Retinoid signaling is essential for patterning the endoderm of the third and fourth pharyngeal arches

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SUMMARY

The requirement of retinoic acid (RA) in the initial formation of the pharyngeal arches was investigated by treating headfold-stage mouse embryos with a pan-RAR antagonist in vitro and in vivo. This results in a complete absence of mesenchyme, arteries, nerves and epibranchial placodes of the 3rd and 4th pharyngeal arches, complete agenesis of the 3rd and 4th pouches and consistent lack of the 6th arch artery. Mesodermally derived endothelial cells are absent from the 3rd and 4th pharyngeal arch region and the distribution domain of EphA2 transcripts in mesodermal cells is shifted caudally. In situ hybridization with CRABPI, kreisler and EphA4 probes and the pattern of expression of a Wnt1-lacZ transgene show that neural crest cells (NCC) normally destined to the 3rd and 4th arches migrate ectopically. Most interestingly, the appearance of the 3rd and 4th arches is prevented by the antagonist only during a very narrow window of time, which does not correspond to the period of post-otic NCC migration. Both the timing of appearance and the nature of the defects in RAR antagonist-treated embryos indicate that migrating NCC and mesodermal cells destined to the caudal pharyngeal arches do not represent primary targets of RA action. Alterations in the endodermal expression pattern of Hoxa1, Hoxb1, Pax1, Pax9, Fgf3 and Fgf8 in response to the antagonist-induced block in RA signal transduction demonstrate for the first time that RA signaling is indispensable for the specification of the pharyngeal endoderm and suggest that this signaling is necessary to provide a permissive environment locally for the migration of NCC and mesodermal cells. Our study also indicates that the formation of the 2nd pharyngeal arch and that of the 3rd and 4th pharyngeal arches probably involve distinct RA-dependent developmental processes.

Key words: Retinoic acid receptor, Endoderm, Pharynx, Neural crest, Hox gene, Pax gene, Fgf, Embryo culture, Mouse

INTRODUCTION

Pharyngeal arches (PA) are transient bulges of the embryonic surface that develop in a cranial to caudal sequence between the head and the heart and are separated by evaginations of the pharyngeal endoderm, the pharyngeal pouches. Adult organs originating from caudal (i.e. post-otic) PA and 3rd pouch, which include the thymus, parathyroid glands and arteries destined to the head and lungs, are all partially derived from neural crest cells (NCC) (Larsen, 1993; Le Douarin, 1982).

Transduction of retinoic acid (RA) signals by nuclear receptors, the RARs and RXRs, plays key roles in development (Kastner et al., 1995, 1997; Chambon, 1996; Mascrez et al., 1998). Mouse fetuses carrying targeted inactivations of both the RARα and RARβ genes recapitulate the defects generated in the chick by ablation of large portions of the neural crest destined to the caudal pharynx, namely thymus and parathyroid gland ageneses or ectopias, aberrant pattern of the great cephalic arteries, and absence of the pulmonary arteries and aortico-pulmonary septum (Bockman et al., 1989; Kirby and Waldo, 1990; Mendelsohn et al., 1994; Ghyselinck et al., 1997). RARα−/− / RARβ−/− embryos have fused 3rd and 4th PA but do not display obvious defects in the number and migration paths of NCC (Dupé et al., 1999). The defects of RARα−/− / RARβ−/− mutants do not reflect a state of complete retinoic acid (RA) deficiency, possibly because the loss of these two receptors is partially compensated by RARγ (Dupé et al., 1999). The generation of RARα−/− / RARβ−/− / RARγ−/− mutants is technically difficult and, additionally, such triple null mutants would probably die prior to the onset of organogenesis, as RA and RARs are present in embryos as early as E6.5 (Ang et al., 1996; Ang and Duester, 1997). Thus, in order to study the formation of the embryonic pharynx in a situation where retinoid signaling is severely impaired, we designed a culture system in which wild-type mouse embryos, collected at the headfold stage, were exposed to a pan-RAR antagonist BMS493, which competitively blocks the activation of all three RAR isotypes (α, β and γ) by agonistic retinoids.

MATERIALS AND METHODS

Embryo culture, retinoid treatments and mouse lines

Embryos were collected at various time points between the early
headfold, presomite stage and the 14-somite stage and cultured as described by Copp and Cockroft (1990). All-trans-RA (Sigma) or the pan-RAR synthetic retinoid antagonist BMS493 (Bristol-Myers-Squibb, Princeton, NJ, USA), diluted in ethanol, was added to the culture medium to 0.1%. In control embryo cultures, the ethanol vehicle was added at the same final dilution. For in vivo treatments, a solution of BMS493 at $10^{-2}$ M in ethanol was diluted in sunflower oil (1:2.5 v/v) and administered by oral gavage twice at 10-hour intervals to pregnant females at a concentration of 10 mg/kg body mass. Embryos heterozygous for the mRARb2-lacZ (Mendelsohn et al., 1991), the RAREhsplacZ (Rossant et al., 1991) and the Wnt1-lacZ (Echelard et al., 1994) transgenes were processed for X-gal staining as described by Mendelsohn et al. (1991).

**Histology, histoenzymology, whole-mount immunohistochemistry and in situ hybridisation**

Histology was performed according to standard procedures. Whole-mount in situ hybridisation (ISH) was performed according to Décimo et al. (1995) using digoxigenin-labeled riboprobes for Hoxa1, Hoxb1, Pax1, Pax9, Fgf8, Fgf5, EphA4, EphA2, kreisler, CRABPI and Twist. Whole-mount immunohistochemistry using the 2H3 neurofilament-specific antibody (Developmental Studies Hybridoma Bank), the anti-Phox2a epibranchial placode-specific antibody (Tiveron et al., 1996), and the anti-PECAM-1 (Pharmingen, San Diego, CA, USA), as well as whole-mount TUNEL labeling, were used as described (Mark et al., 1993; Davis et al., 1991; Schlaeger et al., 1995; Conlon et al., 1995). TUNEL staining on histological sections was performed according to Ghyselinck et al. (1998) with a 3-minute proteinase K treatment.

**Ink injections**

Embryos cultured for 48 and 60 hours were injected with Pelikan Ink n°17 via the yolk-sac vasculature, using a 1 mm capillary tube, then fixed and cleared according to Waldo at al. (1990).
RESULTS

Unless otherwise mentioned, embryos collected at E7.5 (i.e. early headfold, presomite stage), E8.0 i.e. 2-4 somite stages (ss), E8.5 (10-14 ss) were cultured in a medium supplemented with either BMS493 at a concentration of $10^{-6}$ M or vehicle alone for 6-60 hours. For the sake of simplicity, these cultured embryos are referred to as Ex+y hours BMS493-treated embryos or controls respectively, x corresponding to the age of the embryos in days at the time of explantation and y to the hours spent in culture.

The competition between retinoic acid and its antagonist can be monitored in mRARβ2-lacZ transgenic embryos

The effects of the RA antagonist were tested on cultured embryos harboring an RA-inducible mRARβ2-lacZ transgene (Mendelsohn et al., 1991). A dose-response curve was established on E8.5+36 hours embryos treated with BMS493 at $10^{-5}$ M, $4 \times 10^{-6}$ M, $10^{-6}$ M, $10^{-7}$ M, $10^{-8}$ M and $10^{-9}$ M. The last three concentrations did not affect the extent or the intensity of β-galactosidase staining. However, expression of the transgene was strongly decreased at $10^{-6}$ M and abolished at $4 \times 10^{-6}$ M (data not shown). Even at this latter concentration, the embryos did not show external malformations. However, exposure to BMS493 at $10^{-5}$ M resulted in severe morphological alterations, which varied greatly between embryos and could not be prevented by simultaneous administration of RA, thus raising the possibility that they were caused by toxic (e.g. detergent-like) effects of the retinoid (data not shown). Therefore, all subsequent cultures were carried out in the presence of BMS493 at $10^{-6}$ M: at this antagonist concentration, the RARβ2 promoter activity was already decreased after 6 hours and this decrease was reverted by the simultaneous addition to the culture medium of RA at $10^{-7}$M (Fig. 1a-c and data not shown).

Long-term treatments with BMS493 of early somite stage embryos result in defects restricted to the caudal portion of the pharynx, the fifth rhombomere and the lungs

The external morphology of the vast majority (80%) of E8.0+24 hours (n=70) and E8.0+48 hours (n=110) controls was identical to that of E8.75 and E9.5 embryos in vivo, respectively (Kaufman, 1992; Van Maele-Fabry et al., 1997 and references therein). Dysmorphogenetic embryos exhibiting abnormal body turning, forebrain hypoplasia or caudal truncations, which occurred at the same frequency in the presence and absence of BMS493 (about 20%), were discarded. The majority (70%) of E8.0 embryos cultured for 60 hours (n=20) were dead. The surviving E8.0+60 hours embryos were comparable to E9.75 embryos in vivo. Embryos treated with BMS493 at E8.0 differed from controls only by the constant agenesis of the 3rd and 4th PA, enlargement of the second branchial pouch and near-absence of primary lung buds, as well as frequent increase of the size of rhombomere 5. The lungs and hindbrain defects will be described elsewhere.

Treatments at E8.0 selectively impair the formation of the 3rd and the 4th pharyngeal arches, arch arteries and branchial pouches

Externally, all E8.0+48 hours controls displayed bulges corresponding to the first three PA (B1-B3, Figs 1a, 2a). On histological sections, the 3rd PA (B3, Fig. 1d) was delimited by the 2nd and the 3rd branchial pouches (P2 and P3), and contained an arch artery (A3). This histological analysis also revealed the presence of a 4th PA (B4, Fig. 1d), containing an artery in the process of canalisation (A4) and delimited caudally by a 4th pouch (P4). In E8.0+48 hours BMS493-treated embryos (n=110),...
the bulge of the 3rd PA was never seen (Figs 1b, 2b). Histological analysis of seven embryos revealed a complete, bilateral, absence of 3rd and 4th PA, corresponding arch arteries and branchial pouches (compare Fig. 1d and e). Moreover, the treated embryos consistently showed a markedly enlarged 2nd pouch (P2, Fig. 1e). The absence of mesenchyme corresponding to the 3rd PA and the enlargement of the 2nd pouch in BMS493-treated embryos was confirmed by whole-mount ISH using twist and Pax1 probes, respectively (compare Fig. 2a,b). The first two PA (B1 and B2) of the BMS493-treated embryos were indistinguishable from their counterparts in controls (Fig. 1a,b,e).

In order to rule out the possibility that the alterations observed in E8.0+48 hours BMS493-treated embryos reflected regionally restricted developmental retardations, embryos were analyzed after 60 hours in culture. In E8.0+60 hours controls, the 4th PA was externally visible (data not shown), the 4th arch artery (A4, Fig. 1g) was now connected to the dorsal aorta and aortic sac, and the 4th pouch (P4) had enlarged. In contrast, the pharyngeal region of E8.0+60 hours BMS493-treated embryos did not show any morphological changes (compare Fig. 1e and h). Ink injections allowed further investigation of the pattern of arch arteries (Fig. 2c-f). The first three (A1-A3) and the first four (A1-A4) arch arteries were readily visualized in controls cultured for 48 and 60 hours, respectively. Injections of BMS493-treated embryos confirmed the absence of the 3rd and 4th arch arteries and also showed an enlargement of the 2nd arch artery (A2, Fig. 2d,f), probably reflecting an attempt to functionally compensate the absence of the 3rd and 4th arch arteries.

BMS493-induced defects of the caudal pharynx can be prevented by all-trans retinoic acid and are determined during a narrow period of time

70% of the E8.0 embryos simultaneously exposed to 10⁻⁶ M of BMS493 and 10⁻⁷ M RA for 48 hours (n=21) developed 3rd and 4th PA and pouches (Figs 1c,f, 2i). That an RAR physiological ligand could prevent defects caused by the synthetic antagonist demonstrates that they arose as a consequence of impaired retinoid signaling. Moreover, the intensity of β-galactosidase staining was increased in the ‘rescued embryos’ (Fig. 1b,c), indicating that the competition between RA and its antagonist most probably took place at the transcriptional level, as expected.

Embryos were treated with BMS493 for 6 hour periods (during which 2-3 additional pairs of somites were formed), then washed and cultured for additional 36 or 42 hour periods in medium without added retinoids in order to reach approximately 30 ss. At least three groups of 4-5 embryos were analyzed in each culture experiment. Embryos treated for 6 hours always developed normal 3rd and 4th PA and pouches when collected at 3-4 ss (Fig. 3b), in contrast to embryos collected at 7-8 ss, in which these PA always failed to form (Fig. 3c). In embryos explanted at 10-11 ss and exposed to BMS493 throughout the 36 hour culture period, normal 3rd and 4th PA structures were formed (Fig. 3d and data not shown). Thus the RA antagonist can disturb the ontogeny of the post-otic pharynx only during a narrow window of time, between 7-10 ss.

Pan-RAR antagonist treatments at E8.0 selectively impair the formation of the post-otic branchial nerves and of their distal ganglia

The patterning of the nerves and ganglia related to the 1st and 2nd PA (e.g. G5, G7/8 and N7; Fig. 4a,b), were indistinguishable in control (n=3) and BMS493-treated (n=3) embryos. The IXth and the Xth nerves, which develop within the 3rd and 4th PA, respectively, were fused in controls (N9 and N10; Fig. 4a), mimicking a condition rarely observed in normal embryos in vivo (Ghyselinck et al., 1997). In BMS493-treated embryos, the bundle of axons corresponding to IXth and Xth nerves, was absent or appeared markedly thinner than in controls (Fig. 4b, and data not shown); the common proximal ganglion of the IXth and Xth nerves, which is exclusively derived from NCC, was normally developed (PG9/10, Fig. 4a,b), in contrast to the distal ganglia of these nerves, which were small, fused and never connected to the rhombencephalon (DG9/10, compare Fig. 4a and b). Neurons of the distal ganglia of the IXth and Xth nerves arise from ectodermal thickenings, the 2nd and 3rd epibranchial placodes (E2 and E3; Fig. 4c) located at the anterior margin of the 3rd and 4th PA, respectively (Begbie et al., 1999 and references therein). Immunostaining of E8.0+48 hours BMS493-treated embryos with antibodies against Phox2a showed a fusion of the 2nd and 3rd placodes and a decrease in the number of their neuronal cells (E2/3; Fig. 4d). The 1st epibranchial placode was unaffected by the treatment (E1; Fig. 4c,d).

Neural crest cells and angioblasts are not quantitatively deficient, but fail to colonize the lateral portion of the caudal pharynx

The absence of caudal PA in BMS493-treated embryos might reflect a deficiency in post-otic NCC since these cells normally provide mesenchyme to the 3rd and 4th PA (Lumsden et al., 1991). Therefore, the status of the rhombencephalic NCC was analysed by ISH with probes to cellular retinoic acid binding protein one (CRABPI), kreasler and EphA4 and by studying apoptosis in the PA using the TUNEL technique. These experiments were carried out on embryos cultured for 24 or 30 hours, i.e., prior to and at the onset of formation of the 3rd PA, respectively and, in any event, before the earliest appearance of the 4th PA.

CRABPI is a general marker of migrating and early post-migratory NCC (Maden et al., 1992) which, in E8.0+24 hours controls, is detected in three streams populating the 1st PA (B1, Fig. 5a), the 2nd PA (B2) and the prospective 3rd and 4th PA (B3/4). In E8.0+24 hours BMS493-treated embryos, NCC were as numerous as in controls (Fig. 5a,b), but the posterior stream migrated more caudally (Fig. 5b). EphA4 and kreasler are expressed in NCC destined for the 3rd PA and originating from rhombomeres 5 and 6 (R5 and R6), respectively (Nieto et al., 1992; Cordes and Brash, 1994). ISH for these two markers on E8.0+30 hours BMS493-treated embryos showed similar caudal deviations in the migration paths of NCC (Fig. 5c,d,g,h).

In situ detection of DNA nicks was performed at E8.0+24 hours and E8.0+48 hours, i.e. at developmental stages when NCC have already left the hindbrain neurectoderm, prior and after the appearance of the 3rd PA, respectively. The number of apoptotic cells was increased in the ectoderm overlying the dorsal portion of the 2nd branchial pouch of E8.0+24 hours BMS493-treated embryos (arrowheads in Fig. 5i,j). In contrast, BMS493-induced changes in the amount of apoptotic cells were not observed in the mesenchyme after
The endoderm of the caudal pharynx responds to physiological concentrations of RA

The mRARβ2-lacZ transgene used to monitor the general level of retinoid responsiveness in cultured embryos is not expressed in the pharynx. In order to provide direct evidence that pharyngeal tissues respond to RA, we used transgenic embryos harbouring three copies of a retinoic acid response element (RARE) inserted upstream of a hsplacZ construct (Rossant et al., 1991). In these embryos, the pattern of β-galactosidase activity matches closely the distribution of endogenous RA (Ang et al., 1996; Wagner et al., 1992; Maden et al., 1998). RAREhsplacZ embryos collected at E7.5 were cultured for 24 hours to reach the 8-10 ss. In control embryos, strong β-galactosidase activity was detected in the endoderm (E; Fig. 5a), and in its anterior counterpart. The staining of the endoderm was abolished in BMS493-treated embryos, demonstrating that the hsp promoter was not constitutively active in this tissue (Fig. 6b). Conversely, simultaneous addition of 10−7 M RA and 10−6 M BMS493 to the culture medium prevented the inhibition of β-galactosidase activity in the pharyngeal endoderm (Fig. 6c). These results indicate that the cells in the prospective 3rd and 4th PA region respond to physiological concentrations of RA, and that the absence of these PA in BMS493-treated embryos results from a local inhibition of RA signaling.

The endoderm of the caudal pharyngeal arches is not correctly specified in BMS493-treated embryos

The products of the 3’ Hox genes are essential for the morphogenesis of the pharynx (reviewed in Rijli et al., 1998). Expression of Hoxa1 and Hoxb1 in the foregut endoderm and its associated mesoderm and ectoderm is regulated through RAREs (Frasch et al., 1995; Huang et al., 1998). In E8.0+24 hours and E8.0+30 hours controls, Hoxa1 and Hoxb1 transcripts were detected only in the caudal pharynx (Fig. 6d), in accordance with in vivo data (Murphy and Hill, 1991). In BMS493-treated embryos, the pharyngeal expression of Hoxb1 was undetectable and that of Hoxa1 was markedly decreased (Fig. 6e, g).

Pax1 and Pax9 are paired-box-containing genes whose expressions in the endoderm correlate with focal increases in cell proliferation that are responsible for the growth of the branchial pouches (Müller et al., 1996). The expression patterns of Pax1 and Pax9 in E8.0+30 hours and E8.0+48 hours controls were comparable to those reported in vivo (Figs 2g, h and data not shown) (Wallin et al., 1996; Peters et al., 1998). In E8.0+30 hours BMS493-treated embryos, the domain of strong Pax9 expression in the 2nd pouch was broader than in controls (data not shown). These data and the patterns of Fgf8 expression at E8.0+24 hours (see the text below and Fig. 7a) indicate that the enlargement of the 2nd pouch represents the first detectable morphological defect caused by inhibition of RA signaling, the absence of the 3rd PA being consistently observed only after 48 hours in culture. In E8.0+48 hours BMS493-treated embryos, Pax9 was only weakly expressed at the axial level corresponding to the 3rd pouch (large arrows in Fig. 7c). Additionally, the caudal extremity of the lateral pharyngeal endoderm displayed a persistent expression of Pax1 whereas the 4th pouch, its counterpart in the control embryos, does not express Pax1 (Fig. 2h and data not shown).

Thus, BMS493-induced alterations in expression patterns of Hoxa1, Hoxb1, Pax1 and Pax9 are consistent with the histological observation that the pharyngeal endoderm caudal to the 2nd PA has adopted a fate resembling that of a normal 2nd pouch.

Fgf8 and Fgf3 signalings are locally altered in the pharyngeal endoderm following treatment with BMS493

The expression pattern of Fgf8 in the developing PA has suggested that this signaling molecule could trigger the formation of the pharyngeal pouches, as well as interactions between endoderm or ectoderm and migrating NCC (Crossley and Martin, 1995; Wall and Hogan, 1995). When compared to controls, E8.0+24 hours BMS493-treated embryos showed an enlarged domain of Fgf8 expression in the endodermal caudal to the 2nd PA (P2; Fig. 7a). In contrast, the strong Fgf8 expression in the prospective 3rd pouch of E8.0+30 hours controls was undetectable following treatment with BMS493 (brackets in Fig. 7b). Interestingly, this treatment did not affect the intensity nor the pattern of Fgf8 expression in the PA ectoderm. The transient Fgf3 expression in the endoderm of the caudal-lateral pharynx (Wilkinson et al., 1989) was also strongly decreased in E8.0+30 hours BMS493-treated embryos (data not shown). These data indicate that treatment with BMS493 inhibits the synthesis by endodermal cells of secreted factors thought to play important roles in their specification and/or in signaling pathways controlling cell migration within the prospective PA.

BMS493 treatment at the presomitic stage in utero does not affect the formation of the first two pharyngeal arches, but the 3rd and 4th arches are lacking

When compared to E9.0 embryos in vivo, E7.5+48 hours BMS493-treated embryos (n=70) displayed multiple external
malformations (data not shown). The majority of these abnormalities were also observed in E7.5+48 hours controls \((n=70)\), albeit at lower frequencies. The 3rd PA was consistently absent in all treated embryos, but only in two control embryos. In contrast, the first two PA were not conspicuously altered following BMS493 treatment.

As cultures of presomitic stage embryos resulted in relatively high rates of spontaneous malformations, we complemented our observations by in vivo experiments performed with mice harbouring a \textit{Wnt1-lacZ} transgene that is expressed in migrating and post-migratory NCC (Echelard et al., 1994). BMS493 was administrated twice to two pregnant mice at both E7.0 and E7.5, i.e. prior to the appearance of the 1st PA and to the onset of NCC migration (Serbedzija et al., 1992 and references therein), whereas two control mice were given the vehicle (i.e. ethanol and oil) only. At E10.5, ten living embryos, and six living embryos and four resorptions, were recovered from the two BMS493-treated dams. Control embryos were morphologically normal, whereas all embryos exposed to BMS493 in utero showed identical phenotypes: the first two PA were normal (B1 and B2; Fig. 8a,b), the 2nd branchial pouch (P2) extended caudally and NCC accumulated at its ventral side (large arrow in Fig. 8b); the 3rd and 4th PA were absent (compare Fig. 8a and b, and data not shown). Quite strikingly, the 3rd, 4th and 6th arch arteries (A3, A4 and A6; Fig. 8c) were bilaterally absent, so that the only connection between the aortic sac (AS; Fig. 8c,d) and aortas was represented by an enlarged 2nd arch artery (A2; Fig. 8d). These findings strongly suggest that the in vivo formation of the 1st and 2nd PA and

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**Fig. 5.** Abnormal cell migrations and apoptosis in BMS493-treated embryos. Whole-mount ISH (a-h) for detection of migrating neural crest cells, (a-d, g, h) and/or mesodermal cells (e,f), and Tunel staining of whole embryos (i,j) and of histological sections (k,l). B1-B3 branchial arches 1-3; ‘B3’, prospective branchial arch 3; ‘B3/4’, prospective branchial arches 3 and 4; N, notochord; OP, otic placode; P2, 2nd pharyngeal pouch; R3, R5 and R6, rhombomeres 3, 5 and 6. The large black and green arrows point to NCC (or mesodermal cells) migrating caudally or accumulating in the ventral region of the pharynx, respectively. The arrowheads indicate apoptotic cells in the ectoderm (i, j) or in the mesenchyme (k,l).

**Fig. 6.** Distribution of the \textit{RAREhsplacZ} transgene and of \textit{Hoxa1} and \textit{Hoxb1} transcripts in control and retinoid-treated embryos. The embryos were cut into halves and the medial side is displayed. B1 and B2, branchial arches 1 and 2; E, endoderm; M, dorsal mesocardium.


DISCUSSION

Unliganded RARs can transrepress the basal promoter activity of target genes in transfected cells through binding to corepressors (reviewed in Chambon, 1996). The fact that the pan-RAR antagonist BMS493 stabilizes the association of RAR/RXR heterodimers with such corepressors in vitro (our unpublished results) raises the possibility that it could not only block the effect of agonistic retinoid signals, but also act as an artificial silencing signal in our cultured embryos. However, this is most probably not the case, as defects in the pharyngeal arches (PA) and pouches similar to those observed here upon BMS493-treatment of wild-type embryos are exhibited by embryos lacking RARs (Dupé et al., 1999), RXRs (Wendling et al., 1999) or their physiological agonistic ligands (White et al., 1998; Niederreither et al., 1999). Therefore, our present study of embryos with a block in retinoid signal transduction (BRST) definitively demonstrates that retinoid signaling is essential for the formation of 3rd and 4th PA structures during a narrow developmental window corresponding to the 7-10 somite stages.

Migrating post-otic neural crest cells and angioblasts are not primary targets of the RA signal

In normal mouse embryos, neural crest cells (NCC) arising from the post-otic hindbrain yield part of the mesenchyme of the 3rd and 4th PA and all the cells of the common proximal ganglion of the IXth and Xth nerves. Later in ontogeny, NCC of these two PA differentiate into connective tissues of the neck glands (e.g. parathyroid and thymus) and smooth muscle cells of the great cephalic arteries and aortico-pulmonary septum (Le Douarin, 1982). The present data indicate that a block in retinoid signaling (BRST) specifically alters the migratory behaviour of post-otic mesenchymal NCC, which are derouted towards the most caudal portion of the pharynx, instead of invading the 3rd and 4th PA. In the post-otic region of the embryonic head, the choice of the migration path is not an NCC-autonomous property (Couly et al., 1998). Therefore, the ectopic, caudal, migration of mesenchymal NCC observed in embryos with a BRST must reflect the existence of RA-dependent guidance cues emanating from the other tissues of the PA. Along the same lines, we note that neurogenic post-otic NCC, which do not interact with PA, migrate normally in embryos with BRST, as assessed from the normal position and size of the common proximal ganglion of the IXth and Xth nerves.

We have previously concluded that the defects in the neck of the 1st branchial pouch require less RA than that of the 3rd and 4th PA and branchial pouches.
glands and cephalic arteries observed in all RARα+/RARβ−/− mutant fetuses cannot be accounted for by a decrease in the number of NCC destined to invade the 3rd and 4th PA (Ghyselinck et al., 1997; Dupé et al., 1999). The present analysis of the CRABP1, kreisler and EphA4 transcript distribution, of the expression of the Wnt1-lacZ NCC-specific transgene and of apoptosis in NCC, reveals that a severe BRST that leads to a complete agenesis of these two PA (1) has no obvious effect on the production of NCC by the post-otic hindbrain neurectoderm, (2) does not affect the survival of migratory and early post-migratory NCC and, most importantly, (3) acts during a very narrow critical developmental period (7-10 ss), which does not coincide with that of post-otic NCC migration as the last NCC are leaving this region of the neural tube only at the 14-somite stage (Serbedzija et al., 1992). Moreover, a severe BRST completely inhibits the formation of all 3rd, 4th and 6th arch arteries. This inhibition cannot be accounted by a generalized failure of angiogenesis, but most probably reflects the inability of the mesodermally derived angioblasts to populate the region of the pharynx caudal to the 2nd PA. Altogether these data indicate that the migrating post-otic NCC and angioblasts do not represent primary targets of RA action.

RA signaling plays an essential role in the specification of the pharyngeal endoderm by regulating the expression of Hox1 and Hoxb1

The 3rd and 4th pharyngeal pouches are hypoplastic in RARα+/−/RARβ−/− mutant embryos (Dupé et al., 1999), but absent here in embryos treated with the pan-RAR antagonist, demonstrating that RA signaling is more severely impaired in this latter condition. The molecular marker analyses of these treated embryos with Hoxa1, Hoxb1, Pax1, Pax9, Fgf3 and Fgf8 probes support the conclusion that the single lateral evagination of the endoderm caudal to the 2nd PA is analogous to an enlarged 2nd pouch.

It is well established that Hox gene products impart distinct morphological identities to neurectodermal and mesectodermal derivatives (for reviews, see Krumlauf, 1993; Mark et al., 1997; Favier and Dollé, 1997; Rijli et al., 1998), but their role in patterning endodermal structures has received little attention. In embryos with BRST, Hoxa1 expression is downregulated and Hoxb1 expression is abolished in the endoderm of the caudal pharynx at developmental stages preceding the appearance of the 3rd and 4th pharyngeal pouches. Both Hoxa1 and Hoxb1 contain retinoic acid response elements (RAREs) that are functional in vivo (Marshall et al., 1994; Studer et al., 1994; Dupé et al., 1997; Huang et al., 1998). In addition one of the Hoxb1 RARE is indispensable for initiating expression of this gene in the pharyngeal endoderm at E8.0 (Huang et al., 1998). Altogether these data strongly suggest that the RA-dependent expression of Hoxa1 and Hoxb1 in the endoderm is crucial for the specification of the 3rd and 4th pharyngeal pouches and that the endoderm is a primary target for endogenous RA. Along these lines it is noteworthy that Hoxa1+/−/Hoxb1−/− fetuses consistently exhibit an agenesis of the thymus and parathyroid glands as a result of abnormal patterning of their pharyngeal pouches (Rossel and Capecchi, 1999). The decrease in Pax1 and Pax9 expression in the caudal portion of the pharyngeal endoderm of embryos treated with the pan-RAR antagonist is in keeping with our proposal that this tissue is a primary target of RA action, as the expressions of both of these genes in the endoderm are regulated cell-autonomously, as demonstrated by heterotopic grafting of prospective foregut endoderm (Müller et al., 1996).

Interestingly, Pax9 is indispensable for the formation of the thymus and parathyroid glands (Peters et al., 1998) and agenesis of these glands is observed in RARα+/−/RARβ−/− fetuses (Ghyselinck et al., 1997). Thus, it is most likely that the lack of the Pax9 expression at the level of the presumptive 3rd pouch under conditions of impaired RA signaling accounts for the cases of thymus and parathyroid gland agenesis observed in RARα+/−/RARβ−/− fetuses.

The ectopic migration of post-otic NCC in embryos with BRST could be caused by a lack of RA-dependent guidance cues originating from the endoderm, e.g. Fgf3 and Fgf8 are specifically and strongly downregulated in the endoderm of the caudal pharynx prior to the formation of the 3rd and 4th PA. Alternatively, the misshapen pharyngeal endoderm might prevent the normal ventral movement of the neural crest.

Epibranchial placodes are induced by the endoderm (Begbie et al., 1999). Therefore, the extensive apoptosis in the ectoderm corresponding to the prospective 2nd epibranchial placode, the subsequent fusion of the 2nd and 3rd placodes, the decrease in the number of their neurons, and the hypoplasia of the distal ganglia of the 1Xth and Xth nerves, are probably all related to the improper specification of the pharyngeal endoderm in embryos with BRST.

The formation of the 2nd pharyngeal arch and that of the 3rd and 4th pharyngeal arches are likely to involve distinct RA-dependent developmental processes

Previous studies have shown that the formation of the first PA and of its derivatives appear essentially independent of RA. In contrast, RA is clearly required for the formation of the 2nd and more caudal PA (Lohnes et al., 1994; Mark et al., 1995; Maden et al., 1996; Niederreither et al., 1999).

Under our culture conditions, BRST induces rapid changes in the expression patterns of the mRARβ2-lacZ transgene and of several endogenous genes (Chazaud et al., 1999; O. W., P. C. and M. M., unpublished data). Thus, the finding that blocking the RA signal transduction prior to the earliest appearance of the foregut (i.e. at E7.5) has no effect on the subsequent formation of the 2nd PA suggests that this formation is less dependent on RA than that of more caudal PA. This view is supported by several other lines of evidence: (1) RALDH2, a major RA-synthesizing enzyme, has an anterior expression boundary that is immediately adjacent to the prospective 4th PA, but distant from the prospective 2nd PA (Niederreither et al., 1997); (2) endogenous RA induces the expression of two distinct RA-responsive transgenes in the caudal, but not in the rostral pharyngeal region (Rossant et al., 1991; Balkan et al., 1992; the present report); in both of these transgenic mouse lines, the availability of RA is a limiting factor, as cells in the 2nd PA express both lacZ reporters in response to teratogenic concentrations of RA; (3) compound knockouts of either RARα and RARβ or RARα and RARγ cause drastic alterations of the 3rd and 4th PA, but have no apparent effect on the 2nd PA; and (4) RA deficiency begun at the time of conception in rat embryos leads to agenesis of the 3rd and 4th PA, without altering the formation of the 2nd PA (White et al., 1998). It is clear, however, that the formation of the 2nd PA
requires RA, as it is totally inhibited in RALDH2-null mutant mice (Niederreither et al., 1999) as well as in vitamin A-deficient quails (Maden et al., 1996). In our culture system, the agenesis of the 2nd PA induced by high BMS493 concentrations (i.e. 10^{-5}M; data not shown) is difficult to interpret because of possible non-specific embryotoxic effects.

In any event, the above observations strongly suggest that the formation of the 2nd PA and that of the 3rd and 4th PA rely on distinct subsets of RA receptors, RA-responsive elements and developmental mechanisms. Along these lines, it is noteworthy that: (1) RARβ is apparently expressed at higher levels in the endoderm of the caudal pharynx than in its rostral counterpart (Ruberte et al., 1991; Smith, 1994); (2) distinct RA-responsive elements control the expression of Hoxb1 in the endoderm and in NCC migrating from rhombomere 4 (Huang et al., 1998 and references therein) and (3) the extensive apoptosis reported in the NCC destined to the 2nd and more posterior PA in RALDH2-null mutant mice and vitamin A-deficient quail embryos (Niederreither et al., 2000; Maden et al., 1996) is not observed in our embryos with BRST.

Implication for CATCH22 syndromes

Absent or hypoplastic thymus, absent or hypoplastic parathyroid glands, atresia of the aortic arch and absent or abnormal aorticopulmonary septum are observed in human CATCH22 syndromes characterized by monoallelic microdeletion of chromosome 22q11.2. That this spectrum of defects can be due to agenesis of the thymus (Weber, T. W. and Duester, G., 1996). Retinoic acid synthesis in mouse embryos during gastrulation and craniofacial development linked to class IV alcohol dehydrogenase gene expression. J. Biol. Chem. 271, 9526-9534. and craniofacial development linked to class IV alcohol dehydrogenase gene expression. J. Biol. Chem. 271, 9526-9534.


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