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Immunohistochemical Study of 78kDa Glucose-regulated Protein (Grp78) and Cripto in the Spheno-occipital Synchondrosis

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Abstract: The synchondroses in the cranial base are important structures in craniofacial growth, and the spheno-occipital synchondrosis (SOS) is representative of a typical growth site. Endoplasmic reticulum (ER) stress is associated with multiple biological processes and is a critical factor in chondrogenesis. It has been reported that 78kDa Glucose-regulated protein (Grp78) plays an important role in suppressing regulators in ER stress-mediated apoptosis in chondrogenesis, and Cripto is the cell surface signaling partner of Grp78 in the transforming growth factor-β (TGF-β) signaling pathway. We attempt to clarify the immunolocalization of Grp78 and Cripto in the SOS. The mice head at embryonic day 17.5 (E17.5) were collected and embedded in paraffin. The serial sections were stained with hematoxylin and eosin, Alcian blue, lectin, and immunostaining. The SOS structure containing the resting, proliferative, and hypertrophic zone were identified with Alcian blue staining, wheat germ agglutinin, and Type II Collagen immunostaining. Immunostaining of Grp78 in the SOS revealed positive immunoreactivity in all the chondrocytes of the SOS. However, the chondrocytes of the proliferating zone were weakly immunopositive to Cripto, while the chondrocytes of the hypertrophic zone were strongly immunopositive. Since the immunolocalization of Grp78 and Cripto was different in cartilage zone, these data suggest that Grp78 and Cripto would be involved in the regulation of hypertrophic chondrocyte differentiation and may be related with ER stress in the SOS.

Keywords: Spheno-occipital Synchondrosis (SOS), Grp78, Cripto

1. Introduction

The craniofacial skeleton is composed of the neurocranium, which surrounds the brain, and the viscerocranium, which encompasses the facial bones. The neurocranium is subdivided into the cranial vault (calvaria) and the cranial base. The cranial base is characterized by the presence of the synchondroses, cartilaginous structures persisting between ossification centers, which play an important role in the harmonized development of the neurocranium and the viscerocranium. There are several synchondroses in the cranial base, including the spheno-occipital synchondrosis (SOS) located between the posterior border of the body of the sphenoid and the anterior edge of the basiocciput. Because fusion of this synchondrosis starts at approximately 7 years of age and ends at approximately 14 years in humans [1], the SOS contributes markedly to anterior–posterior cranial base elongation during adolescence. Thus, premature closure of the SOS has been associated with shortening and flattening of the anterior cranial base, midface hypoplasia, Apert syndrome [2], and Crouzon syndrome [3]. The cranial base is formed by endochondral ossification, and the histological structure is different from skeletal bone, which has a growth

plate consisting of a resting zone of chondrocytes on one side and a hypertrophic zone on the other side. However, synchondroses consist of a central resting zone of chondrocytes, flanked by proliferative and hypertrophic zones of chondrocytes on each side.

78kDa glucose-regulated protein (Grp78) is a member of the heat shock protein 70 family and is considered to be an endoplasmic reticulum (ER) chaperone facilitating the transport of newly synthesized proteins into the ER lumen, protein folding, protein quality control, Ca2+ binding, and the regulation of ER stress signaling [4-6]. It has been increasingly recognized that ER stress is associated with multiple biological processes, including inflammation, bone loss, cell apoptosis, and extracellular matrix degradation [7], and that ER stress is a critical factor in chondrogenesis [8]. In chondrogenesis, the expression of Grp78 protein was increased in spheroid formation using mesenchymal stem cells [9]. It has been reported that Grp78 plays a critical role as a suppressing regulator in ER stress-mediated apoptosis in chondrogenesis [10]. In chondrocyte cells derived from human articular cartilage, Grp78 expression was increased by stimulating IL-1β [11]. To date, few studies have been conducted to investigate the function and expression profile of Grp78 in cartilage during craniofacial development.

Cripto (Cripto-1, TDGF1) is a small glycosylphosphatidyl- inositol (GPI)-anchored cell surface signaling protein with several essential physiological roles during embryogenesis related to cell survival, proliferation, differentiation, and migration. It is known that Cripto is an accessory receptor based on its ability to bind TGF-β ligands as a regulator of the TGF-β signaling pathway and its requirement for Grp78 as a cell surface signaling partner [12]. There are studies reporting the role of Cripto in tumor cell proliferation, but its function in mesenchymal cells and its role in cell differentiation are still unclear [13]. The present study aimed to clarify the immunolocalization of Grp78 and Cripto in the development of synchondroses, and in the SOS in particular.

2. Materials and Methods

C57/BL6 mice (Kyudo, Fukuoka, Japan) at embryonic day 17.5 (E17.5) were used. All animal studies were approved by the Animal Experiment Committee of Fukuoka Dental College, Fukuoka, Japan. The mice embryos were collected and fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS), pH 7.4 for 24 h. After fixation, samples were dehydrated in a graded series of ethanol, embedded in paraffin, and serially sectioned into 4-μm-thick sagittal sections. The sections were stained with hematoxylin and eosin using standard protocols. Serial adjacent sections were stained with Alcian blue (pH 1.0) and lectin, and immunostained with collagen type II, Grp78, and Cripto.

The primary antibodies used in this study were anti-mouse Type II Collagen (LB-1297, LSL, Japan), anti-mouse GRP78 (ab21685, Abcam, Cambridge, UK) and Cripto1 (ab19917, Abcam). For immunostaining with Grp78 and Cripto, sections were deparaffinized with xylene and rehydrated in an alcohol gradient. After washing with PBS, sections were blocked with 1% bovine serum albumin in PBS for 30 min, and incubated with each primary antibody diluted from the original solutions of Type II Collagen (1:100), GRP78 (1:50) and Cripto1 (1:100) overnight (16 h) at 4 °C. The immunoreaction was visualized on sections with goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA) diluted to 10 μg/mL at room temperature for 1 hour in the dark. After washing in PBS, to visualize the cartilaginous matrices, sections were incubated with wheat germ agglutinin (WGA) conjugated fluorescein isothiocyanate (FITC) (J-Chemical, Tokyo, Japan) diluted to 10 μg/mL for 10 min at room temperature. The sections were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and immediately mounted for observation.

3. Results

The SOS at E17.5 was present directly under Rathke's pouch developing to pituitary gland (Figure 1A, Rp) and showed chondrocyte differentiation in the central resting zone of chondrocytes, flanked by two proliferative and hypertrophic zones of chondrocytes (Figure 1A). The extracellular matrices of all the chondrocytes in the SOS were stained with Alcian blue (Figure 1B) and were immunoreactive to collagen type II (Figure 1C) where they were stained with WGA lectin (Figures 1D and 1E).

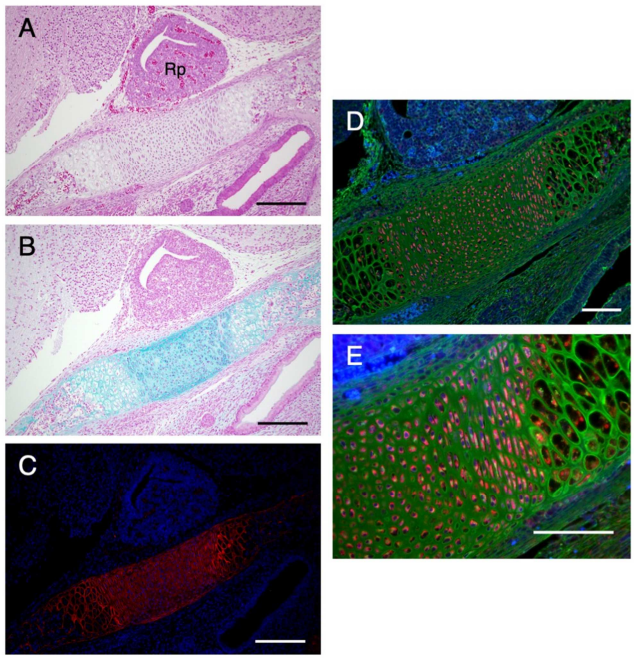

Figure 1. *Morphological and immunoreactive findings of the spheno-occipital synchondrosis at embryonic day 17. A: Hematoxylin and eosin (H-E). B: Alcian blue staining. C: Immunostaining of collagen type II (red). D and E: Wheat germ agglutinin lectin staining (green) and immunostaining of Grp78 (red). Counterstaining was performed with DAPI (blue) in C to E. Bar in A to C = 200 μm, bar in D and E = 100 μm.*

Immunostaining with Grp78 revealed that the chondrocytes in all zones were immunoreactive to Grp78, and weak immunoreactivity was observed in perichondrium cells (Figures 1D and 1E).

Immunostaining with Cripto showed that the chondrocytes of the resting zone were immunoreactive and the chondrocytes of the hypertrophic zone were strongly immunoreactive to Cripto (Figures 2D and 2F). However, the serial sections revealed that the chondrocytes of the proliferating zone were weakly immunoreactive to Cripto (Figure 2F, arrow), and were immunoreactive to Grp78 (Figure 2E, arrow).

4. Discussion

Grp78 is an ER-resident molecular chaperone that facilitates the correct folding and assembly of newly synthesized protein [5]. Excessive ER stress results in apoptosis, and Grp78 controls the activation of transmembrane ER stress sensors [4]. It has been reported that Grp78 is involved in chondrocyte differentiation induced by BMP2 [14, 15] and acts as a negative regulator of ER stress-mediated apoptosis in chondrocyte differentiation [10]. Cripto is a multifunctional signaling protein involved in embryogenesis and in cell proliferation and differentiation. Cripto is a GPI-anchored cell surface signaling protein and co-receptor for Nodal and Activin, belonging to the TGFβ superfamily [12]. Grp78 is also a cell surface receptor and co-factor required for Cripto signaling via Nodal and Activin receptor IB (ActR-IB, ALK4) [16]. It has been reported that Grp78 is present in the cartilage matrix surrounding proliferating chondrocytes [17]. Cripto expression has been reported in adult mouse muscle tissue, which regulates myostatin signaling in myoblasts [18]. However, no studies have elucidated the expression pattern in the SOS. This study attempts to reveal the localization of Grp78 and Cripto in the SOS.

A previous study reported that Grp78 was present in the cartilage matrix in developing long bones at postnatal day 3 and day 5, but no expression of Grp78 was observed in either the chondrocyte or cartilage matrix at 4 weeks [17]. Our study of Grp78 immunostaining revealed that the chondrocytes of all the cartilage development zones (i.e., resting, proliferative, and hypertrophic zones) were immunoreactive in the SOS at E17.5. This result is consistent with a previous immunohistochemistry study that found positive nuclear staining of Grp78 during the entire chondrogenic developmental stage in both the proliferating and hypertrophic zones of the tibial growth plate at birth [10]. Indeed, in several primary mesenchymal-derived cells (odontoblasts, osteoblasts), periodontal ligament cells, and in the C3H10T1/2 mouse mesenchymal cell line [17], localization of Grp78 in the nucleus of each cell was revealed in vitro by immunostaining. Our study indicates that Grp78 would be present in the chondrocytes in all the cartilage development zones of the SOS as well as in the cartilage located in long bones, because both are formed by mesenchymal cells.

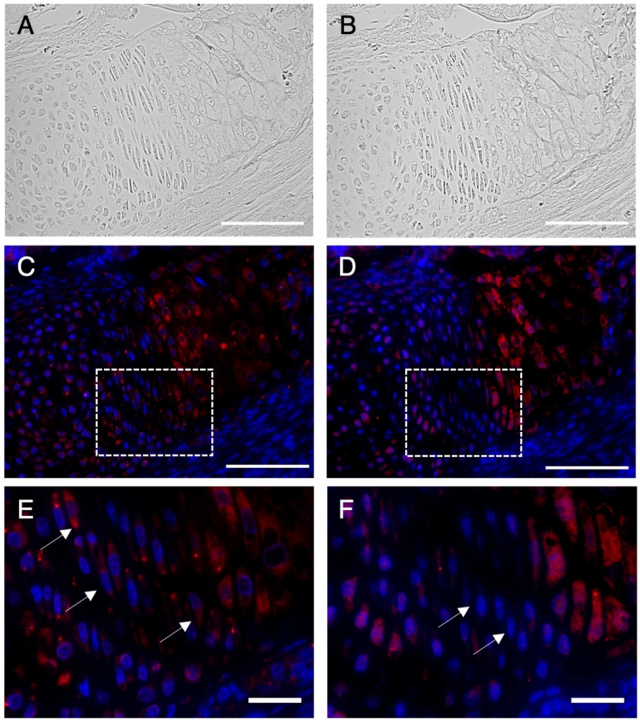

Figure 2. *Immunoreactive findings of Grp78 and Cripto in the spheno-occipital synchondrosis. A and B: Phase contrast image corresponding to C and D respectively. C and E: Immunostaining of Grp78 (red). D and F: Immunostaining of Cripto (red). Counterstaining was performed with DAPI (blue) in C to F. Areas boxed with a dashed line in C and D correspond to E and F respectively. Arrows in E and F indicate chondrocytes in proliferating zone. Bar in A to D = 100 μm, bar in E and F = 25 μm.*

Apart from the expression pattern of Grp78, our results showed that the chondrocytes of the proliferating zone were weakly immunopositive to Cripto, while the chondrocytes of the hypertrophic zone were strongly immunopositive to both Cripto and Grp78. A recent study has shown that immunofluoroscene labeling positivity to Cripto was found at the boundaries of the area containing hypertrophic chondrocytes in a metatarsal organ culture derived from E15.5 to E17.5 mouse hind limb [19]. This result indicates that the protein expression pattern of Cripto in the hypertrophic chondrocytes of the SOS would be similar to that of chondrocytes in the endochondral ossification of long bones. Because increased Cripto gene expression was observed during chondrogenic differentiation of mouse prechondrocytes in vitro, it has been suggested that Cripto could stimulate chondrocyte hypertrophy [19]. Various studies have shown that Grp78 is localized in the plasma membrane and the complex of Cripto and Grp78 [12, 17, 20]. Cripto is not only present in the cell membrane, but can also be released from the cell in a soluble form, acting as a secreted growth factor [21]. Although our results did not show the presence of Cripto in the extracellular matrix of the SOS or on the cell surface of chondrocytes, the Grp78 and Cripto complex could regulate the differentiation of cells into hypertrophic chondrocytes because Grip78 and Cripto would be co-localized in these cells as revealed by immunohistochemistry using serial sections. The

chondrocytes of the SOS originate from neural crest-derived cells in the rostral portion of this cartilaginous structure, but none in the caudal portion, and mesoderm-derived cells contribute to all parts of the SOS [22]. Previous studies showed that Grp78 and Cripto play an important role in chondrogenesis derived from the mesoderm [10, 17]. Further detailed investigation is needed into the regulation of Grp78 and Cripto in chondrogenesis derived from neural crest cells.

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