In vivo inhibition of programmed cell death by local administration of FGF-2 and FGF-4 in the interdigital areas of the embryonic chick leg bud

Abstract The formation of the digits in amniote vertebrates is accompanied by a massive degeneration process that accounts for the disappearance of the interdigital mesenchyme. The establishment of these areas of interdigital cell death (INZs) is concomitant with the flattening of the apical ectodermal ridge (AER), but a possible causal relationship between these processes has not been demonstrated. Recent studies have shown that the function of the AER can be substituted for by implantation of beads bearing either FGF-2 or FGF-4 into the apical mesoderm of the early limb bud. According to these observations, if the onset of INZs is triggered by the cessation of the AER function, local administration of FGFs to the interdigital tissue prior to cell death should delay or inhibit interdigit degeneration. In the present study we have confirmed this prediction. Implanting Affi-gel blue or heparin beads pre-absorbed with either FGF-2 or FGF-4 into the interdigital tissue of the chick leg bud in the stages prior to cell death stimulates cell proliferation and causes the formation of webbed digits. Vital staining with neutral red confirmed an intense temporal inhibition of interdigital cell death after FGF treatment. This inhibition of interdigital cell death was not accompanied by modifications in the pattern of expression of Msx-1 or Msx-2 genes, which in normal development display a domain of expression in the interdigital tissue preceding the onset of degeneration.

Key words Apoptosis · Limb morphogenesis · Growth factors

Introduction

Formation of the digits takes place in the distal segment of the limb bud (autopod) in the later stages of limb morphogenesis. In amniote vertebrates, digits appear as diverging chondrogenic rays delimiting interdigital areas in which the mesenchymal cells maintain an undifferentiated state for a considerable period. In subsequent stages this interdigital mesenchyme undergoes massive cell death by apoptosis, creating the interdigital necrotic zones (INZs). Differences in the pattern of cell death between species with free and those with webbed digits, and the absence of interdigital cell death in syndactylous mutants, indicates a sculpturing morphogenetic role for this interdigital cell death (see review by Hurle 1988).

Recently, knowledge of the molecular basis for the patterning of the limb has advanced considerably (reviewed by Tickle and Eichele 1994). Studies suggest that the formation of the digits is controlled by the temporal and spatial expression in the early limb bud of several regulatory genes and signaling molecules (Tickle and Eichele 1994). However, the molecular basis for the formation of the interdigital areas, and their subsequent degeneration, awaits clarification. Although several genes exhibit temporal and spatial domains of expression in the interdigital areas in the stages preceding the onset of cell death, their significance in the establishment of INZs is obscure (Ros et al. 1994). These genes include the homeobox-containing-genes Msx-1 and Msx-2 (Hill et al. 1989; Coelho et al. 1991a; Suzuki et al. 1991), genes related to retinoic acid (Ruberte et al. 1992), and the gene encoding for the bone morphogenetic protein 2 (Lyons et al. 1990; Francis et al. 1994). Early morphological studies showed a temporal correlation between the onset of interdigital cell death and the cessation of the activity of the apical ectodermal ridge (AER; Pautou 1978). This structure is a specialized region of the ectoderm arranged along the distal margin of the limb (Fallon and Kelley 1977); it is responsible for the proximodistal growth of the limb. Experimental removal of the AER arrests limb outgrowth and only the proximal parts of the limb al-
moral was also analyzed. The marginal ectoderm of the third interdigital area was removed with a fine tungsten needle as previously described (Hurle and Gañan 1986) and an Affi-gel blue bead soaked in FGF-2, or in PBS for controls, was implanted in the interdigital mesenchyme subjacent to the wound.

FGF application to beads

Beads with a diameter of 100–200 µm were selected. The beads were washed in PBS and soaked for 1 h at room temperature (20°C) or at 37°C in a solution of 1 µg/µl of recombinant human FGF-2 or FGF-4 (both from R&D Systems). Control beads were incubated in PBS.

Morphological analysis of the limb

The morphology of the limbs was initially analyzed in specimens stained for cartilage. The embryos were sacrificed 3 or 4 days after the implantation of the first bead, fixed either in Bouin’s fluid or 5% trichloroacetic acid, stained with Alcian blue BGX and Alcian green respectively (Hurle and Gañan 1986; Niswander et al. 1993), and cleared in xylene or methyl salicylate. Absence of interdigital tissue regression in these specimens was confirmed by scanning electron microscopy. For this purpose, the cleared specimens were transferred to acetone, dried by the critical-point method and sputter-coated with gold. Observations were made with a Jeol T-100 microscope.

Detection of cell death

The distribution of cell death was analyzed at 6–8 h intervals after the implantation of the first bead by vital staining with neutral red according to the method of Hinchliffe and Ede (1973).

Measurement of cell proliferation

The extent of cell proliferation in the interdigital mesenchyme was analyzed by anti-bromodeoxyuridine immunolabeling at 10-, 15- and 24-h intervals after implantation of the bead. For this purpose 100 µl of bromodeoxyuridine (BrdU) solution (100 µg/µl) was pipetted directly over the limb. After 30 min of further incubation, the embryos were fixed in 70% ethanol. The autopod was then dissected free, dehydrated and embedded in diethylene glycol (Poly-science) or in paraffin wax. Immunocytochemistry to detect BrdU incorporation into DNA was carried out in tissue sections according to the directions of the manufacturers, using anti-BrdU antibody (Becton Dickinson) and a fluorescein-conjugated secondary antibody. Observations were made with a standard fluorescence microscope.

In situ hybridization technique

Hybridization with msx1- (obtained from M. Solursh) msx2- (obtained from A. Kuroiwa) and Sonic Hedgehog (Shh; obtained from J.C. Izpisu-Belmonte) probes was performed at 8 h intervals after the implantation of the first bead, as described in Ros et al. (1994). The limbs were fixed in 4% paraformaldehyde (freshly prepared) in PBS and embedded in paraffin wax. The specimens were serially sectioned (6 µm), and the hybridization was performed in adjacent sections for each of the three probes used. Hybridization was carried out at 50°C for 16 h in 50% deionized formamide, 0.3 M NaCl, 20 nM TRIS (pH 7.4), 5 mM EDTA, 10 mM sodium phosphate (pH 8), 10% dextran sulfate, 1x Denhardt’s solution, 50 g/ml of yeast RNA, with 50,000–75,000 cpm/µl of complementary RNA labeled with 35S-labeled UTP (>1,000 Ci/mmol, Amersham, UK). Washing was at 65°C in 50% formamide, 2xSSC, and 10 mM STT. Slides were then treated with RNase A (20 µg/ml; Sigma) for 30 min at 37°C. After washes, slides were processed for standard autoradiography with Kodak NTB-2 nuclear track emul-

Materials and methods

Experimental manipulation of the chick limbs

Fertile Rhode Islands chick eggs were incubated at 38°C until they reached stages 28–30 (Hamburger and Hamilton 1951). Each egg was windowed and the leg bud was exposed. An Affi-gel blue bead (Bio-Rad) or a heparin acrylic bead (Sigma, H5263) soaked in FGF-2 or FGF-4, or in PBS, was then implanted into the mesenchyme subjacent to the AER of the third interdigital area and the embryo returned to the incubator. In some cases a second bead was implanted 15 h later, with the same procedure. The effect of FGF application to limbs subjected to local interdigital ectoderm re-