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Retinoic Acid Induces Malformations Related to Cell Death in the Developing Mouse Embryo

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Abstract. Intraperitoneal administration of all-trans retinoic acid (RA) to pregnant mice on 8.5 or 10.5 days post coitum (dpc) resulted in malformed fetuses in all litters in a dose-dependent and developmental stage-specific manner. The pregnant mice were injected with RA dissolved in dimethyl sulfoxide, at a dose of 25 or 50 mg/kg of body weight on 8.5 or 10.5 dpc. In the 8.5 dpc-50 mg/kg treatment group, all fetuses had tail anomalies and vertebral malformations, and some fetuses showed craniofacial anomalies such as exencephaly, spina bifida, micrognathia and agenesis of auricles. Excessive cell death was seen in the tail bud 24 h after RA injection. In the 8.5 dpc-25 mg/kg treatment group, however, fetuses showed palliation of these defects. In the 10.5 dpc-50 mg/kg treatment group, limb disorders were induced, but the tail showed normal morphology. The present results indicate that RA-inducible malformation in mouse embryos is dose-dependent and developmental stage-specific, and these teratogenicity may correlate with excessive cell death. RA is considered to affect rapidly growing parts of embryonic tissues and causes disorders in normal pattern formation of embryonic tissues.

Key words: Retinoic acid, Mouse embryo, Cell death, Malformation, Tail and limb buds.

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Vitamin A is required for several critical life processes, including reproduction, vision, maintenance of tissues and overall survival. All-trans retinoic acid (3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid; RA) is an active vitamin A derivative, since RA administration can prevent most of the defects generated by a vitamin A-deficient diet [1]. On the other hand, maternal administration of excess RA can induce morphological abnormalities in mammalian embryos such as exencephaly, micrognathia, agnathia, cleft palate, spina bifida, tail anomalies, axial skeletal anomalies etc. [2–5]. RA is considered to be a signaling molecule involved in pattern formation, and may provide information for understanding the mechanism of pattern formation in mammalian embryos.

The discovery of retinoic acid receptor (RAR) and retinoid X receptor (RXR), both of which belong to the nuclear steroid/thyroid hormone receptor superfamily, has advanced our understanding of the mechanism of several responses to RA [6, 7]. These receptors each have a DNA-binding domain and ligand-binding domain, so that RA is thought to exert its effects by modifying the transcriptional activity of specific genes through binding to their receptors. RXRs are activated by 9-cis retinoic acid, a isomer of all-trans retinoic acid, and form heterodimers with several other nuclear receptors including RARs [8, 9]. Therefore, retinoids may support some physiological functions of hormones. Since these discoveries, several lines of RAR-knock out mice have been generated in order

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to analyze their genetic functions.

In the present study, we focused on tail and limb anomalies in mouse embryos after maternal administration of RA. Differences in teratogenic patterns in mouse embryos between the RA doses and the developmental stages of the embryos at RA treatment were compared. We also observed the dynamics of cell death in embryos after RA administration to clarify the mechanisms underlying the morphological anomalies induced by RA treatment.

Materials and Methods

Animals and chemicals

Female jcl:ICR mice aged 6 weeks in each treatment group were purchased from Clea Japan (Tokyo, Japan), and housed under controlled conditions (lights on between 7:00 and 19:00; temperature 22 ± 2 C; humidity 70 ± 5%). The mice were mated with mature male jcl:ICR mice (Clea Japan) overnight. At noon of the following day, mice with a vaginal plug were considered as 0.5 day post coitus (dpc). All-trans retinoic acid (Sigma Chemicals, St. Lewis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) at concentrations of 7.5 and 15 mg/ml for 25 and 50 mg/kg of body weight administration groups, respectively. These RA solutions were intraperitonealy administered to the pregnant mice at 8.5 or 10.5 dpc. All RA treatments were performed under dark yellow light. In each experimental group, six maternal mice were examined.

Morphology and histology

At 9.5, 10.5, 13.5 and 18.5 dpc, pregnant mice were sacrificed under ether anesthesia, and their litters were collected from the uterus into phosphate buffered saline (PBS). Embryos were observed and photographed under a dissection microscope (Olympus, Tokyo, Japan), and then their tail length was measured.

For morphological evaluation of the embryonic skeleton, skeletal samples of 18.5 dpc embryos were prepared by conventional alcian blue and alizarin red procedure, which stains cartilage blue and ossified tissue red. Briefly, 18.5 dpc embryos were fixed in 80% ethanol for one week at room temperature (RT), skin and organs were removed under a dissection microscope, and dehydrated in 96% ethanol for one day and acetone for 3 days. They were stained for a day at 37 C in staining solution consisting 1 volume of 0.3% alcian blue 8GX (Chroma-Gesellscaft Scmid, Köngen, Germany) in 95% ethanol, 1 volume of alizarin red S (Wako Pure Chemical, Osaka, Japan) in 95% ethanol, 1 volume of acetic acid anhydride and 97 volumes of ethanol. The specimens were rinsed in 96% ethanol for 1 h at RT, and kept in 1% KOH solution for 2 days for clearing. Then, they were transferred into graded glycerol (20, 50 and 80%) in 1% KOH one week for each step. Finally, specimens were transferred into 100% glycerol for storage, and observed under a dissection microscope.

For histological evaluation, 13.5 dpc embryos collected as described above were fixed with 20% (v/v) neutral-buffered formalin in 0.1 M phosphate buffer (pH 7.4) for 7 days at RT, dehydrated through graded ethanol, and embedded in paraffin. Whole embryo sections, 5 μm thick, were deparaffinized in xylene, rehydrated through graded ethanol, and then stained with hematoxylin and eosin (HE).

For detection of cell death, 9.5 dpc and 10.5 dpc embryos after administration at 8.5 dpc were collected as described above. The whole embryo specimens were stained with Nile blue sulfate (NBS; Chroma-Gesellscaft Scmid) vital staining procedure to detect cell death. Briefly, whole embryos were immediately transferred into NBS staining solution containing 0.005% (w/v) NBS in lactated Ringer's solution for 30 min at 37 C, and then examined and photographed using a dissection microscope.

Statistical analysis

Analysis of variance (ANOVA) was carried out with the StatView IV program (Abacus Concepts, Berkeley, CA, USA) using a Macintosh computer. Differences with a probability of $P<0.05$ were considered to be statistically significant. Results are expressed as means ± SD.

Results

Dose-dependency of malformations induced by RA

In the 8.5 dpc-50 mg/kg group, malformations such as transformation of the craniofacial or trunk region were observed, e.g. no tail (100%), disap-

pearance of auricles (30.8 ± 8.8%) and exencephaly (11.5 ± 4.2%), on the neonates with good reproducibility. Particularly, none of the embryos had a intact tail. Further, vertebral skeletons posterior to the lumbal vertebrae were seriously malformed, and additional or fused ribs and deformed skulls were also observed (Fig. 1). The posterior neural tube seems to be folded inside the deformed tail upheaval (Fig. 2). In the 8.5 dpc-25 mg/kg group, such malformations were less marked. However, many embryos had a shrunken and/or truncated tail. In the vehicle control group, no malformations were seen (Fig. 1).

Tail lengths of embryos in each group are shown in Fig. 3. In the 8.5 dpc treatment groups, the values of 0 (vehicle control), 25 and 50 mg/kg groups were 12.4 ± 1.2, 6.8 ± 1.8 and 0.0 ± 0.0 mm, respectively. No significant difference in tail length was observed between vehicle control (12.3 ± 1.6 mm) and 10.5 dpc RA-treated groups (9.9 ± 1.9 and 9.7 ± 2.2 mm in 25 and 50 mg/kg groups, respectively).

Stage-dependency of malformations induced by RA

In the 10.5 dpc-50 mg/kg group, truncated forelimbs and bent hind limbs were observed, but the

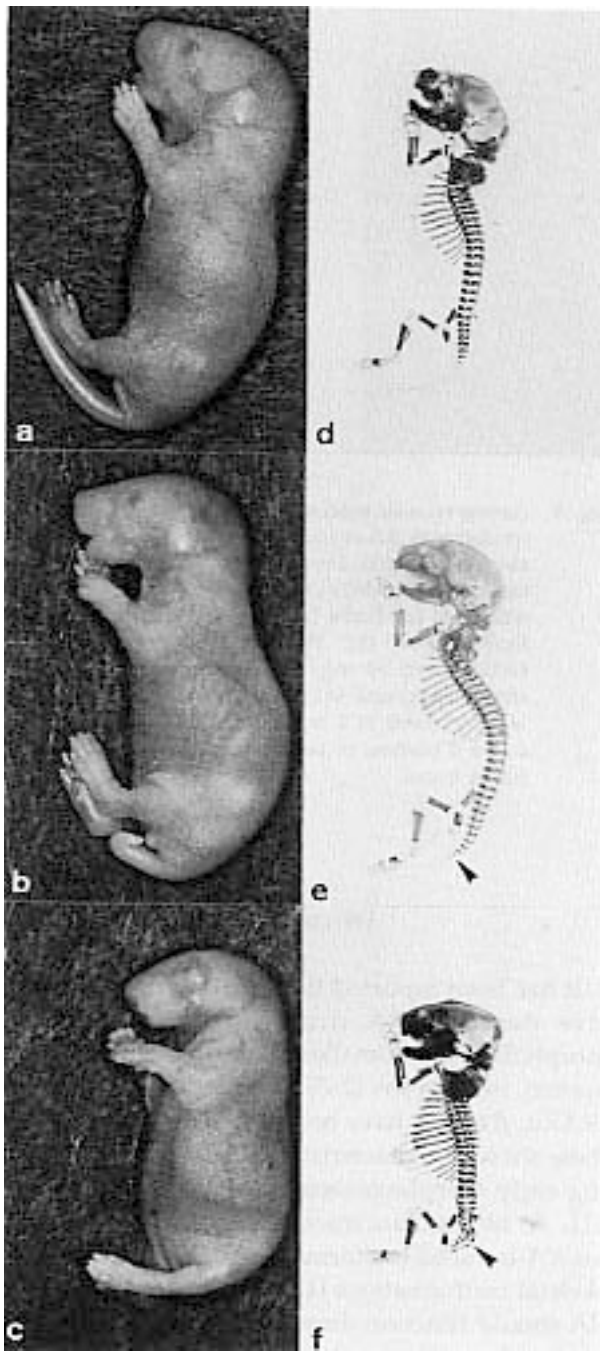

Fig. 1. Appearance of 18.5 dpc embryo following maternal administration of RA at 8.5 dpc (a–c) and their skeletal samples (d–f). Vehicle control embryo showed a normal appearance (a), but 25 mg/kg RA-treated embryo had a truncated tail (b). The embryo treated with 50 mg/kg had no tail, and micrognathia and agenesis of auricles were observed (c). In comparison with the control skeletal specimen (d), the 25 mg/kg-treated embryo had additional ribs and truncated posterior vertebrae (arrow head) (e), and the 50 mg/kg-treated embryo showed disrupted lumbar and sacral vertebrae (arrow head), additional ribs and a malformed cranium (f).

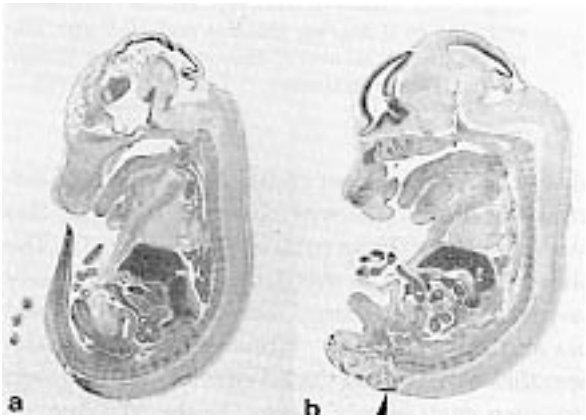

Fig. 2. Sagittal sections of 13.5 dpc embryo. Vehicle control (a) and 50 mg/kg RA-treated embryos (b). The bent posterior neural tube is folded up in the upheaval region in the RA-treated embryo (arrow head) (b).

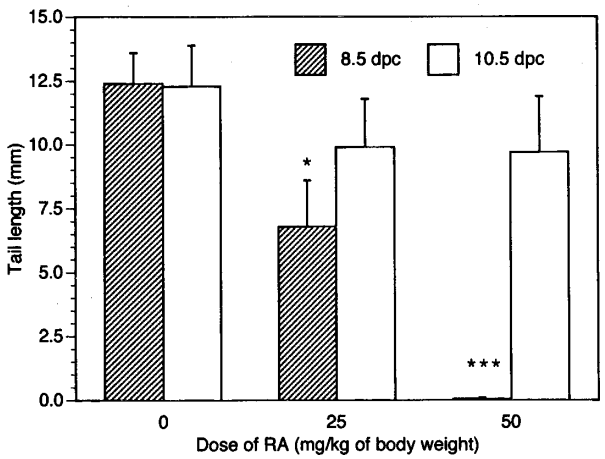

Fig. 3. RA dose and tail length of newborn mouse embryos following maternal treatment with RA on 8.5 and 10.5 dpc. Tail length in the 8.5 dpc RA-treated group (62, 58, and 60 embryos in 0, 25 and 50 mg/kg groups, respectively) was significantly shorter with increasing RA dose. No significant difference in tail length was observed between vehicle control (63 embryos in 0 mg/kg groups) and 10.5 dpc RA-treated groups (61 and 57 embryos in 25 and 50 mg/kg groups, respectively). * $P<0.05$, *** $P<0.001$.

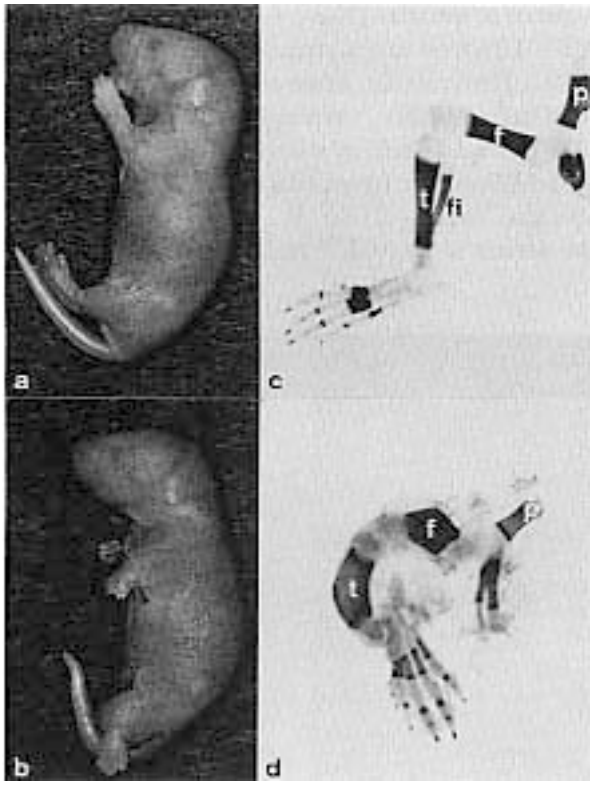

Fig. 4. Appearance of murine 18.5 dpc embryo maternally treated with RA at 10.5 dpc. Vehicle control embryo showed normal development (a). Embryo with maternal administration of 50 mg/kg RA had truncated forelimbs (arrow dead) and twisted hind limbs (arrow) (b). Hind limb skeletal samples of vehicle and 50 mg/kg RA-treated embryos are shown in (c) and (d), respectively. No fibula bone was observed in 0 mg/kg RA-treated embryo. In c and d photos, p: pelvis, f: femur, t: tibia, and fi: fibula bones.

tail was normal in most cases. No significant difference in tail length was observed between this group and the vehicle control group (Fig. 3). The bones of the limbs in this group were completely disordered, i.e. truncated and bent femur, fused tibia and fibula (Fig. 4). None of the craniofacial anomalies observed in the 8.5 dpc-50 mg/kg group were seen even in this group. In the 10.5 dpc, no malformations were observed in the embryos with administration of 25 mg/kg RA, and there was no significant difference in tail length between RA treatment group and control group (Fig. 3).

Cell death in the tail bud

Using the NBS staining technique, we visualized apoptotic cell death in the embryos. Excessive cell death was seen in the tail buds of 9.5 dpc embryos treated with 50 mg/kg RA on 8.5 dpc as compared with the control group (Fig. 5). The 10.5 dpc embryos treated with 50 mg/kg RA on 8.5 dpc had disrupted tails. Though in this period, no excessive cell death was detected in the tail (Fig. 5). Equally, in the 11.5 dpc embryos treated with 50 mg/kg RA on 10.5 dpc, excessive cell death was seen in the limb bud mesenchyme.

Discussion

It has been reported that treatment with excessive doses of RA during gestation induces morphological anomalies, mainly parietal malformation, in embryos [2–5]. Three subtypes of RAR (RARα, β and γ) have been identified, and each of these shows a characteristic expression pattern during early morphogenesis in mouse embryos [10, 11]. As null mutant mice to RARγ show resistance for RA-induced malformations such as embryonic skeletal malformations [12], so it is considered that RA should function through RAR which is one of nuclear transcriptional factors.

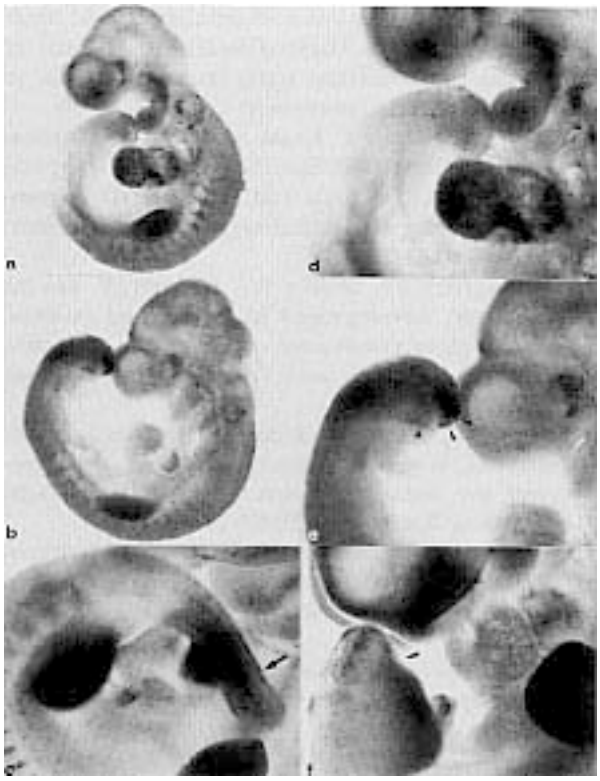

Fig. 5. Cell death in the tail bud detected with Nile blue sulfate staining in 9.5 and 10.5 dpc mouse embryos following RA treatment on 8.5 dpc. In comparison with 9.5 dpc vehicle control embryos (a, enlargement in d), excessive cell death (dark stained part: arrow heads in e) occurred in the tail bud in RA-treated embryos (b, enlargement in e). In the 10.5 dpc groups, vehicle controls (c) had an elongated tail (arrow), while RA-treated embryos had no tail (large arrow head) (f).

The present results showed that the malformations in the tail and limbs induced by maternal administration of RA is developmental stage-specific. Early in development (8.5 dpc), RA treatment induced truncation of the tail in a dose-dependent manner, but no such malformation was observed with treatment later stage (10.5 dpc). In contrast, no morphogenic effect of RA on limb formation was observed in the early stage embryos, but malformations of limbs induced by RA administration were observed in the later stage embryos (Figs. 1 and 4). These results indicate that embryonic limb and tail buds have stage-specific RA-sensitive periods, these results agree with previous reports [2–5, 13–15]. In the present study, we showed exogenous RA inhibits normal cell growth in embryos and induces excessive cell death in the tail and limb buds, so tail and limb defects may correlate with cell death. It is considered that the tail bud grows rapidly early in development while the limb buds proliferate at a later stage, therefore maternal treatment with RA may affects proliferating cells in the tail and limb buds. Such developmental stage-specificity of limb malformations induced by exogenous RA administration has also been observed in human embryos with maternal administration of thalidomide [16] and cyclophosphamide [17]. In human embryos with limb malformations induced by cyclophosphamide, excessive cell death in the alar plate was detected, similarly to the present results induced by maternal RA treatment [17]. It is supposed that the fundamental mechanisms responsible for such drug-induced teratogenicity should be the same as those of RA-induced malformation. Maternal administration of exogenous RA is a good animal model for studying human teratology, and the physiological functions and metabolism of teratogenic drugs should be examined using this model.

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