Abstract The process of Meckel’s cartilage development was examined with regard to expression of p53, a tumor suppressor gene product and hsp70, a stress protein (heat-shock protein), in association with the occurrence of programmed cell death (apoptosis). Balb C mice embryos from embryonic days E13, E14, E15, E16, E17, E18 and 1- and 3-day-old pups were used. P53-positive cells were detected first at E15, and were found in the perichondrium of the distal part of Meckel’s cartilage. During the degeneration process chondrocytes also became p53-positive. In contrast to p53, the expression of hsp70 was high and widespread in the early stages of development (E13–E15); however, it decreased with age, except for Meckel’s cartilage, where hsp70 was found in the cytoplasm or nuclei of the hypertrophic cells. Apoptosis was first detected at E14–E15 in the perichondrium of the distal parts of Meckel’s cartilage. The number of apoptotic bodies increased with age and the ongoing resorption of Meckel’s cartilage. From the present study it can be concluded that expression of p53 and hsp70 varied during the development of Meckel’s cartilage and that both proteins showed nuclear location in hypertrophic cells. No direct spatial or temporal correlation was observed between the expression of p53 and hsp70 and the occurrence of apoptotic bodies.

Key words Chondrocytes · Apoptosis · Degeneration · Immunohistochemistry

Introduction Meckel’s cartilage is derived from embryonic neural crest cells that migrate to the mandibular arch, where they give rise to the cartilage rod (Chai et al. 1994). The development of Meckel’s cartilage starts with a condensation of the cells to form a precartilaginous blastema. The maturation process advances in the intermediate area of the mandible. Later, the distal parts demonstrate signs of focal degeneration, which is followed by extensive resorption. The proximal part undergoes endochondral ossification and is converted to the bony structures of the middle ear – the maleus and incus (Zschäbitz et al. 1995). The disappearance of the cartilage occurs without any morphologically detectable reactions in the neighboring mesenchyme (Granström et al. 1988). However, the molecular mechanisms and the acting genes regulating the development and the degeneration of Meckel’s cartilage have not yet been identified.

Genes involved in morphogenesis are often associated with control of cell proliferation, cell differentiation and cell death. A tumor suppressor protein, p53, was shown to be related with the programmed cell death, apoptosis, by causing cell cycle arrest in a number of tissues, in different cell types (lymphocytes, fibroblasts). Moreover, p53 plays an important role in controlling the cellular response to DNA-damaging agents and mutation. Mutation of p53 leading to inactivation has been found in several forms of cancer (Lee and Bernstein 1995). Wild type and mutant p53 were also shown to bind to heat-shock protein 70 (hsp70), which also causes a loss of function. The hsp70 or cognate protein (hsc70) is widely expressed during embryogenesis. The main function of hsp is to control protein folding and protein transport between organelles (Agard 1993). Hsp is a molecular chaperone in various aspects of protein maturation, but has also been shown to play a role in cell proliferation, survival and apoptosis in tumor cells (Wei et al. 1994, 1995). As implied by the name, the protein is expressed in response to thermal stress and also after other types of cell injury, e.g., exposure to metals, mechanical forces.
and hormones. Thus both p53 and hsp70 are sometimes connected with apoptosis.

Apoptosis provides a powerful regulatory mechanism for many aspects of normal growth and function of tissues. It is a physiological process that, in contrast to necrosis, is not combined with inflammatory responses (Kerr et al. 1972). The initiation of apoptosis is regulated by different signals, such as cellular damage inflicted by ionizing radiation or viral infection, cell interactions, and hormones (Raff 1992; Raff et al. 1993; Steller and Grether 1994). In the process of apoptosis, the chromosomal DNA is degraded into oligonucleosomal fragments (Roy et al. 1992). The nucleus and the cytoplasmin condense into cell fragments and membrane-bound apoptotic bodies that are rapidly phagocytozed (Steller 1995).

In the present study, we have analyzed the occurrence of apoptotic bodies during the resorption of Meckel’s cartilage. In order to better understand the mechanisms underlying the process of disappearance of this transitory cartilage, we have analyzed the expression of genes known to be connected with the regulation of cell proliferation and death, p53 and hsp70.

Materials and methods

The experiments have been conducted in conformity with the laws and regulations controlling experiments and procedures in live animals, as described in the Principles of Laboratory Animal Care (NIH Publication no. 85–23, revised 1985). BALB C mice embryos from embryonic days E13, E14, E15, E16, E17, E18 and 1- and 3-day-old pups were used. A whole E13 embryo and the heads of E14–E17 embryos were fixed for 4 h in 4% paraformaldehyde (PA)/phosphate buffer (PB) at pH 7.4. After dehydration in graded ethanol the specimens were embedded in paraffin. Serial frontal sections were cut at 6 µm on a conventional microtome.

In addition, heads of E16, E18 and whole Meckel’s cartilages from E15 and E17 were microdissected, fixed in 4% PA and frozen. Longitudinal sections (10 µm) were cut on a cryostat. In general, 2–3 specimens of each developmental stage were sectioned and an average of 10–15 sections per embryo/pup were stained in two independent experiments.

Detection of p53 and hsp70

For detection of p53 and hsp70 a mouse monoclonal antibody to p53 from Oncogene Science (Cambridge, Mass.) and a mouse monoclonal antibody to hsp70 from Sigma (St. Louis, Mo.) were used. The hsp70 antibody detects two forms of hsp, one that is constitutive (heat-shock cognate protein) and one that is heat or stress inducible, hsp70.

The immuno-staining was carried out as follows: the sections were deparaffinized and the endogenous peroxidase activity was blocked by incubation with 0.3% H₂O₂ in methanol for 15–20 min. Frozen sections were post-fixed in methanol/acetic acid (1:1) before incubation with H₂O₂ in methanol. After that, 0.1% Triton/PBS+5% normal swine serum was applied to the sections for 15 min. The primary antibodies were diluted in phosphate buffer (PB) (p53, 1:50 and hsp70, 1:100) and applied to the sections for 45–60 min, at room temperature. The secondary, biotinylated antibodies and the Vectastain Elite-Kit for detection of mouse IgG were used (Vector Labs, Burlingame, Calif.). After incubation with ABC-Horse Radish Peroxidase (HRP), the staining was developed in diaminobenzidine tetrahydrochloride (DAB). In the control sections, the primary antibody was omitted or an irrelevant antibody was used, which resulted in no staining. Some of the sections were counterstained in hematoxylin. The specificity of antibodies was analyzed on Western blots in cultured myoblasts and in paraffin, and cryosections of different tissues. The sections were observed and photographed in the light microscope using 80A and 80B blue filters and Fuji 200 ASA color film or Kodak T64 color reversal film.

Whole Meckel’s cartilages from E15 and E18 embryos were microdissected and were processed for whole mount immunocytochemistry. The same protocol as for the sections was used, except that in some steps of the preparation the incubation times were prolonged. The cartilages were incubated with the blocking serum (0.1% Triton/PBS+5% normal swine serum) for 30 min and with the primary antibodies (p53 or hsp70) 60 min. The incubation with the secondary, biotinylated antibodies and the ABC-reagent was for 60 min in each case. Each PBS wash was for 10–15 min.

Detection of apoptosis

Apoptosis was detected by direct digoxigenin labeling of 3′-OH DNA ends that are generated by DNA fragmentation occurring during cell death. The terminal deoxynucleotidyl transferase (TdT) and Digoxigenin-dUTP from the “Apoptag™” in situ Apoptosis Detection Kit – Peroxidase” were used (Oncor Gaitherburg, Md.). Sections embedded in paraffin, were deparaffinized in xylene and incubated in 2% H₂O₂ in PBS for 5 min. All the steps were done according to manufacturer’s instructions. The reaction product was detected with anti-digoxigenin peroxidase-conjugated antibodies. The peroxidase activity was visualized by immersion of the slides in a solution containing DAB and H₂O₂ for 3–7 min. The reaction was stopped by a rinse in distilled water for 5 min. No staining was detected in controls in which TdT or the anti-digoxigenin antibody were omitted.

In a separate experiment, whole Meckel’s cartilages from E15 and E18 embryos were microdissected, fixed in 4% PA for 1 h and transferred to PBS prior to detection of apoptosis. In the whole mount preparations, the incubation in 0.2% H₂O₂ in PBS was for 5 min. After this, the cartilages were incubated in PBT (PBS+0.1% Triton) for 15 min. Thereafter, they were washed with PBS (3×5 min) and incubated in Equilibration Buffer for 20 min. The labeling time (TdT+reaction buffer) was 90 min at 37° C. The reaction was stopped by incubation with Stop-wash (45 min at 37° C) and afterwards the specimens were rinsed in PBS (3×7.5 min). The anti-digoxigenin-HRP antibodies were applied to the cartilage for 45 min at room temperature. Incubation with DAB was for 5–10 min.

Results

p53 and hsp70

The p53 staining was not detectable in the Meckel’s cartilage at E13 or E14 (Fig. 1A). At E15, in the whole-