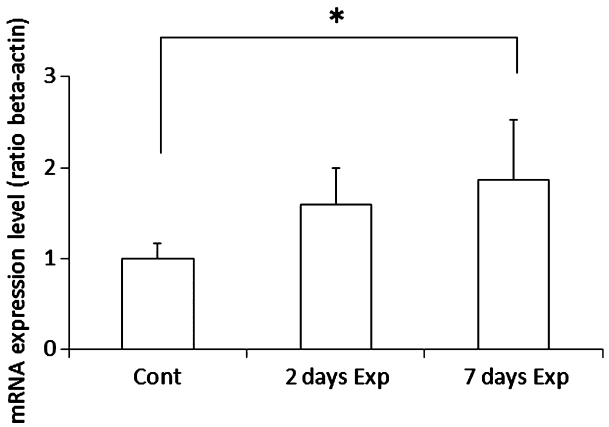

Fig. 4. Osteopontin mRNA expression Osteopontin mRNA expression in the experimental group at 7 days was significantly higher than in the control group. *: significantly difference ($P < 0.05$).

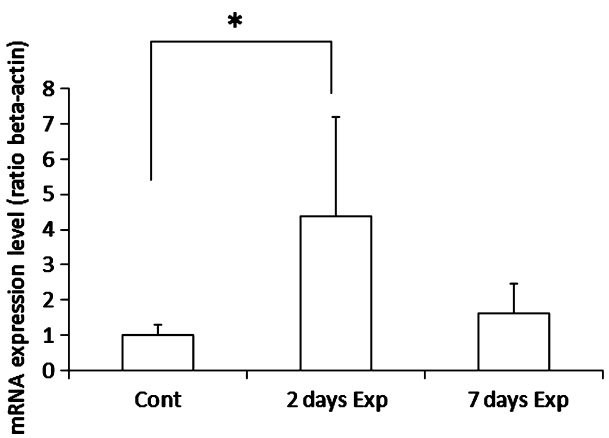

Fig. 5. Heat shock protein 27 mRNA expression HSP27 mRNA expression in the experimental group at 2 days after tooth fracture was significantly higher than in the control group. *: significantly difference ($P < 0.05$).

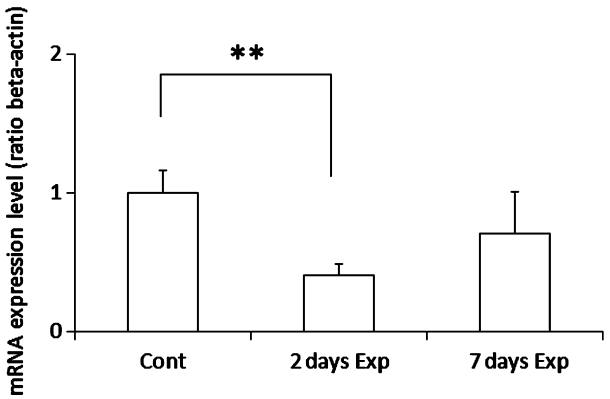

Fig. 6. Nestin mRNA expression Nestin mRNA expression in the experimental group at 2 days was significantly lower than in the control group. **: significantly difference ($P < 0.01$).

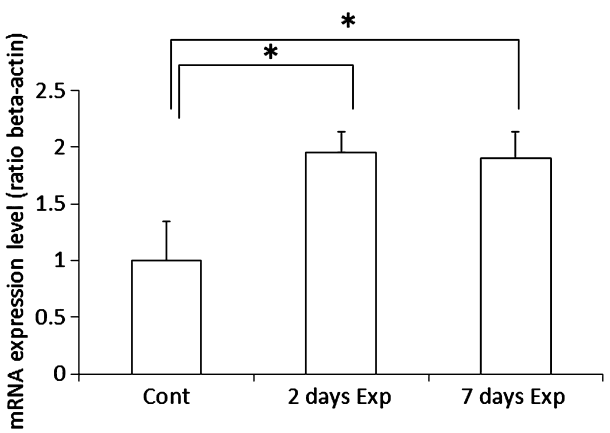

Fig. 7. $p57^{kip2}$ mRNA expression $p57^{Kip2}$ mRNA expression at 2 and 7 days was significantly higher in the experimental group than in the control group. *: significantly difference ($P < 0.05$).

This phenomenon is different form human dental pulp, and so, in this study, only the coronal portion of the dental pulp was used.

Although calcified materials were observed in this study, the tooth fracture stimulates dental pulp cells to differentiate to osteogenic or odontogenic cells. Yamada and Matsumoto (16) suggested that significant damage to the pulp plays a special role to start the signal switch for the formation of bone-like materials. In this study, the fractured surface was completely enclosed with dentin at 7 days.

Osteocalcin is a vitamin K-dependent and non-collagenous dentine and bone matrix protein. Osteocalcin is synthesized by osteoblasts and odontoblasts and is a well-known marker of viability, differentiation, and osteogenic ability in those cells (17). Osteocalcin has an important role in inhibiting excessive mineralization. Osteopontin is also known as a bone matrix protein, but many kinds of cells such as osteoclasts, placenta, renal cells, and so on secrete it. It is well known that BSP is a bone-specific protein while DSP is a dentin-specific protein. This study demonstrated that the mRNA expression of hard tissue-related proteins in the experimental group was higher than those in the control group. This means that stimulation such as a tooth fracture makes pulp cells differentiate to osteogenic cells.

Heat shock protein 27 is a protective protein for cells, serves as a molecular chaperone in maintaining homeostasis, and is expressed physiologically in response to stress (18). VEGF is an angiogenic growth factor that elicits cellular responses to injury (19). HSP27 and VEGF expression play extremely important roles in the early stage of wound healing (20). In this study, HSP27 and VEGF mRNA expression was higher in the experimental group than in the control group. This means that pulp cells are recovering against the tooth fracture.

ATP-binding cassette transporter G2 is a member of the ABC transporter super family and was first described in drug-resistant MCF-7/Adr cells (21). ABCG2 is a half-transporter and is responsible for the Hoechst dye efflux pattern, which is enriched in stem and/or progenitor cells in various tissues (22). In this study, ABCG2 mRNA

expression in the experimental group tended to decrease compared with the control group. This means that the tooth fracture provokes stem cells in the pulp to differentiate. Nestin is a marker for neural stem cells (23) and has previously been detected in odontoblasts and in the tooth germ (24, 25). This study revealed that the expression of nestin mRNA in the experimental group at 2 days was significantly lower than in the control group, but in the experimental group at 7 days, it had increased again similar to the control. This phenomenon coincided with immunohistochemical observations, in which nestin was not detected in the experimental group at 2 days.

Maintenance of stem cells in the G0/G1 phase is controlled by p57 (26, 27). In this study, p57 mRNA expression in the experimental group both at 2 and at 7 days was significantly higher than in the control group. This means that the tooth fracture causes stem cells to stop proliferating and to begin differentiating. Casasco et al. (28) reported that approximately 90% of dental pulp tissue is in the G0/G1 phase, although p57 mRNA expression in the experimental group was higher than in the control group, which means that the tooth fracture causes stem cells in the dental pulp to proliferate through the cell proliferation cycle.

In conclusion, this study reveals that many stem cells are contained in the fractured dental pulp and can be used for tissue engineering because of their capability to differentiate into osteogenic and/or odontogenic cells.

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