

Analysis of cell proliferation kinetics during the secondary palate development in quail

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Summary. A study was undertaken to analyze the spatio-temporal pattern of mesenchymal cell proliferation in the developing palate of quail. Quail embryos were grown in shell-less culture. The developing palates were labelled with ^3H -thymidine between culture days 2-6 (which corresponded *in vivo* incubation days 5-9), and processed for light microscopic autoradiography. Percent labelled mesenchymal cells were determined. The data showed that, as in mammals, a high rate of random cell proliferation in mesenchyme was a major component of early palate development in quail. As the palate morphogenesis advanced, the rate of cell proliferation declined. Segmental analysis, however, indicated that, in contrast to mammals, the mesenchymal cell proliferation rates continually changed in various regions of quail palate during morphogenesis. It was suggested that the spatio-temporal changes in the distribution of dividing cells may reflect differences in the timings of cell cycles between various segments, thus resulting in a heterogeneous population of cells in the developing palate of quail. Further, the differences in the segmental pattern of cell proliferation between birds and mammals may form the basis for differences in the morphogenesis of their palates.

Key words: Palate morphogenesis, Quail, Cell proliferation, ^3H -thymidine, Autoradiography

Introduction

Although much of our current understanding of vertebrate secondary palate development comes from studies in mammals, other animals, such as fish (Shah et al., 1990, 1995a), alligator (Ferguson, 1981; Shah and Ferguson, 1988) and birds (Shah and Crawford, 1980; Koch and Smiley, 1981; Greene et al., 1983; Shah et al., 1985, 1987; 1988, 1994a; Forman et al., 1991; Benkhaial

et al., 1993; Benkhaial and Shah, 1994; Young et al., 1994) have also been examined. These studies have clarified some of the biological basis for differences in the secondary palate development among various classes of vertebrates.

In a developing structure, spatio-temporal distribution of proliferative pools of cells is one of the crucial factors for achieving correct size (volume) and shape (form) (Yasuda et al., 1991; Garcia-Bellido et al., 1994). The pattern of cell proliferation is, however, tissue/organ specific (Oates and Morgan, 1989). Previous studies have shown that during the formation of the vertebrate secondary palate one of the major differences is the class-specific variation in the rate of DNA synthesis (indicative of cell proliferation). For example, in fish, where palatal shelves grow as vertical projections with no further morphogenesis (Shah et al., 1990), the rate of DNA synthesis increases only after the palatal form is established (Shah et al., 1995b). On the other hand, in mammals, where palatal shelves initially grow vertically and then reorient to a horizontal plane and fuse, both DNA synthesis and cell proliferation rates were high during the initial half of vertical shelf development but declined to a lower, steady rate during the subsequent steps of morphogenesis (Nanda and Romeo, 1975; Cleaton-Jones, 1976; Burdett et al., 1988; Luke, 1989; Shah et al., 1989a,b, 1991, 1994b; Zenk and Stiller, 1990). In birds, where palatal shelves grow horizontally *ad initium* and approximate in the midline but never fuse, there was a steady decline in the rate of DNA synthesis (Shah et al., 1994a). Clearly, these ontogenetic differences in the temporal pattern of rates of DNA synthesis, along with those of extracellular matrix (ECM) molecules (Brinkley and Morris-Wiman, 1984; Knudsen et al., 1986; Forman et al., 1991; Benkhaial et al., 1993; Benkhaial and Shah, 1994; Young et al., 1994; Shah et al., 1995b; Singh et al., 1994), have significant impact on how palate morphogenesis progresses in various classes of vertebrates. Except for mammals (Mott et al., 1969; Hudson and Shapiro, 1973; Jelinek and Dostal, 1974; Nanda and Romeo, 1975; Cleaton-Jones, 1976; Brinkley, 1984; Brinkley and Bookstein, 1986; Luke, 1989;

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Amwayi and Luke, 1990; Singh and Moxham, 1993; Shah et al., 1994b), however, information on cell proliferation during palate formation in any other vertebrates is non-existent. An understanding of cell proliferation kinetics during palatogenesis is significant because: (a) achieving a critical number of cells may be important to complete palate morphogenesis; (b) most cases of human cleft palate patients shows severely hypoplastic palatal shelves; (c) in most studies on teratogen-induced cleft palate in laboratory rodents the palatal shelves were of small size due to growth retardation; (d) X-linked deviant morphology in human, which sometimes includes cleft palate, may originate as mitotic error (Schwartz et al., 1986) possibly leading to changes in the cell replication time; and (e) it may contribute to our understanding of comparative biology of the vertebrates secondary palate development.

In the present study, we have analyzed kinetics of cell proliferation in the developing palate of quail grown in shell-less culture. The shell-less culture facilitates serial observations of the developing embryo, and allows the accessibility to the embryo *ad libitum* (Dunn et al., 1981). The historical aspects of the development of shell-less culture technique and its strengths and limitations have been discussed earlier (Dunn et al., 1981). Also, the details of morphological, cellular and biochemical aspects of *in vivo* and *in vitro* development of quail palate have been described earlier (Shah et al., 1985, 1994a; Shah and Cheng, 1988; Benkhaial et al., 1993; Benkhaial and Shah, 1994; Young et al., 1994d), and, for the sake of brevity, are not repeated here.

Materials and methods

Random bred, wild type quail eggs, *Coturnix japonica*, were obtained from The Quail Genetic Stock Center, The University of British Columbia, Vancouver, Canada. On day 3 of incubation, embryos along with their intact membranes and yolk sac were isolated, placed individually in a cellophane basket, which was suspended in an inverted plastic beaker with the bottom removed (also see Fig. 1 in Dunn et al., 1981). These «*in vitro*» embryos were incubated at 37 °C and 5% CO₂ under humidified conditions as described earlier (Shah and Cheng, 1988). In order to assess whether the embryogenesis in shell-less culture condition resembled that *in vivo*, a preliminary study was undertaken in which 30 cultured embryos were obtained on days 2, 3, 4, 5, and 6 of culture, weighed, measured for crown-rump length (CRL) and staged according to the method described by Hamilton and Hamburger (HH; 1951). A corresponding group of *in vivo* developing embryos, i.e., days 5, 6, 7, 8, and 9 of incubation were similarly processed. The comparison between the two groups revealed (data not shown) that, although the cultured embryos weighed slightly less, and were smaller in CRL in contrast to the corresponding *in vivo* developing embryos, they resembled each other on the basis of HH developmental stages, which correlated with specific day

of incubation. Hence, in the subsequent cell proliferation study, the embryos were grouped according to the HH developmental stages. The data in the Result and Discussion sections of the text are adjusted to reflect appropriate *in vivo* days of incubation so that adequate comparison can be made with the data from the previous studies on quail as well as mammals.

In a separate experiment, embryos were labelled on day 2, 3, 4, 5 and 6 of culture with 20 µCi of ³H-thymidine (ICN, Montreal, Canada; Specific Activity 50.8 Ci/mmol) for one hour. The labelled embryos were then rinsed three times in 0.2M phosphate buffered saline (pH 7.2), and immersed in Bouin's fixative for at least 48 hours. Subsequently, the embryos were developmentally staged by HH method (1951), and dehydrated in ethanol for paraffin embedding. Frontal sections (6 µm) from the anterior third of the developing secondary palate were used for autoradiography because the morphogenesis of quail secondary palate begins in the anterior third and proceeds posteriorly (Shah et al., 1985). The sections, mounted onto gelatin coated glass slides, were dipped in Kodak NTB-2 solution and kept in a dark, airtight box at 4 °C for 8 weeks. The slides were developed with Kodak D-19 (1:1 dilution), and then stained with Haematoxylin and Eosin.

The proliferation indices of mesenchymal cell were determined from the palatal shelves at different times during development. Three sections from each embryo were counted, simultaneously ensuring that the adjacent section was not used in the analysis, to determine the average for each embryo. A total of 15 embryos were counted between days 5 and 9 of incubation (HH stages 27-36) to determine the percent mesenchymal cells undergoing proliferation. The proliferation index was defined as the number of labelled cells expressed as a percent of the total nuclei. A cell was considered labelled when the number of silver grains was five or more above the background level. The boundaries of the developing palatal shelf was determined by points where the nasal and oral epithelia changed direction (see Fig. 1 in Shah et al., 1994a).

In order to further determine whether the dividing cells in the developing shelf were distributed randomly or were localized in a particular area, the palate sections were divided into four segments with the aid of a 10x10 marked glass grid inserted into the eyepiece of the microscope. These segments were constructed from a line dividing (1) the medial (ME) half (towards the growing tip of the shelf) and a lateral (LA) half (away from the growing tip of the shelf), or (2) an upper (U) half (away from the tongue) and a lower (LO) half (adjacent to the tongue). The mesenchymal cell proliferation index was determined in each of the four segments (U-ME, U-LA, LO-ME, LO-LA). The data were evaluated by Friedman two-way analysis of variance, non-parametric Wilcoxon test or Student's t-test (Zar, 1984).

Results

Figures 1-3 shows ^3H -thymidine labelled cells at different times during quail palate development. It is obvious that there are spatio-temporal changes in the distribution of labelled cells in the developing palate.

The data on mesenchymal cell proliferation indices during quail palate formation are summarized in Fig. 4. One may deduce that approximately one-third of the mesenchymal cells were dividing on days 5 and 6 of incubation (HH stages 27-29). Subsequently, as the palate morphogenesis advances, the rate of proliferation declined, and on day 9 of incubation (HH stages 34-36) it was reduced by approximately a third of that seen on day 5 of incubation ($p < 0.01$).

The spatio-temporal distribution of dividing cells is summarized in Fig. 5. The data show that on day 5 of

incubation, i.e., at the time of appearance of the palatal primordia, fewer dividing mesenchymal cells were present in the LO-ME segment than in the other three segments (U-ME=U-LA=LO-LA) where they were equally distributed ($p < 0.01$). On day 6 of incubation, the percent dividing cells remained unchanged in the U-ME and LO-LA segments. It, however, approximately doubled in LO-ME and decreased by 28% in the U-LA segments ($p < 0.01$). The rank order of the proliferation rate was LO-ME, LO-LA, U-ME, and U-LA. On day 7 of incubation, the distribution pattern of dividing mesenchymal cells changed dramatically. Although in all four segments the percent labelled cells were decreased, the dramatic differences were seen only in the LO-ME and both lateral segments ($p < 0.01$). The rank order of proliferation rate was U-ME, LO-LA=U-LA, and LO-ME. Subsequently, on day 8 of incubation, when quail palatal shelves approximate (Shah et al., 1985), the percent labelled cells decreased further in the U-ME segment ($p < 0.01$). Consequently, the dividing cells were distributed equally among all four segments. On day 9 of incubation, the percent labeled cells, although increased in all four segments, were distributed equally in all segments ($p < 0.01$).

Discussion

The two major components of avian palatal growth are cell proliferation and synthesis of ECM molecules. Previous studies have outlined the contribution made by some of the ECM molecules in achieving the critical (volume) mass during quail palate formation (Benkhaïal

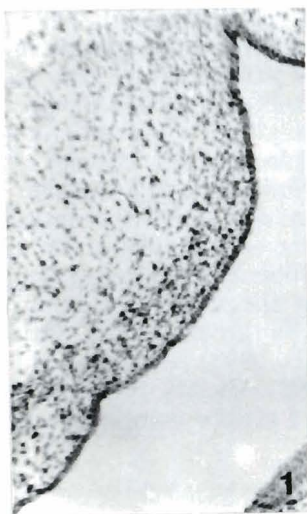


Fig. 1. Frontal sections of developing quail palate showing distribution of ^3H -thymidine labelled cells. Palatal primordium on day 5 of incubation (culture day 2; HH stage 27). The labelled cells appear as dark dots. x 190

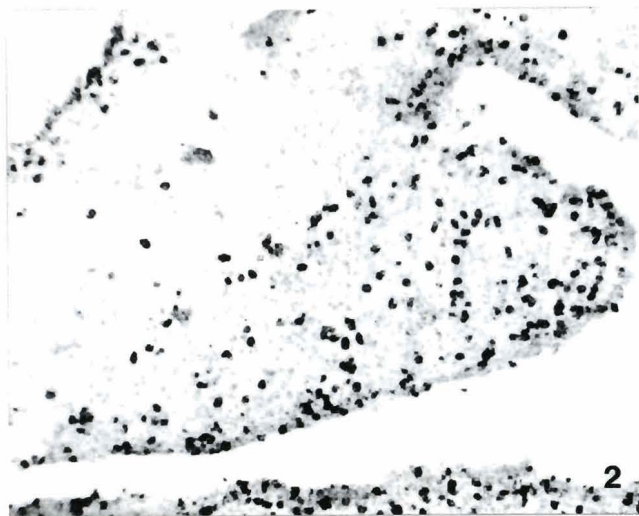


Fig. 2. Frontal sections of developing quail palate showing distribution of ^3H -thymidine labelled cells. Horizontal palatal shelf on day 6 of incubation (culture day 3; HH stage 29). The labelled cells appear as dark dots. x 190

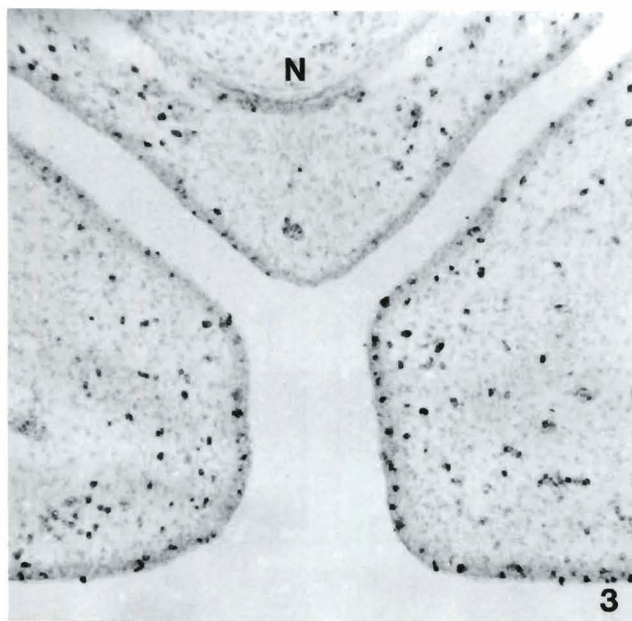


Fig. 3. Frontal sections of developing quail palate showing distribution of ^3H -thymidine labelled cells. Approximating palatal shelves on day 9 of incubation (culture day 5; HH stage 35). Nasal septum (N). The labelled cells appear as dark dots. x 190

et al., 1993; Young et al., 1994). The present study provides details of growth rates of cells entering the cell cycle at different times during palate morphogenesis, and thus on the formation of heterogeneous pools of cells as well as on the location of dividing cells within the developing palate. These basic data should be useful in evaluating, both qualitatively and quantitatively, the roles of various growth factors and hormones, which are implicated in the regulation of palate formation, and of teratological perturbations of the developing palate.

The data of the present study show that the general trends of cell proliferation rates during quail palate morphogenesis parallel the biochemically determined DNA synthesis rates described earlier (Shah et al., 1994a). Initially, the rate of cell proliferation was high. However, it declined as palate morphogenesis in quail advanced. A similar temporal pattern of cell proliferation activity (DNA synthesis) during embryonic development have been noted in facial primordia, eye, limb, pharyngeal plate, etc (Ede, 1971; Searls and Janner, 1971; Minkoff and Kuntz, 1977, 1978; Summerbell, 1977; Truby, 1983; Bailey et al., 1988; Miller et al., 1993). During palate formation in mammals, the rate of cell proliferation was also high at the time of primordial formation, and it declined as the vertical palate morphogenesis advanced (Shah et al., 1994b). During reorientation and fusion of mammalian palatal shelves the proliferation rates remained unchanged (Mott et al., 1969; Jelinek and Dostal, 1974; Nanda and Romeo, 1975; Cleaton-Jones, 1976; Singh and Moxham, 1993). Thus, it appears that at least between birds and mammals (information on other vertebrates is lacking) there is a similarity in the temporal sequence of cell proliferation activity even though the phylogenetic outcome of their

palate morphogenesis is different. This may indicate that various putative factors or molecules regulating cell proliferation, and thus possibly the entry or exit of cells through the cell cycle, during palate morphogenesis may be similar in both classes of vertebrates. Recently, it was shown that various second-messenger independent protein kinases (for example, mitogen activated protein kinase, p34^{cdc2} and casein kinase II) are activated during both mammalian and avian palate morphogenesis corresponding to the high cell proliferation activities (Hehn et al., 1994, 1995; Paddon et al., 1994; Pelech et al., 1994; Young et al., 1994b, 1995), and are modulated by growth factors (Izadnegahdar et al., 1995; Shah et al., 1995a). Also, as noted previously, a variable rate of cell proliferation during palate morphogenesis may provide greater flexibility in achieving the necessary shape and volume of palatal shelves (Shah et al., 1994b) and allowing positioning of cells for the temporally regulated production of ECM molecules (Benkhaial et al., 1993; Young et al., 1994a). This would contrast a system in which the cellular decisions for position and lineages as well as for the creation of microenvironment were specified by some pre-localized determinants (Kimmelman, 1993). The usefulness of this concept of declining rate of cell proliferation may be further substantiated by the observations that following prenatal treatment with growth inhibitors, which inhibited DNA synthesis, both the shape and volume of the shelves and the production of ECM in them, during palate morphogenesis, were affected (Burdett et al., 1988; Shah et al., 1989a; Benkhaial et al., 1993; Benkhaial and Shah, 1994; Young et al., 1994). Clearly, differential rates of cell proliferation is a crucial component of

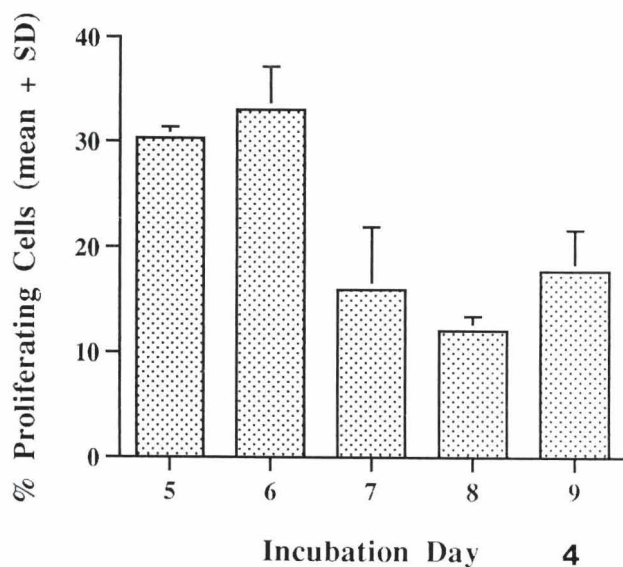


Fig. 4. Profile of the rate of mesenchymal cell proliferation in the developing palate of quail at different times during incubation (culture).

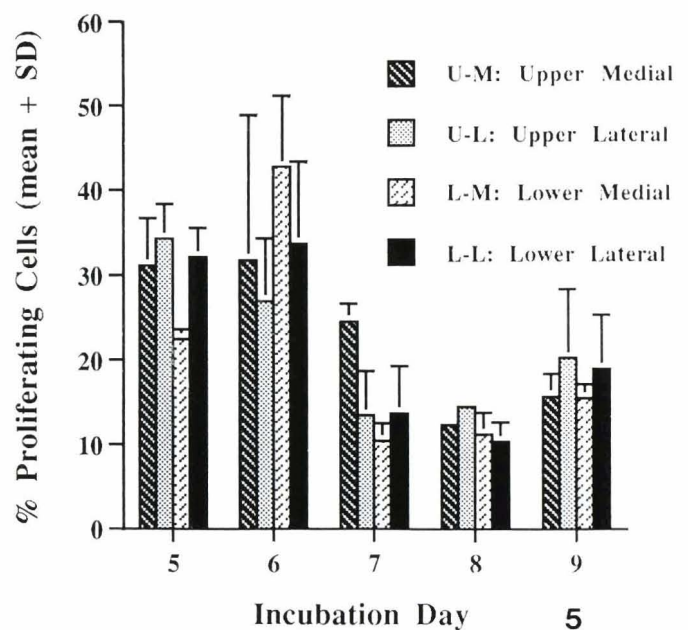


Fig. 5. Profile of the rate of mesenchymal cell proliferation in different segments of the developing palate at different times during incubation (culture).

advancing morphogenesis of vertebrate palate.

The data of the present study further demonstrate that there are spatio-temporal differences in the pattern of proliferation of mesenchymal cells indicating that, as in mammals (Shah et al., 1994b), the role of proliferating pools of cells during quail palate development may also be very complex. Segmental analysis (upper and lower, medial and lateral) revealed that initially, at the time of appearance of the palatal primordia, the cell proliferation was random. Subsequently, as the palate morphogenesis advanced, however, the proliferation gradient changed. When the data of the segmental analysis for days 5 and 6 of incubation (the period when the labelling index was high) were pooled the proliferation gradient was LO-ME=LO-LA<U-ME<U-LA suggesting that the lower (oral) segment showed higher rate of cell proliferation than the upper (nasal) segments. Subsequently, between days 7 and 9 of incubation, however, the pattern reversed and the proliferation gradient was U-ME<U-LA<LO-LA<LO-ME. The changing pattern of proliferation may be indicative of differences in the duration of cell cycle in each segment during quail palate development. This may contribute to formation of a heterogeneous pool of cells in the developing palatal shelf. It, however, does not indicate whether the different populations of proliferating cells have a self-organizing capacity (autonomy) to differentiate into appropriate phenotypes. On the other hand, in mammals (Shah et al., 1994b) there appears to be a successive wave of cell proliferation from the base (equivalent to the lateral half in quail) toward the growing tip (equivalent to the medial half in quail), thereby segregating cells for necessary phenotypic commitment during subsequent morphogenesis. It is possible that the diversity in the pattern with regard to the differential rates of cell proliferation may contribute to different morphogenesis of palate in mammals and birds. It is still unclear, however, whether the species-specific difference in the proliferation pattern is a part of a temporal program of palate development, or is determined by the «positional information» of cells (Wolpert, 1969), or by some other physical (volume, length and shape of the palatal shelves) or chemical (growth factors, hormones) factors.

Acknowledgements. The work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

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