

Wing Bud Apoptosis in Embryonic Chick Development

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I. Introduction

Apoptosis, a form of programmed cell death (PCD), in the developing wing bud of a chick embryo was studied in this experiment. This cellular suicide process is distinguished by a specific regulation of cell death as well as the maintenance of intact cells (Browder and Iten, 1998). Apoptosis acts as a developmental mechanism in avian digit remodeling. During embryonic chick wing bud maturity, apoptosis targets the soft tissue connecting the digits eradicating solely these cells. If PCD fails in molding these tissues, the chick will develop an external dysmorphology known as webbed wings. The wing bud begins development with the outward proliferation of the lateral plate mesoderm followed by the formation of the apical ectodermal ridge (AER) from the thickening of the distal ectoderm (Hosung et al., 1998). Cooperating signaling pathways differentiate the course of each limb tissue on all three axis (dorsal-ventral, anterior-posterior and proximal-distal). Sonic Hedgehog (Shh) has been indicated in signaling anterior-posterior patterning of each limb bud specified by the zone of polarizing activity (ZPA) (Hosung et al., 1998).

Sanz-Ezquerro and Tickle (2001) suggest that Shh prepares cells for digit formation and the Bone Morphogenetic Proteins (BMPs) sequentially signal PCD of mesoderm cells in the interdigital space of chick wings. Each individual digit is determined by different concentrations of BMPs thereby controlling which type of digit develops at a particular place. Major changes in morphogenesis can arise from miniscule changes in the genome. Gremlin protein, a known inhibitor of BMP, could induce webbed-wings upon a small change in gene regulation. Apoptosis purposely doesn't occur in some species, such as the duck. Morphogenic changes can also result from grafting duck ectoderm from the hind limbs to chick wing mesoderm. Saunders' performed this grafting experiment (1966) and observed that PCD fails and the digits remain joined. This implicates that the ectoderm provides the signal to trigger PCD.

Apoptosis induces cell death by inhibiting and interfering with several growth factors responsible for regulating the proliferation and survival of cells. Numerous growth factors are needed for the development of cells and also for cell division to proceed normally. It is believed that BMP signaling inhibits growth factors Fgf-8 and Fgf-10, which have the appropriate temporal and spatial expression that might direct limb bud initiation (Hosung et al., 1998). This results in patterns of decreased regions of cells in the developing wing bud, which will be measured in this experiment.

Current research of apoptosis is of considerable interest, mainly in respect to oncogenesis, the formation and development of tumors. Apoptosis has great potential for therapeutic purposes. The inhibition of uncontrolled apoptosis and subsequent promotion of cell survival could curb oncogenesis (Browder and Iten, 1998). On the other hand, triggering cell death could eliminate tumor cells. Cures for diseases such as cancer, autoimmune disorders, and viral infections appear to be in sight with this mechanism identified. The acquisition of tumor necrosis factor (TNF), which triggers apoptosis in target cells, could potentially revolutionize cancer research and eventually patient

care (Browder and Iten, 1998).

In this study, we tested the hypothesis that apoptosis in the embryonic chick wing bud causes digit separation. A chick model was used because of the ability to stain its naturally occurring necrotic wing bud cells. The loss of membrane integrity in dead and dying cells allows for uptake of the Nile Blue A stain. In order to observe the apoptotic patterns on the developing wing bud of a chick embryo, the normal embryo was explanted and separated from its yolk to be exposed to Nile Blue A stain at day six of development. The pattern and the areas of cell death was then measured using digital imaging.

II. Materials and Methods

Explanting the Embryo

The lab bench, utensils and hands were sterilized with 70% ethyl alcohol (EtOH). This semi-sterile working environment was meticulously maintained throughout the experiment (Armstrong et al., 1994). Forceps, paper bowls, weigh boats and 3 eggs at day 9 of development were collected. Everything was procedurally sprayed with 70% EtOH and allowed to air dry. The egg shell at the wider end of the egg was gently cracked and peeled away using forceps, exposing the air-space. Caution was taken not to puncture the egg's shell membrane. The egg was held, smaller-end-up, as close to the weigh boat as possible. The shell on the smaller end was lightly pierced with a tip of the forceps, releasing the contents of the egg out the wider end into the weigh boat.

Chicks require warmth, moisture, and a bacteria-free environment for development so they were housed in two different incubators. The one in the ICUC was used for temporary storage while we were conducting the experiments. This one was neither sufficiently large nor humid enough to accommodate the developing chicks. The second incubator, located directly across the hall from the ICUC, served as a long-term storage with trays of water to ensure 100% humidity. Using this system, the chick embryos were never out of the incubators for more than 15 minutes at a time.

Staining with Nile Blue A

The embryo was removed from the yolk by placing a piece of filter paper on the wet blastoderm so that it stuck to the membrane surrounding the embryo (Grey et al., 1982). With small scissors the area opaca was cut outside the paper. The paper did not stick and the embryo on the filter paper was transferred using forceps to a Petri dish of warm Tyrode's, a physiologically based saline solution, to wash away any remaining yolk. The embryo was exposed by carefully tearing the intact amnion with the forceps. The embryo was then transferred to a Petri dish containing a 1:1,000 dilution (20 microliters in 20 milliliters) of Nile Blue A in Tyrode's solution. Embryo stayed in the stain for 15-30 minutes. The embryo was then transferred back to a dish of Tyrode's solution for about 30 minutes.

Analysis

Pictures were taken at 4x magnification of the wing buds on the standard microscope in the ICUC. The icon for a picture of a stained wing bud was dragged into Adobe Photoshop 7. The red, green and RGB channels were unselected, leaving only the blue channel highlighted. Image was selected from the toolbar, then Mode and Greyscale. This new image was saved to our personal folder and was then dragged into the ImageJ program. A rectangular stained region was selected, followed by the Histogram under the Analyze option on the toolbar. The List option on the Histogram was selected and copied into Excel. The average blueness of an inter-digital region and a digital region was calculated on the developing wing bud on the 7 day embryo and the 9 day embryo. A higher average means lower blueness because the computer reads white to have a value of 255 and black a value of 0, so blue translated into grayscale would be greatest at the lower numbers.

III. Results

The week 1 batch of chicks yielded no stainable subjects out of 8 embryos. We successfully explanted 2 eggs out of 7 during week 2, one on day 7 and another on day 9. The wing buds on the day 7 embryo had apoptosis concentrated on the tip of the limb (See Fig.1), while the day 9 wing buds stained dead cells in the inter-digital region (See Fig.3). The variation in quantitative blueness between the apoptotic region and the healthy cells region was greater in day 7 with a difference of 68 units in the left wing bud and 73 units in the right wing bud than day 9 with a difference of 50 units in the left wing bud and 59 units in the right wing bud. Figure 2 shows the gradient of blueness from the digital

region of healthy cells to the inter-digital region of apoptotic cells in the left wing bud of the day 9 chick. The spike from 210.1 units of blue to 183 units represents the crossing from the healthy cells to the dead cells. The higher the units of blue represents a lower blue stain (see analysis section of methods).

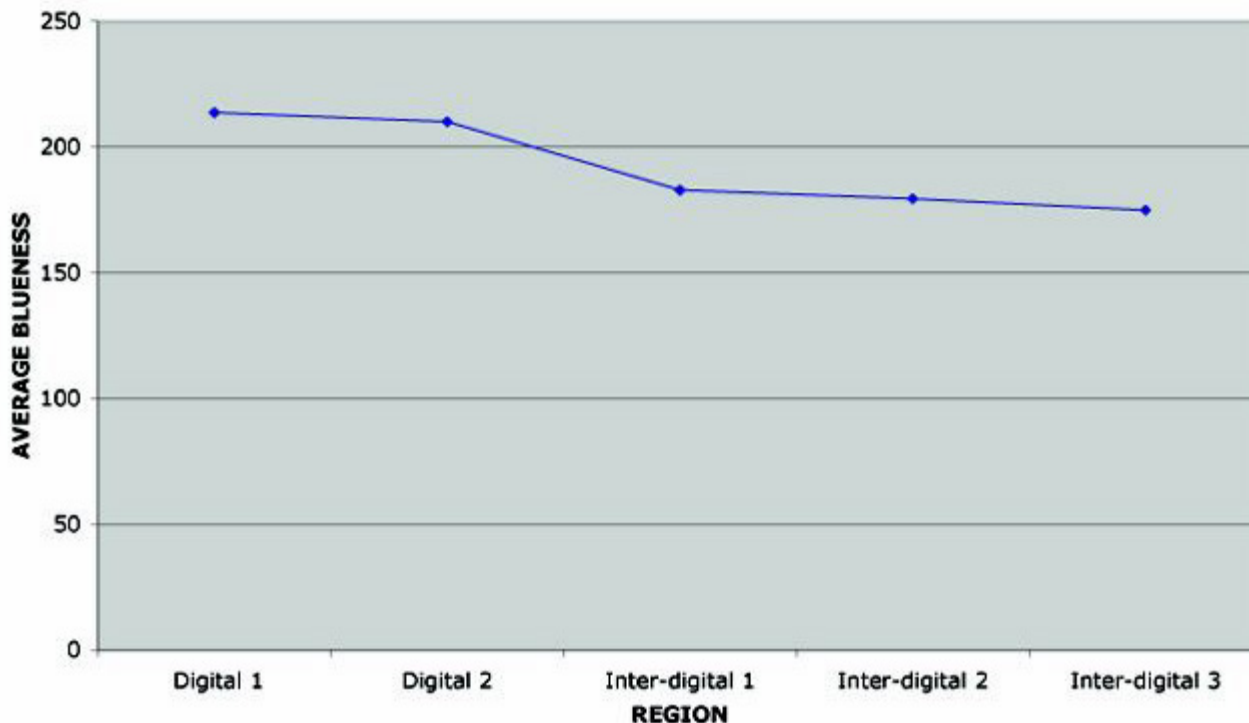
FIGURE 1: APOPTOSIS IN CHICK EMBRYO WING BUD AT DAY 7 OF DEVELOPMENT



This figure shows the apoptosis in the wing bud of an embryonic chick at day 7 of development at 10X magnification on the stereoscope. The dead cells at the wing tip took up the Nile Blue A stain in the beginning stages of digit morphogenesis.

FIGURE 2: Change in Blueness From the Digital Region to the Inter-digital Region

Change in Average Blueness From the Digital to the Inter-digital Region of Day 9 Chick Left Wing Bud



This figure shows the sharp change in blueness from the digital region of the left chick wing bud at day 9 of development to the inter-digital region. The higher unit of blueness represents lesser stain-uptake, so the declining slope is the transition from non-stained healthy cells to apoptotic cells in the inter-digital region.

The day 7 embryo had high apoptosis in the wing tip region with an average blueness of 152.0 in the left wing and 136.7 in the right wing (See Table 1). The non-apoptotic region of the wing buds, or the digital region, had an average blueness of 220.5 in the left wing and 209.9 in the right wing. This is comparatively close to the 255 or no-blue end of the spectrum. On day 9 of embryonic development, the wing buds exhibited cell death in the inter-digital regions with an average blueness of 175.3 in the left wing and 130.1 in the right wing. The area of healthy cells of the wing buds, had an average blueness of 225.6 in the left wing and 189.8 in the right wing.

TABLE 1: Average Blueness for Apoptosis in Developing Chick Wing Buds

	LEFT WING DAY 7	RIGHT WING DAY 7	LEFT WING DAY 9	RIGHT WING DAY 9
AVE. BLUENESS OF INTER-DIGITAL REGION	152.0	136.7	175.3	130.1
AVE. BLUENESS OF DIGITAL REGION	220.5	209.9	225.6	189.8

FIGURE 3: APOPTOSIS IN CHICK EMBRYO WING BUD AT DAY 9 OF DEVELOPMENT

FIGURE 4



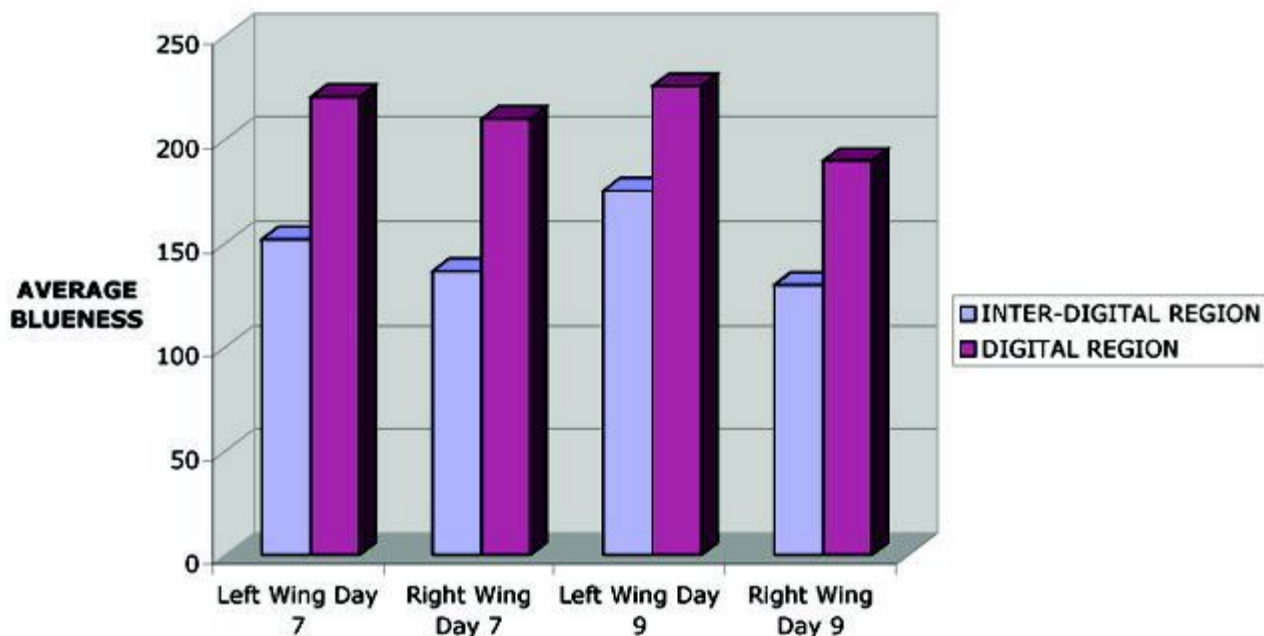
This figure shows the apoptosis in the wing bud of an embryonic chick at day 9 of development at 4X magnification on a Nikon Eclipse E200 scope. The dead cells in the inter-digital region took up the Nile Blue A stain, whereas the digital regions composed of healthy cells did not.

On day 9 the left wing bud had the highest average units for blueness, 175.3, in the inter-digital region meaning that it did not take up as much stain as other apoptotic regions in the other wing buds. The left wing bud also had the highest units for blueness in the digital region, 225.6, because it was the least stained in the area of healthy cells. The right wing bud on day 9 of development displayed opposite qualities. It had the lowest average units for blueness, 130.1, in the inter-digital region meaning that it took up a lot of stain in comparison to other apoptotic regions in the other wing buds. This wing bud also had the lowest units for blueness in the digital region, 189.8, because it took up the most stain in the healthy cells. The range of blueness for the inter-digital regions was 130.1-175.3 and for the digital regions was 189.8-225.6. The day 7 left and right wing buds fell in between both of these ranges, whereas the day 9 wings held the extreme ends of the spectrum.

There is a significant consistency in the lower average blueness (152.0, 136.7, 175.3 and 130.1) of the inter-digital regions in each of the four maturing wing buds and a respective higher average blueness (220.5, 209.9, 225.6 and 189.8) in the digital regions (See Fig 4).

FIGURE 4

AVERAGE BLUENESS OF INTER-DIGITAL AND DIGITAL REGIONS OF EMBRYONIC CHICK WING BUD



This figure compares the average blueness of the inter-digital regions to the digital regions of embryonic chick wings. The high blueness of the digital regions represents failure to take up the Nile Blue A stain, whereas the low blueness of the inter-digital regions represents the dead cells ability to take up the stain.

IV. Discussion and Conclusions

The data was overall conclusive that apoptosis in the embryonic chick wing bud causes digit separation. Nile Blue A, a stain for cell death, showed that cell death progressed from the wing tip to the inter-digital region in the developing wing buds of a 7 day and 9 day chick embryo. The lower units of blueness represent the areas where cells underwent programmed cell death and took up the Nile Blue A stain. The higher numbers were the areas where apoptosis did not occur and so the healthy cells did not take up that stain. On day 7 apoptosis was concentrated in the wing tip and the inter-digital region was not yet defined. On day 9 it was distinguishable where each digit would eventually form. The only source of error was that there were only two testable subjects. If the experiment was to be done again, more chick embryos would have to be successfully explanted to gain harder evidence of apoptosis as a tool for molding digits in the wing bud. The data collected on these day 7 and day 9 subjects were supportive to my hypothesis.

The range of blueness for the inter-digital regions, 130.1-175.3 and for the digital regions, 189.8-225.6, do not represent significant differences. A higher blueness for the digital region means that the healthy cells took up absolutely no stain at all and were thoroughly washed by the saline solution. A lower blueness for the digital region means that the healthy cells still had a little bit of stain left over and this could be due to insufficient washing. A high blueness in the inter-digital region means that the cells took up the stain, but not as much as other apoptotic regions. This occurs in the day 9 chick because the inter-digital regions have undergone apoptosis, but not to its full extent. A lower blueness in the inter-digital region represents high apoptosis. This happened in the right wing of the day 9 chick, which also had the highest stain-uptake in the non-apoptotic region. I do not think that there was more apoptosis in the inter-digital regions, but rather the stain failed to wash away properly.

The variation in quantitative blueness between the apoptotic region and the healthy cells region was greater in day 7 than day 9 means that the dead cells in the wing tips of the day 7 chick were more blue in comparison to the rest of the healthy wing than the apoptotic cells in the inter-digital region of the day 9 embryo. The wing tip region is the first to undergo cell death and had matured to full extent, whereas the cells in between the digits had not all experienced

programmed cell death yet. Had there been a further developed embryo available to be stained, I believe we wouldn't have found this divergence.

V. Bibliography

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