Prevention of retinoic acid-induced early craniofacial abnormalities by folinic acid and expression of endothelin-1/dHAND in the branchial arches in mouse

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Prevention of retinoic acid-induced craniofacial abnormalities by folinic acid, and endothelin-1 (ET-1)/dHAND protein and mRNA expression were investigated in mouse embryos using the whole embryo culture, streptavidin–biotin peroxidase complex method, and whole-mount *in situ* hybridization. In the whole embryo culture, 1·0 and 0·1 mM-folinic acid dose dependently prevented branchial region malformations and decreased defects by 93 % and 77 %, respectively. Folinic acid at concentrations of 1·0 and 0·1 mM significantly increased ET-1 and dHAND protein expression levels compared to retinoic acid-exposed values in embryonic branchial areas. Folinic acid also increased ET-1 and dHAND mRNA levels in the same region. The present results suggest that folinic acid may prevent retinoic acid-induced craniofacial abnormalities via increasing ET-1 and dHAND levels in the branchial region during the organogenic period.

Branchial arch: dHAND: Endothelin-1: Folinic acid: Whole embryo culture

Retinoic acid (RA) plays an important role in vertebrate embryonic development, regulating morphogenesis, cell proliferation, differentiation and extracellular matrix production (Maden, 2001; Clagett-Dame & Deluca, 2002; Tahayato *et al.* 2003). It is well known that RA acts by binding either of two nuclear receptor families: RA receptors or retinoid 'X' receptors (Bonilla *et al.* 2000; Husson *et al.* 2003). However, when administered at high doses during the first trimester of pregnancy, RA acts as a potent teratogen that causes severe birth defects. These defects include malformations of the central nervous system, heart, inner and external ear, limbs and craniofacial primordia (Tsuiki & Kishi, 1999; Frenz & Liu, 2000; Yu *et al.* 2005). One commonly studied defect produced by excessive RA is craniofacial abnormalities. Craniofacial abnormalities may include one or more of the following: mid-facial hypoplasia, a short nose with anteverted nostrils, a broad depressed nasal bridge leading to redundant inner canthal skin folds, a long phitrum ending in a bowed upper lip, and cleft lip and/or cleft palate (Gelineau-Vanwaes *et al.* 1999). The pathogenesis of retinoid-induced craniofacial anomalies is known to differ with the developmental stage of the embryo at the time of RA exposure. It is well known that the developing hindbrain and branchial region are particularly sensitive target tissues to RA based on ample experimental and clinical evidence (Zhu *et al.* 1997; MacLean *et al.* 2001; Tahayato *et al.* 2003; Menegola *et al.* 2004).

The branchial arch system is a transient segmental structure that is conserved in all vertebrates and mainly contributes to craniofacial development during embryogenesis (Noden, 1998; Ozeki *et al.* 2004). Each branchial arch is covered by ectoderm- and endoderm-derived epithelium and is populated by cranial neural crest- and paraxial mesoderm-derived mesenchyme (Chang & Hemmati-Brivanlou, 1998). During neural tube closure, cranial neural crest cells migrate ventrolaterally from the hindbrain and caudal midbrain in distinct streams and populate corresponding branchial arch segments, where they surround a central core of mesoderm (Fukuhara *et al.* 2004). Neural crest cells originated from rhombomere (r) 1/r2 and r4 mainly contribute to the first (BA1) and second (BA2) branchial arches, respectively. BA1 gives rise to the lower jaw and part of the upper jaw, as well as to the lateral part of the skull and some of the components of the middle ear. In mouse embryos, BA1 first becomes visible on gestational day (GD) 9·0, just as the cranial neural crest cells migrate into and condense within the arch. This neural crest-derived mesenchyme, termed ectomesenchyme, interacts with epithelial and mesodermal cell populations within the arch and differentiates into cartilaginous (chondrocranial) and osseous (dermatocranial) structures (Kontges & Lumsden, 1996; Noden, 1998). The outgrowing BA1 develops into the mandibular and maxillary arches, which in turn contribute to the formation of the lower and upper jaws, respectively.

The molecular basis for tissue organization relies on epitheliomesenchymal interactions, which involve many different factors in a dynamic spatio-temporal cascade. Initial patterning of the mandibular arch involves epithelial signalling factors that include endothelin-1 (ET-1), dHAND, bone morphogenic protein-4 and fibroblast growth factor, as well as regionalized expression of homeobox genes, such as *Dlx*, *Msx*, *Barx1* and *Pax9*.

ET-1, a 21-amino acid peptide, exerts a variety of biological effects including vasoconstriction and cell proliferation via the G protein-coupled endothelin receptors type A (ETAR; Kurihara *et al.* 1999; Yokota *et al.* 2001). Gene targeting experiments have shown that ET-1/ETAR signalling is necessary for the development of cranial neural crest-derived structures including BA1 (Kurihara *et al.* 1994; Clouthier *et al.* 2000; Fukuhara *et al.* 2004; Ozeki *et al.* 2004).

Subsequent studies have revealed that several transcription factors appear to serve as downstream targets for ET-1/ETAR signalling in BA1. For example, it has been shown that expression of *dHAND* and *eHAND*, members of the basic helix–loop–helix protein family, in branchial arch mesenchyme is significantly downregulated in *ET-1$^{-/-}$* or *ETAR$^{-/-}$* embryos (Thomas *et al.* 1998; Ozeki *et al.* 2004). Specifically, disruption of *dHAND* leads to severe craniofacial and cardiovascular abnormalities in mice. RA can suppress ET-1 gene expression at the transcription level in endothelial cells (Yokota *et al.* 2001). However, reports concerning the relationship between RA and dHAND are unavailable.

Studies have shown that folic acid (FA) supplementation can decrease the degree and occurrence of neural tube defects (NTD) in genetically predisposed and teratogen-induced mouse models (Scott *et al.* 1994; Sadler *et al.* 2002). FA can prevent orofacial clefts in human subjects (Andrew *et al.* 1999). Folinic acid, a metabolite of FA, has also been shown to alleviate cleft palate frequencies in genetically susceptible mice (Paros & Beck, 1999). FA and methionine can lessen the occurrence of RA-induced cleft palate in mice (Reynolds *et al.* 2003). However, reports on the effect of folinic acid on branchial regions were not found. Furthermore, the mechanism by which folinic acid prevents RA-induced abnormalities needs further research. The present study was undertaken to expand upon previous studies by: (1) identifying the potential of prenatally administered folinic acid in reducing the incidence of RA-induced craniofacial anomalies in mice by using a whole embryo culture system on GD8·5 *in vitro*; and (2) investigating the mechanisms by which folinic acid reduces malformations. The overall goal of this research was to expand our understanding of the role played by nutrient supplementation in the prevention of birth defects in man.

Materials and methods

Experimental animals

Mice from the Institute of Cancer Research were used. Each male was caged with two nulliparous females overnight. Pregnancy was confirmed the following morning by the presence of a vaginal plug and this was considered as GD0. On GD8·5, pregnant mice were killed by cervical dislocation and uteri were removed to allow preparation of embryos for culture. The females would be considered 0·5 d pregnant at

noon on that day, since copulation was assumed to have occurred within 2 h either side of midnight. The use of animals was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Mice were housed under controlled conditions of temperature (23 ± 1°C), humidity (40–60 %) and lighting (12 h light–12 h dark cycle) and fed *ad libitum* solid diet and tap water.

Mouse whole embryo culture

The culture medium was rat serum (from healthy adult Sprague–Dawley rats weighing 250–280 g), which was immediately centrifuged, heat-inactivated (56°C for 30 min), filter-sterilized and supplemented with 100 IU/ml penicillin G (Sigma, St Louis, MO, USA) and 100 μg/ml streptomycin (Invitrogen Life Technologies, Beijing, China).

Embryos were explanted according to the method developed by New (1978) and adapted by Van *et al.* (1990). GD8·5 embryos displaying three to five pairs of somites were selected for culture. After removal of decidua and Reichert's membrane, those with an intact visceral yolk sac and ectoplacental cone were placed randomly in sealed culture bottles (three embryos per bottle) containing 3 ml culture medium. Cultures were incubated at 37°C and bottles were rotated at 40 rpm. The culture bottles were initially gassed for 2·5 min with a mixture of 5 %O_2–5 %CO_2–90 %N_2. Subsequent gassing for 2·5 min duration occurred at 16 h (20 %O_2–5 %CO_2–75 %N_2) and 26 h (40 %O_2–5 %CO_2–55 %N_2). All embryonic mouse cultures were terminated at 48 h and the embryos were evaluated for viability by the presence of a yolk sac circulation and heart beat. The embryos were examined using a dissecting microscope and specifically processed for the different purposes. Viable embryos were assessed for development by the morphological scoring system developed by Van *et al.* (1990). Reduction of BA1 and BA2 are determined as abnormalities, so the amount of branchial arch and fusion between BA1 and BA2 were recorded for malformation analysis. The scoring index for branchial arches in Van's morphological scoring system is the amount of branchial arches, the present study added one index of mandibular length based on Van's system. On GD10, the outgrowing BA1 develops into the mandibular and maxillary arches, so mandubular length measured using a microscope is regarded as the length of BA1.

Treatments

RA powder (Sigma) was dissolved in dimethyl sulphoxide and added to culture medium at time 0. Folinic acid (Sigma) was dissolved in Hank's buffer and added to culture medium at time 0 at different concentrations. Control cultures received dimethyl sulphoxide (0·1 %, v/v) only. The dose of RA was referred to our previous laboratory work (unpublished results). We selected five doses for RA (0·01, 0·1, 0·4, 1·0, 10·0 μM) in our previous study researching teratogenicity of RA with a whole embryo culture model, and we found that RA at a dose of 0·4 μM could result in abnormal development of mice embryos but not death, which was consistent with the literature. As for folinic acid, the doses used in the present study

were referred to the study of Sadler *et al.* (2002). Thus, there were one control (rat serum + dimethyl sulphoxide) and four experimental groups: (1) embryos exposed to RA only, at a concentration of 0·4 μM; (2) embryos exposed to 0·4 μM-RA and 1·0, 0·1 and 0·01 mM-folinic acid, respectively.

Immunohistochemistry

After being cultured for 48 h, the first branchial arch explants were dissected and fixed in 4 % paraformaldehyde, dehydrated by ethanol series, embedded in paraffin and serially sectioned (5 μm thick) in the horizontal plane. Deparaffinized and rehydrated sections from control, RA-treated only, and RA with 0·1 mM-folinic acid-treated embryos were treated with the Histostain-Streptavidin Peroxidase Kit according to specifications (Zymed Laboratories, San Francisco, CA, USA). The sections were soaked in 3 % H_2O_2 to block endogenous peroxidase. Sections were incubated with normal serum and then incubated at 4°C overnight with the primary antibody in a humidified chamber. The antibodies used were as follows: rabbit polyclonal anti-ET-1 (Phoenix Pharmaceuticals Inc., Phoenix, AZ, USA) was used at a dilution of 1:500, and the dHAND antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used at a 1:100 dilution. After incubation with the primary antibody, sections were incubated for 15 min with biotinylated goat anti-rabbit Ig G, and finally with streptavidin–horseradish peroxidase complex for 15 min at 37°C. The peroxidase reaction was visualized with diaminobenzidine buffer. All sections were countered with dilute haematoxylin to differentiate the nuclei. Finally, sections were washed, mounted, dried, dehydrated, cleared and sealed. We substituted PBS (pH 7·4) for the primary antibody to provide the negative controls; all negative controls showed no immunostaining.

Antigen–antibody complex localization was qualitatively evaluated by the density of the peroxidase reaction product, with relative intensity rated on a scale of 1−4 + . Evaluations were performed on ten sections from four embryos per treatment group. Representative sections were photographed.

Whole-mount in situ hybridization

Whole-mount fixation and *in situ* hybridization were performed according to the procedure described by Wilkinson (1992). Embryos per group cultured for 48 h were fixed for 2 h in 4 % paraformaldehyde in PBS. After dehydration–rehydration with methanol, the embryos were bleached for 1 h in 6 % H_2O_2 and then washed again in phosphate-buffered saline-Tween 20 three times. The samples were treated with 10 mg/ml proteinase K for 20 min, washed with 2 mg/ml glycine in phosphate-buffered saline-Tween 20, and post-fixed in 4 % paraformaldehyde and 0·2 % glutaraldehyde for 20 min. After this pretreatment, the samples were pre-hybridized for 1 h at 70°C in 50 % formamide, 5 × SSC (1 × SSC is 0·15 M-NaCl plus 0·015 M-sodium citrate), 1 % SDS, 50 mg/ml heparin and 50 mg/ml yeast tRNA. With a 1 mg/ml digoxygenin-labelled RNA probe in the hybridization buffer, the samples were hybridized overnight at 70°C. They were then washed three times in 50 % formamide and 5 × SSC for 30 min at 70°C, then in 0·5 M-NaCl, 10 mM-Tris-HCl (pH 7·5), 0·1 % Tween-20 for 5 min and treated with 100 mg/ml RNase for 30 min. After a final wash in 50 % formamide,

$2 \times SSC$ for 1 h at 65°C, the samples were pre-blocked with sheep serum, incubated with alkaline phosphatase-conjugated anti-digoxygenin antibody and stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate. Riboprobes for ET-1 and dHAND were prepared from plasmids containing each cDNA fragment obtained by RT–PCR. For each probe, four embryos per group were analysed.

Statistics

All statistical analyses were performed with the Statistical Package for Social Sciences for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). Effects on embryonic growth between groups were evaluated using an ANOVA followed by a least significant difference test as a *post hoc* test or Dunnett's T3 test. To evaluate whether RA and folinic acid supplementation had a significant effect on the frequency of craniofacial abnormalities, differences between all groups were compared using Fisher's exact test. For all statistical tests $P < 0.05$ was considered significant.

Results

Morphology

At the end of 48 h of culture, all embryos were examined. Data on the effects of folinic acid on RA-induced malformations of morphological features in brain areas and craniofacial areas are given in Table 1.

Embryos cultured in control medium grew normally and showed no malformations, as has been described previously for this stage of development (Liu *et al.* 2005). During this time in culture, early somite-stage embryos completed closure of the cranial neural folds, and established a rapid heartbeat with circulation in the visceral yolk sac and embryo. The otic vesicles were formed, three branchial arches were visible and forelimb buds appeared. However, 100 % of embryos which were exposed to RA at a concentration of 0·4 μM for 48 h produced malformations. Malformations consisted of: (1) cranial NTD in which the neural folds had failed to fuse in the midline in one or more regions of the forebrain, midbrain and hindbrain; (2) branchial arch abnormalities in which the amount of branchial arches decreased and mandible failed to fuse (Fig.1). Supplementation of folinic acid at doses of 1·0 and 0·1 mM containing medium with RA reduced the severity of frontonasal hypoplasia and decreased the incidence of NTD and craniofacial anomalies to 8·6 % and 22·7 %, respectively. But folinic acid at a concentration of 0·01 mM showed no reduction of NTD and craniofacial anomalies (Tables 1 and 2).

Effect of folinic acid on the expression of endogenous endothelin-1 and dHAND protein in the branchial arch

Endothelin-1. To investigate the mechanism through which folinic acid may act on craniofacial abnormalities induced by RA, we performed immunohistochemistry on adjacent sections through the first branchial arch *in vitro*. At embryonic age GD10·5, which corresponds to the fusion of the branchial arch to form the mandibular prominence (Menegola *et al.* 2004), the distribution of endogenous ET-1 and dHAND was examined in the branchial arch of RA-exposed mouse

Table 1. Effect of folinic acid on retinoic acid (RA)-induced malformations of brain and craniofacial region of 10·5 d old mouse embryos‡

(Mean values and standard deviations)

| | Control (n 25) | | RA (0·4 μM) (n 23) | | RA (0·4 μM) + folinic acid (mM) | | | | | |
| | | | | | 1·0 (n 23) | | 0·1 (n 22) | | 0·01 (n 20) | |
Parameter	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Forebrain score	4·96	0·20	3·56	1·23*	4·61†	0·72	4·68†	0·47	4·05	1·05
Midbrain score	5·00	0·00	3·56	1·23*	4·60†	0·83	4·54†	0·91	4·10	1·07
Hindbrain score	4·96	0·20	3·60	1·46*	4·65†	0·71	4·68†	0·56	4·20	1·10
Otic system score	4·80	0·40	2·21	0·12*	4·21†	1·04	4·18†	1·18	3·25	1·55
Branchial arch score	3·00	0·0	1·86	0·81*	2·86†	0·45	2·72†	0·55	2·25	0·78
Mandibular score	2·80	0·40	1·47	0·66*	2·17†	0·65	2·36†	0·72	1·80	0·83
Mandibular length (mm)	0·37	0·06	0·21	0·07*	0·32†	0·04	0·29†	0·10	0·25	0·08

Mean values were significantly different from those of the control group (one-way ANOVA): $*P < 0.05$.
Mean values were significantly different from those of the RA-treated group (one-way ANOVA): $†P < 0.05$.
‡ For details of procedures, see pp. 419–420.

embryos and folinic acid-added mouse embryos, and compared to control embryos of the same developmental stages. Control specimens of GD10·5 embryos exhibited dense immunolabelling (4 +) for ET-1 over the cytoplasm of the mesenchyme and epithelium surrounding the developing branchial arch (Fig. 2(A)). By contrast, in RA-exposed embryos, the mesenchyme surrounding the developing GD10·5 branchial arch, which was reduced in size, was only lightly labelled (1 +) for the presence of ET-1 (Fig. 2(B)). However, in folinic acid-added specimens, a moderate staining deposition (3 +) localized over the mesenchyme and epithelium for the presence of ET-1 (Fig. 2(C)).

dHAND. The transcription factor dHAND showed a similar tissue and cellular pattern of expression when compared to ET-1. As indicated in Fig. 2(E), RA decreased protein expression of dHAND (1 +) (Fig. 2(E)). In folinic acid-added embryos, this factor increased to normal level (4 +) (Fig. 2(F)).

Expression level of endogenous endothelin-1 and dHAND mRNA in the branchial arch

Previous reports have demonstrated that RA can suppress *ET-1* gene expression in endothelial cells (Yokota *et al.*

2001). However, the precise region of *ET-1* expression affected by RA in the branchial region has not been well characterized. Also, it remains unknown whether the expression of *dHAND*, which is essential for the specification of intramandibular arch patterning in co-operation with *ET-1*, is downregulated. How folinic acid affects *ET-1* and *dHAND* expression is also uncertain. To ascertain whether and how the expression of *ET-1* and *dHAND* is affected by folinic acid in the branchial region of embryos, we performed whole-mount *in situ* hybridization of GD10·5 mouse embryos.

In control embryos, *ET-1* and *dHAND* were expressed in a similar pattern in the first and second branchial arches (Fig. 3(A, D)). In contrast, RA-exposed embryos demonstrated significantly decreased of *ET-1* and *dHAND* in both arches (Fig. 3(B, E)). In folinic acid-added embryos, expression of both genes increased in the branchial region (Fig. 3(C, F)). The present results suggested that folinic acid could increase *ET-1* and *dHAND* expression in the branchial region.

Discussion

The teratogenic potential of RA on post-implantation mouse whole embryo culture was confirmed by the present study.

Table 2. Frequency and distribution of the observed abnormalities‡

| | Control | RA (0·4 μM) | | RA (0·4 μM) + folinic acid (mM) | | | | | |
| | | | | 1·0 | | 0·1 | | 0·01 | |
		n	%	n	%	n	%	n	%
Examined embryos	25	23		23		22		20	
Abnormal embryos	0	23*	100	2†	8·6	5†	22·7	11	55
Microcephaly		3							
Neural schisis		2							
First branchial arch reduced		1							
First and second branchial arches fused		8		1		4		7	
Second branchial arch reduced		9		1		1		4	

RA, retinoic acid.
Mean values were significantly different from those of the control group (χ^2): $*P < 0.05$.
Mean values were significantly different from those of the RA-treated group (χ^2): $†P < 0.05$.
‡ For details of procedures, see pp. 419–420.

Fig. 1. Morphological appearance of mouse embryos after 48 h culture. For details of procedures, see pp. 419–420: (A), Control (→ , three distinct branchial arches); (B), retinoic acid-exposed embryo (→ , only the first branchial arch is visible); (C), folinic acid-added embryo (→ , three branchial arches). Magnification × 50.

All embryos per group were at the same development stage or of the same age in the present study. Only GD8·5 embryos displaying three to five pairs of somites were selected for culture in our model, which could assure that all embryos were at the same development stage at the beginning of culture. The culture time of all embryos in the present study was equal. Previous literature showed mouse whole embryo culture models *in vitro* can simulate the development process of embryo *in vivo* (Van *et al.* 1990). As had been shown by previous work in our laboratory (unpublished results), RA severely inhibited embryonic growth, and reached a maximum effect in our previous study at a dose of 0·4 μM. In spite of some methodological differences (species, culture medium, culture system, embryonic age etc.), the observed specific morphological effects obtained in the present study were consistent with the literature: abnormalities at the branchial apparatus had been, in fact, described after exposure of rat and mouse embryos to RA at concentrations from 0·2 to 5 μM (Lee *et al.* 1995; Menegola *et al.* 2004). These abnormalities included microcephaly, neural schisis, the amount of branchial arches reduced, mandibular arch failed to fuse. The high incidence of defects induced by RA in the present study might be due to a specific effect of RA on certain cell populations (neuroepithelium, neural crest) that causes the alterations of

hindbrain segmentation and the abnormal rhomboencephalic neural crest cell migration, and, consequently, an increase in malformed embryos (Lee *et al.* 1995; Gale *et al.* 1996; Niederreither *et al.* 2000; Menegola *et al.* 2004).

The present work also demonstrated a protective effect of folinic acid on RA-induced early craniofacial abnormalities. The present study showed that the effective concentrations for folinic acid were 1·0 and 0·1 mM, which was consistent with previous data indicating that folinic acid at a concentration of 1·0 mM could prevent fumonisin B1-induced NTD (Sadler *et al.* 2002). Although not completely protective, folinic acid at concentrations of 1·0 and 0·1 mM reduced craniofacial abnormalities by 93 % and 77 %, respectively (Table 2). However, 0·01 mM-folinic acid could not inhibit the effects of RA. The present results support the idea of dose-dependent effects of FA on the prevention of orofacial clefts (Andrew *et al.* 1999). Sadler *et al.* (2002) also reported FA can lessen fumonisin-induced facial defects by 57 %. Reynolds *et al.* (2003) reported that combination therapy with FA and methionine could prevent RA-induced cleft palate in mice. It is well known that GD8·5–10·5 in mice is a critical period for development of the heart, midface, ear, nose, upper lip and palate. To our knowledge, however, the present study is the first to demonstrate the effect of folinic acid on RA-induced
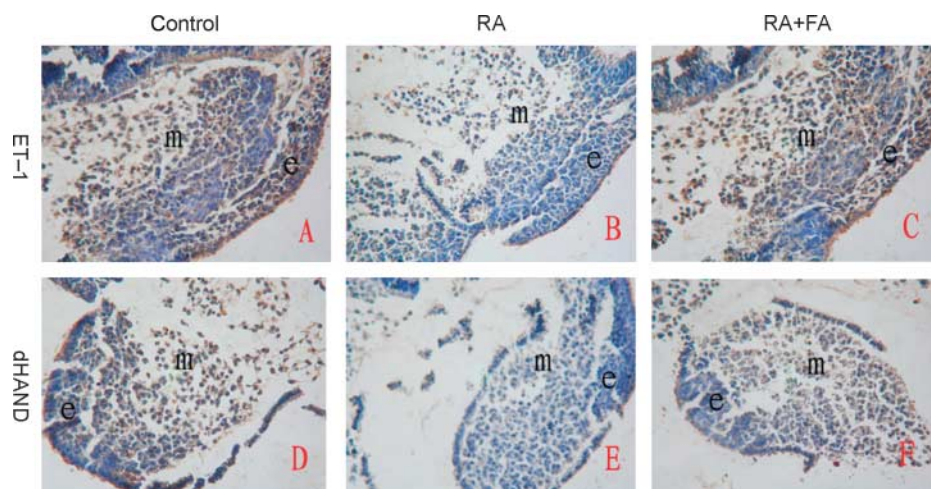

Fig. 2. Gestational day 10·5 mouse first branchial arch, stained for endogenous endothelin-1 (ET-1; A–C) and dHAND (D–F) (coronal section). Photographs are representative of two independent experiments containing two samples for each condition. For details of procedures, see pp. 419–420. (A, D), Control branchial arch demonstrating a dense reaction product (4 +) over the cytoplasm of mesenchyme cells (m) and epithelium (e); (B, E), retinoic acid (RA)-exposed specimen, demonstrating only a pale immunolabelling (1 +); (C, F), folinic acid (FA)-added specimen, immunostaining for both ET-1 and dHAND (immunolabel 3–4 +). Magnification × 400.
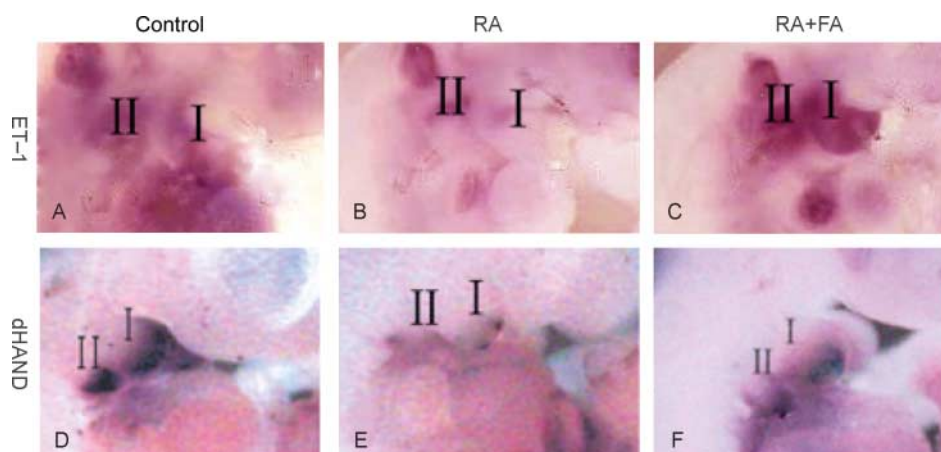
Fig. 3. Whole-mount *in situ* hybridization on control (A, D), retinoic acid (RA)-exposed (B, E) and folinic acid (FA)-added (C, F) embryos at gestational day 10·5 using *ET-1* (A−C) and *dHAND* (D−F) probes. For details of procedures, see pp. 419−420. *ET-1* and *dHAND* are expressed in the first and second (I, II) branchial arches of control and FA-added embryos, whereas *ET-1* and *dHAND* expression decreased significantly in the branchial region of the RA-exposed embryos. Magnification × 50.

craniofacial abnormalities using GD8·5 mouse whole embryo culture *in vitro*. Differences between the present study and our previous study might be due to the fact that in the previous study, data were collected at GD12 *in vivo*, while in the present investigation data were collected at GD8·5 *in vitro*.

The observation that supplemental folate does not completely overcome the effects of RA may be due to the fact that an insufficient number of folate receptors are available to provide all of the necessary intracellular FA. It is also possible that more than one mechanism of action is responsible for RA-induced embryotoxicity.

At present, the mechanism underlying the effect of FA on RA-induced craniofacial abnormalities remains uncertain. It is well known that the segmental migration of hindbrain crest cells is essential to the correct formation of branchial structures (Lumsden *et al.* 1991; Menegola *et al.* 2004). In mouse embryos, BA1 first becomes visible around embryonic GD9·0, just as the cranial neural crest cells migrate into and condense with the arch. Menegola *et al.* (2004) reported that RA could alter segmental migration patterns by inducing ectopic pathways. Other researchers suggested that neural crest cells might be particularly sensitive to FA deficiency (Antony & Hansen, 2000). ET-1 is expressed in the epithelium and paraxial mesoderm core of the branchial arches in mouse embryos. Gene targeting experiments have shown that ET-1/ETAR signalling is necessary for the development of cranial neural crest-derived structures including BA1 in mice (Kurihara *et al.* 1994; Ozeki *et al.* 2004), chick (Kempf *et al.* 1998) and zebrafish (Miller *et al.* 2000). At the same time, dHAND can regulate development of neural crest-derived branchial arch mesenchyme (Ruest *et al.* 2003).

The present study reported that RA treatment produced a significant decrease in immunostaining intensity of ET-1 and dHAND in the branchial arches of mouse embryos compared to the values in the control group ($P < 0.05$) at a concentration of 0·4 μM. The present results were in agreement with the previous data: RA could suppress ET-1 gene expression at the transcriptional level in endothelial cells (Yokota *et al.* 2001) and decrease dHAND level in the mouse embryos (Yasui

et al. 1998). For these reasons, the observed differences in the expression of the two genes were not due simply to the fact that the branchial arches of retinoid-exposed embryos were smaller and less developed than those of control embryos or embryos to which folinic acid was added.

The localization patterns for ET-1 and dHAND mRNA in the developing branchial arches have been well described (Clouthier *et al.* 2000; Ruest *et al.* 2003; Fukuhara *et al.* 2004), and the patterns described here were in agreement with previously published reports (Fig. 3). Simultaneously, the localization of the two proteins in the branchial arches was confirmed in the epithelium and mesenchyme in the present study (Fig. 2). However, without localization data we could not rule out the possibility that RA had a differential effect on transcripts in the epithelium and mesenchyme. Further studies are also needed.

However, information about FA effects on ET-1 expression is considerably less available. In the present study, immunohistochemistry indicated that folinic acid at concentrations of 1·0 and 0·1 mM increased the protein level of ET-1 and dHAND in the first branchial arch. We also showed by whole-mount *in situ* hybridization that mRNA expression of ET-1 and dHAND increased significantly at concentrations of 1·0 and 0·1 mM-folinic acid compared to the RA group ($P < 0.05$). The drawbacks in the present study were that no information about how folinic acid regulated ET-1 and dHAND expression and no clear dose−response relationship between ET-1 or dHAND and folinic acid were provided. The authors thought that there were several possible interpretations for folinic acid increasing ET-1 and dHAND expression: (1) the FA cofactor donates its methyl group to a vitamin B12-dependent enzyme, methionine synthase (Choi *et al.* 2005). Because such methylating reactions are pivotal to DNA synthesis and subsequent expression, it is possible that FA acts on various signalling pathways involved with normal branchial structures. (2) FA can maintain cell function by participating in amino acid and nucleic acid metabolism. Consequently, these cells can secrete cytokines. In spite of these shortcomings, the present results suggested that folinic acid supplementation might prevent craniofacial

abnormalities induced by RA through increasing protein and mRNA expression levels of ET-1 and dHAND in the branchial region. However, further studies will be needed to elucidate the detailed mechanism of action of folinic acid on the ET-1/ETAR signalling pathway in cell function.

In addition, the present study showed that folinic acid supplementation could prevent RA-induced dysmorphogenesis of the auditory system, which was not described in previously published data. However, further studies are needed to investigate the mechanism of the effect of FA on RA-induced otic system abnormalities.

In conclusion, the present study demonstrated the teratogenicity of RA on the mouse embryonic branchial region and prevention of RA-induced craniofacial abnormalities by folinic acid. Moreover, folinic acid increased ET-1 and dHAND expression levels in branchial arches. The present results may contribute to understanding the mechanisms of folinic acid prevention of RA-induced defects. However, further studies are needed.

Acknowledgements

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