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SCIENCE DIRECT

Developmental Biology 278 (2005) 86–102

DEVELOPMENTAL BIOLOGY

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Apoptosis and proliferation in developing, mature, and regressing epibranchial placodes

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Received for publication 18 September 2004, revised 23 October 2004, accepted 25 October 2004
Available online 18 November 2004

Abstract

Epibranchial placodes and rhombencephalic neural crest provide precursor cells for the geniculate, petrosal, and nodose ganglia. In chick embryos and in *Tupaia belangeri*, apoptosis in rhombomeres 3 and 5 helps to select premigratory precursor cells and to segregate crest cell streams derived from the even-numbered rhombomeres. Much less is known about the patterns and functions of apoptosis in epibranchial placodes. We found that, in *Tupaia belangeri*, combined anlagen of the otic placode and epibranchial placode 1 transiently share a primordial low grade thickening with post-otic epibranchial placodes. Three-dimensional reconstructions reveal complementary, spatially, and temporally regulated apoptotic and proliferative events that demarcate the otic placode and epibranchial placode 1, and help to individualize three pairs of epibranchial placodes in a rostrocaudal sequence. Later, rostrocaudal waves of proliferation and apoptosis extend from dorsal to ventral parts of the placodes, paralleled by the dorsoventral progression of precursor cell delamination. These findings suggest a role for apoptosis during the process of neuroblast generation in the epibranchial placodes. Finally, apoptosis eliminates remnants of the placodes in the presence of late invading macrophages.

Keywords: Apoptosis; Proliferation; Epibranchial placodes; Hindbrain; Neural crest; Cranial nerves; Development; 3-D reconstruction; Mammal

Introduction

Epibranchial placodes are serially arranged ectodermal thickenings that develop dorsocaudally adjacent to the branchial membranes. In mammals, three pairs of epibranchial placodes provide sensory neurons for the geniculate, petrosal, and nodose ganglia. Ganglionic glial cells are recruited from the rhombencephalic neural crest (Baker and Bronner-Fraser, 2001; Webb and Noden, 1993). Mechanisms should be in place that coordinate the production, survival, and differentiation of precursor cells in the two distant sources. In fact, in chick embryos (Graham et al., 1993) and in *Tupaia belangeri* (Knabe et al., 2004), large-scale apoptosis of premigratory neural crest cells in rhombomeres 3 and 5 helps to segregate streams of crest cells that emigrate from rhombomeres 1/2, 4, and 6/7. Consequently, crest cell streaming guides axons of placode-derived neurons to the correct afferent location in the hindbrain (Begbie and Graham, 2001; Graham et al., 2004).

Reports on physiologically occurring cell death in the epibranchial placodes are sparse (Adelmann, 1925; Batten, 1957; Sulik et al., 1987; Theiler, 1949). In embryonic mice, highest numbers of apoptotic placodal cells are found in the 33 somite embryo (Sulik et al., 1987). Compared with wildtype embryos, apoptosis in epibranchial placodes or among placode-derived neuroblasts is increased in zebrafish lacking the transcription factor Foxi 1 (Lee et al., 2003), and in embryonic mice exposed to ethanol (Kotch and Sulik, 1992; Sulik et al., 1986), retinoic acid (Sulik et al., 1987), or manipulated by blocking retinoid signal transduction (Wendling et al., 2000). According to Sulik et al. (1987, 1988), the excessive apoptotic loss of cells derived from the

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0012-1606/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.10.016

trigeminal and epibranchial placodes promotes the development of mandibulofacial dysostosis (Treacher Collins)-like syndromes. On the other hand, Dixon et al. (1997, 2000) state that pathologically increased apoptosis of premigratory neural crest cells is the primary cause of the Treacher Collins syndrome. Taken together, these findings encouraged to test whether physiologically regulated patterns of apoptosis in the epibranchial placodes exist, and whether these patterns are temporally and functionally related with the known rhombomere-specific patterns of apoptosis (Graham et al., 1993; Knabe et al., 2004).

Epibranchial placodes develop within larger areas of thickened branchial surface ectoderm (Baker and Bronner-Fraser, 2000; Bartelmez and Evans, 1926; D'Amico-Martel and Noden, 1983; Müller and O'Rahilly, 1988; Verwoerd and van Oostrom, 1979). The existence of a common primordial thickening for most, if not all cephalic placodes of vertebrates, has been postulated or disputed (Schlosser, 2002). Advancing insights into the molecular mechanisms of placode development suggest that, in fact, "different placodes evolved from a common placodal primordium by successive recruitment of new inducers and target genes" (Schlosser and Ahrens, 2004). Thus, in *Xenopus laevis*, otic and epibranchial placodes as well as lateral line placodes share a common primordial thickening and the expression patterns for many different transcription factors (Schlosser and Ahrens, 2004). Accordingly, in chick embryos, the transcription factor cSox3 is initially expressed throughout the epiblast. From Hamburger Hamilton stage 10 (Hamburger and Hamilton, 1951) onwards, cSox3 is secondarily upregulated in two thickened areas which encompass (1) the otic placode and epibranchial placode 1, and (2) developing epibranchial placode 2 as well as the two vagal epibranchial placodes. Finally, isolated patches of cSox3 demarcate the definitive epibranchial placodes (Ishii et al., 2001).

To comprehensively demonstrate developing epibranchial placodes in their cellular and organismic context, we have established a system that performs three-dimensional reconstructions of small cellular events in large objects (Knabe and Kuhn, 1998; Knabe et al., 2000). This system was stepwise improved (1) by introducing "Huge Image", a scanning system which permits data acquisition from semithin sections at maximum light microscopic resolution (Knabe et al., 2002; Süss et al., 2002), (2) by setting up a procedure that realigns vectorized histological serial sections with the help of external fiducials (Knabe et al., 2002), and (3) by implementing triangulation algorithms for the rapid reconstruction of large internal and external embryonic surfaces (Brunnett et al., 2003). In the past, this system helped to identify bands of apoptotic cells that, probably, forward bilateralization processes of the entire forebrain (Knabe and Kuhn, 1998; Knabe et al., 2000). Additionally, a straightforward sequence of rhombomere-specific apoptotic events was observed in the developing hindbrain of the tree shrew *Tupaia belangeri* (Knabe et al., 2004). Tree shrews are diurnal mammals, and their relationship to primates is under debate (Wible and Zeller, 1994).

The present work investigates, whether and how spatially and temporally regulated patterns of apoptosis and proliferation contribute to the morphogenesis and fate of the epibranchial placodes in *Tupaia belangeri*. Since macrophages may either eliminate apoptotic cells (Rabinovitch, 1995) or induce apoptosis (Frade and Barde, 1998), we also investigated whether macrophages invade the epibranchial placodes. Multiparametric three-dimensional reconstructions of cellular events that occur simultaneously in the developing central and peripheral nervous system provide the groundwork for future molecular analyses.

Materials and methods

Animals

Embryos of the tree shrew *Tupaia belangeri* (Scandentia, average gestation period: 43.7 days, Kuhn and Schwaier, 1973), were collected at the German Primate Center (DPZ), Göttingen, and at the Battelle-Institute, Frankfurt/Main, for unrelated projects of the former Sonderforschungsbereich 89 (Cardiology). In accordance with German law, pregnant *Tupaia belangeri* were killed by intraperitoneal injection of an overdose of pentobarbitone sodium (Nembutal), arterially perfused with Macrodex and 0.15% procaine hydrochloride (Novocain), and fixed with 4% glutaraldehyde/3% paraformaldehyde in phosphate buffer (pH 7.3). Embryos from embryonic days 12 to 19 were embedded and sectioned as specified in Table 1. All embryos used for three-dimensional reconstructions were embedded in Araldite (Serva, Heidelberg, Germany), and completely serially sectioned at 1 μm. Consecutive sections were alternately placed on two sets of slides. Sections of the "working series" (Knabe et al., 2002) were stained with Heidenhain's hematoxylin (Romeis, 1989). Sections of the "reference series" remained unstained and, for reasons detailed below, provided external fiducials for the realignment procedure (Knabe et al., 2002). In previous work, 12- to 16-day-old embryos of our collection were classified according to five arbitrary phases of optic cup formation (Knabe and Kuhn, 1998, 1999; Knabe et al., 2000, 2002). This classification and the designation of each embryo by numbers that indicate the ontogenetic day and an alphabetic order of the embryos of each day were maintained and expanded in the present work:

Phase 1: V-shaped optic evagination
Phase 2: Optic vesicle
Phase 3: Onset of the invagination of the optic vesicle
Phase 4: Advanced invagination
Phase 5: Far advanced invagination/optic fissure
Phase 6: Onset of optic fissure closure