Development of the Ectopia Cordis Induced by Hydrocortisone Administration

(ectopia cordis / thorax / development / hydrocortisone)

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Abstract. Our previous study on the development of thorax in chick embryos revealed that mechanical disturbance of the so-called membrana reuniens causes the development of the ectopia cordis (EC). To assess whether membrana reuniens disturbance was really essential for EC development, we employed hydrocortisone, a teratogen known to produce a high incidence of EC. The incidence of EC after the hydrocortisone intraamniotic application on the 4th embryonic day reached 84,8%. It was found that although in the whole course of EC development the membrana reuniens appeared very thin, it nevertheless remained continuous. The morphology of the membrana reuniens in embryos with fully developed EC, studied in classical serial histological sections, was similar to that of the amniotic membrane. Flow cytometry analysis of the cell cycle revealed that EC induced by hydrocortisone administration was associated with a significantly lowered proliferation activity of the prospective body-wall mesenchyme involved in the closure of the anterior wall of thorax. The probable mechanism of EC development is suggested.

When studying the thorax development in the chick embryo (Seichert and Seichertová, 1998), we came to the conclusion that destruction of the so-called membrana reuniens (Rathke, 1838, further MR – see discussion) interfered with closure of the chest wall followed by the appearance of the ectopia cordis (EC). It has been demonstrated that the intact MR is a necessary guiding substrate for the prospective body-wall mesenchyme (PM) migration over the bulging heart loop. In the course of this shifting, PM must perform a small but essential lateral deviation (consider the situation shown in Fig. 1) that is conditioned just by the intact MR. Following disturbance of MR integrity, the medial and cranial shift proceeds normally; however, PM cannot pass laterally over the voluminous heart anlage that becomes gradually displaced in front of the closing body wall. The aim of the present study was to investigate whether the primary MR defect was a prerequisite even for the development of EC of other than mechanical aetiology. We made use of our experience with testing drugs for embryotoxicity, choosing hydrocortisone that in the chick embryos induces a high incidence of EC.

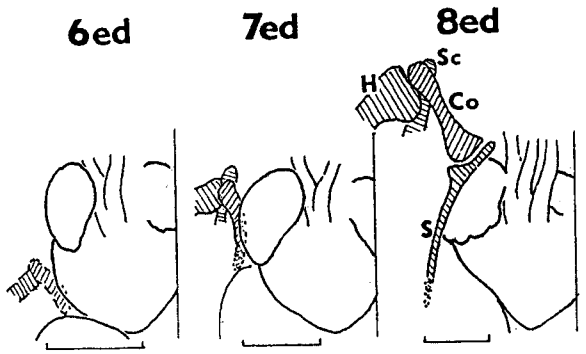

Fig. 1. Topography of the skeletal components of thorax and shoulder relative to the heart in the normal embryos. The medial segment of coracoid and the sternal plate anlagen are, on the 6–7 e.d., slightly covered by the lateral heart margins. Co - coracoid, H – humerus, S - sternal plate, Sc – scapula.

Material and methods

Random-bred Brown Leghorn chick embryos (Dobřenice Farm, Czech Republic), ranging from HH 21 to HH 23 (embryonic day 4), and the standard incubation and window techniques (Jelínek, 1977) were used. EC was induced by single intraamnionic injection of 1.0 μg hydrocortisone (H; Hydrocortisone – Research Institute for Antibiotics, Roztoky u Prahy, Czech Republic) diluted in 3 μl redistilled apyrogenic water. Embryos were reincubated and treated as follows:

Group A: Embryos (46) were sampled on day 10 and examined macroscopically for EC and related malformations prevalence. Thereafter the embryos were fixed for 4–5 days in 70% ethanol, stained with Alcian Blue 8GX (Serva, Heidelberg, Germany) cleared in 1% KOH and

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Abbreviations: EC – ectopia cordis, H – hydrocortisone, MR – membrana reuniens, PM – prospective body-wall mesenchyme.

mounted in glycerol (modified technique of Lundvall, 1927) to study the cartilaginous skeleton morphology.

Group B: Embryos (137) were examined *in ovo* in 12–24 h intervals, and selected specimen were either

Ba: sampled, fixed in Bouin-Hollande fluid, stained by haematoxylin-eosin or with the combination of Alcian Blue, and cut serially into 5–10 μm sections in transversal or sagittal planes, or

Bb: stained with Neutral Red in Ringer saline 1:1000 applied either *in ovo* intraamniotically (on days 5–6) or submerged *ex ovo* supravitally (on days 7–8) for 5 min to detect dead cells in the developing thoracic wall.

Group C: Embryos (10 for each interval) sampled 6, 12 and 24 h after H administration were dissected, and strips of the body wall supposed to contain PM (Fig. 2) were treated for analysis of the cell cycle according to the amount of DNA in the cells (DNA – analysis by flow cytometry). The tissues were dissociated mechanically and enzymatically with 0.1% collagenase-dispase (Sigma, St.Louis, MO) for 2 × 20 min at 36°C. The suspensions of nuclei were treated using a two-step method consisting in one-h incubation of the cells with salt solution containing detergent and RNAase A (Fluka, Buchs, Switzerland) followed by additional treatment with a solution of citric acid and sucrose. Both solutions contained a fluorescent DNA-binding dye, ethidium bromide (Sigma, St.Louis, MO). After staining, the amount of DNA was determined using FACsort cell cytometer (Becton Dickinson IS, San Jose, CA) and the cell-cycle analysis software ModFrit *LT* (Verity, Software House, Topsham, ME). The results were statistically analyzed by both parametric and non-parametric methods (ANOVA with Bonferroni test, and Kruskal-Walis test, respectively), and software STATISTICA (StatSoft, Tulsa, OK).

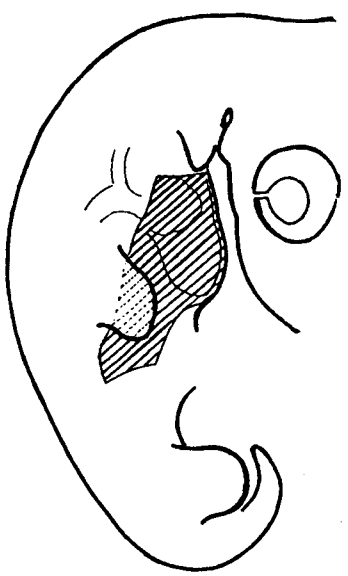

Fig. 2. Schematic drawing showing the range of the body-wall parts sampled for the cell-cycle analysis by flow cytometry (the stippled area).

Results

The incidence of EC in H-treated embryos of all four groups ranged between 87% and 89%. EC was frequently associated with other malformations, most often with the cleft beak (70%), equal to cleft lip (and palate) in mammals. For more details see Table 1, summarizing the results of the experimental group A.

MR remained continuous in the course of the entire observation period. In later stages MR appeared very thin, macroscopically indiscernible from the amniotic membrane (Fig. 3a-c and Fig. 4).

In serial histological sections of embryos with EC, a conspicuously altered transition of the lateral body wall into MR was observed. While in untreated embryos this transition appeared more or less conical, in EC-developing embryos it was found abrupt (Fig. 5a-d). In the latter, at both lateral margins of MR, longitudinal ridges were formed by PM, the medial margins of which were clearly visible even when observed *in ovo*. In between them MR was bulging ventrally (Fig. 3a-c).

Further the development proceeded in several directions:

1. In the first group of embryos the PM ridges fused cranially and in this region the chest was closed (most frequently at the level of differentiating furculae and coracoids – Fig. 3d). At more caudal levels MR appeared further distended and the heart was being displaced into the originating thin-walled sac.

1.1. Even more caudally, the heart displacement was either followed by the liver and other abdominal organs or,

1.2. exceptionally (in two cases), both PM ridges fused below the heart and the umbilicus was formed.

2. In the majority of embryos the PM ridges failed to fuse at all, the thin-walled sac MR replaced the entire front body wall and contained the heart, liver and other abdominal organs (Fig. 3b-c).

In some embryos (about 5%) on days 6–8 we observed a moderately protruding white strip *stained intensively by the Neutral Red* in the ventral midline *(the Group Bb)*. In control specimens the strip always appeared well formed through the whole extent of the closing anterior body wall. In treated embryos, on the contrary, this strip appeared mostly less conspicuous and became limited to the cranial part of the anterior body wall (the embryos mentioned above in group 1 – see Discussion).

The cell-cycle analysis by flow cytometry revealed a significant decrease in the proportion of cells in G2-M phase within treated embryos compared with the controls ($P < 0.01$, Fig. 6). A significant decrease in mitotic cells was encountered 12 h following H administration. In the further 12 h, on the contrary, the proportion of cells in G2-M phase appeared greater, and 48 h after administration normal values were achieved (Fig. 7). The damping of proliferation rate was conditioned by preventing the cells from entering mitosis.