Nutritional factors such as vitamin intake contribute to the etiology of cleft palate. Vitamin A is a regulator of embryonic development. Excess vitamin A can cause congenital malformations such as spina bifida and cleft palate. Therefore, preventive nutritional strategies are required. This review identifies putative biological mechanisms underlying the association between maternal vitamin A intake and cleft palate. Excessive vitamin A may disturb all three stages of palatogenesis: 1) during shelf outgrowth, it may decrease cell proliferation and thus prevent tissue development; 2) it may prevent shelf elevation by affecting extracellular matrix composition and hydration; and 3) during shelf fusion, it may affect epithelial differentiation and apoptosis, which precludes the formation of a continuous palate. In general, high doses of vitamin A affect palatogenesis through interference with cell proliferation and growth factors such as transforming growth factor β and platelet-derived growth factor. The effects of lower doses of vitamin A need to be investigated in greater depth in order to improve public health recommendations.

© 2011 International Life Sciences Institute

INTRODUCTION

Orofacial clefts are the most common craniofacial birth defects in humans. These disorders are caused by impaired fusion of one or more orofacial structures in the embryo. In the etiology of orofacial clefts, both genetic and environmental factors are involved. The genetic background of orofacial clefting is starting to become unraveled because of advances in epidemiological research. However, the environmental factors that are involved, as well as their interaction with genetic factors, are still incompletely understood.

One of the most extensively studied environmental factors in congenital disorders is maternal nutrition. During pregnancy, the nutritional status of the embryo is fully dependent on maternal food intake and metabolism. The maternal diet during the first trimester is crucial for the development of various organs such as the brain and the heart. A nutritional excess or deficiency can lead to birth defects of the neural tube, other neurological abnormalities, congenital heart disease, and intestinal malformations. The maternal diet is also associated with orofacial clefting. For example deficiencies in maternal nutrient intake, such as zinc and myo-inositol, can increase the risk of cleft palate (CP) in offspring. Vitamins are of particular interest because they cannot be synthesized by the body. Maternal folate deficiency may increase the risk of neural tube defects, but it is also reported to increase the risk of CP in offspring. Evidence for this association, however, remains inconclusive. Vitamins are obtained through the diet, and their sources within the diet can be divided into
whole food, foods fortified with synthetic vitamins, and dietary supplements. Vitamins play key roles in growth and development and can, therefore, be considered vital ingredients of embryonic nutrition.

The essential vitamin A, particularly its derivate retinoic acid (RA), is an important regulator of embryogenesis. RA regulates proliferation, differentiation, and apoptosis during the morphogenesis of embryonic structures. RA regulates the specification of cell identity and gene expression through activation of nuclear receptors of the retinoid X receptor and retinoic acid receptor families, which bind to specific DNA elements in the regulatory regions of target genes called retinoic acid response elements. Precursor forms of vitamin A are present in vegetables and animal tissues. Vegetables contain carotenoids, while meat contains retinyl esters. After ingestion, both are transformed into retinol and stored in the liver. In the liver, the stellate cells control blood plasma concentration by retinol storage and mobilization. Retinol in the blood enters the target cell and is oxidized to RA in a two-step process (Figure 1). The first step of RA synthesis is the reversible oxidation of retinol to retinal, which is mainly mediated by alcohol dehydrogenases and short-chain dehydrogenases/reductases. The second step is the irreversible oxidation of retinal to RA by aldehyde dehydrogenase. Alternatively, RA can be directly formed from β-carotene. The availability of RA is controlled by proteins such as cellular retinol-binding proteins and cellular retinoic-acid-binding proteins.

RA receptor knockout studies have illustrated the importance of RA-signaling in palatogenesis. Among other malformations, these knockouts result in orofacial clefting. In general, over- or under-exposure to RA during embryonic and fetal development disturbs the closure of the neural tube and the development of many other organs and limbs. According to the 2001 recommendations of the US Food and Nutrition Board, the recommended dietary allowance (RDA) for vitamin A is 900 μg for adult males, 700 μg for adult females, and 770 μg during pregnancy. Epidemiological studies in humans performed to date have not demonstrated a clear association between vitamin A and CP. However, evidence from animal studies shows a clear increase in the risk of CP related to excess vitamin A. The putative effects of low doses of RA in the human diet are also discussed in this review. The developmental stage at the time of exposure determines the sensitivity to CP induction. In the first weeks of pregnancy, RA-induced apoptosis in cell populations that derive from the first branchial arch leads to CP. However, in humans, RA exposure seems most critical between developmental weeks 4 and 12. This review discusses the role of RA in the regulation of palatogenesis during this particular period and identifies putative biological mechanisms to explain the association between RA and orofacial clefting. The majority of studies in this field are based on mouse models. First, a brief overview of normal facial and palatal development is given. Subsequently, the effects of RA in the different stages of palatogenesis are discussed in detail.

Figure 1  The retinoic acid signaling pathway. Retinol from the diet is converted into retinoic acid through retinal. Binding of retinoic acid to its receptor on the DNA regulates gene transcription. Abbreviations: RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic-acid-binding protein; SDR, short-chain dehydrogenase/reductase.
The development of the face starts in week 4 of human embryonic development, when cells migrating from the neural crest combine with the core mesoderm and the overlying epithelium to establish the facial primordia.27,28 The topographical center of the developing facial structures is the stomodeum, or primitive mouth. It is delimited by five distinct processes (Figure 2). The process rostral to the stomodeum is called the frontal process. Laterally, the stomodeum is delimited by a pair of maxillary processes and caudally by the mandibular processes.

In week 5 of human embryonic development (embryonic day E10 in mice), the epithelium on both sides of the ventrolateral part of the frontal process thickens and gives rise to the nasal placodes. Directed growth and expansion around the nasal placodes results in formation of the nasal pits and the lateral and medial nasal processes (Figure 2).27,29,30 After fusion of the lateral and medial nasal processes, the maxillary and medial nasal processes fuse to form the upper lip. The fusion of the upper lip takes place between developmental weeks 5 and 7 (E10.5–12.5 in mice) and is based on mechanisms similar to those involved in the fusion of the secondary palate. A failure of lip fusion may secondarily affect the later fusion of the secondary palate, which leads to a combined cleft lip and palate (CLP). The secondary palate is formed between weeks 6 and 12 of human development (E12–16 in mice). The inner parts of the maxillary processes develop bilateral shelf-like outgrowths that grow downward on either side of the developing tongue (Figure 3A–D). As palatogenesis progresses, the palatal shelves move upward and grow towards the midline. After contact, the medial edge epithelia (MEE) of the opposing processes adhere and form a midline epithelial seam (MES).31 Subsequently, the MES disappears, but the exact fate of these cells remains controversial. The proposed mechanisms of MES disappearance are discussed later in this review. Palatal confluence is achieved when the MES has completely disappeared and thus mesenchymal continuity across the secondary palate is established. Fusion spreads from the middle third of the palate in anterior and posterior directions and is completed by week 12 of development (E16 in mice).28,30 Subsequently, the ossified hard palate forms out of the anterior two-thirds of the palate, while the posterior one-third of the palate forms the soft palate.32 Interestingly, palatal fusion can also be reproduced in cultured mouse embryonic palatal shelves (Figure 3E–G).

In summary, the development of the human craniofacial complex takes place between weeks 4 and 12 of gestation. The rapid proliferative expansion and complex morphogenetic events that coordinate facial development make it highly sensitive to the effects of gene mutations and to environmental influences. This may explain the relatively high incidence of craniofacial birth defects like CP compared with other craniofacial disorders. In the next sections, palatogenesis is reviewed in detail, and putative biological effects of RA leading to a cleft are identified.

**SHELFL OUTGROWTH: PUTATIVE EFFECTS OF RETINOIC ACID**

The secondary palate develops from bilateral outgrowths of the maxillary processes, generally referred to as the palatal shelves. The shelves increase in size by mesenchymal cell proliferation and by the production and hydration of extracellular matrix (ECM) components.28 In pregnant mice, RA overexposure reduces the growth of the palatal shelves in the embryo.33 This
seems to be a consequence of reduced mesenchymal cell proliferation as well as inhibition of ECM production. Some authors also found evidence of mesenchymal apoptosis in response to RA, but these findings are controversial. The effects of RA on mesenchymal proliferation and ECM production will be discussed in detail in the next section of this review. Proliferation is the main process in shelf outgrowth, while ECM production is crucial in palatal shelf elevation.

The general effects of RA are summarized in Tables 1 and 2. RA inhibits the proliferation of mouse palatal mesenchyme by arresting the cells in the G1 phase. This occurs through upregulation of cyclin-dependent kinase inhibitor p21(Cip1) and subsequent hypophosphorylation of the tumor suppressor Rb. RA also interferes with specific signaling pathways in cell proliferation by modulating platelet-derived growth factors (PDGFs). PDGF-C is downregulated by RA in mouse embryonic mesenchymal palatal cells. During palatogenesis, downregulation of PDGF signaling by RA results in hypoplastic palatal shelves that are not able to fuse, probably due to inhibition of nuclear regulator genes that control cell proliferation, such as members of the signal transducer and activator or transcription family and nuclear factor κB. Moreover, RA stimulates expression of the small proteoglycan decorin, which might inhibit mesenchymal proliferation. In several cell types, decorin activates the epidermal growth factor receptor (EGFR) that induces activation of mitogen-activated protein kinases, mobilization of intracellular calcium, upregulation of p21, and, finally, growth suppression.

In addition, RA interacts with members of the transforming growth factor β (TGF-β) superfamily, which contains, among other items, the bone morphogenetic proteins and the TGF-βs. These are multifunctional growth factors that mediate a variety of biological processes such as the proliferation of mesenchymal cells, the synthesis of ECM proteins, and apoptosis. During palatal shelf outgrowth, bone morphogenetic protein 2 stimulates mesenchymal proliferation, but its expression is decreased by RA. The effect of TGF-β depends highly on the cell type and the developmental stage at the time of exposure. For that reason, the interactions between RA and the TGF-β signaling pathway are often contradictory. Some studies show an increase in TGF-β1 and TGF-β2 signaling in response to RA, while others show a decrease (Table 1). Decreased expression of the TGF-β1 and -β2 isoforms reduces mesenchymal proliferation. The TGF-β3 isoform is mainly involved in a later stage and will be discussed in another section. TGF-β signaling is downregulated by RA through suppression of Smad2/3 phosphorylation, probably by binding of the Smad transcriptional corepressor TGIF (TG-interacting factor).

In conclusion, the major mechanisms through which excess RA may disturb palatal shelf outgrowth include inhibition of mesenchymal proliferation and signaling of growth factors.
The shelves elevate to a horizontal position above the dorsum of the tongue, while the mandible elongates and the tongue moves downward (Figures 3A,B). Excess RA was found to increase apoptosis in the base of the fetal tongue, which might physically impair the horizontal elevation of the palatal shelves.37 Furthermore, excess RA prevents tongue withdrawal by disturbance of tongue muscle development.48 As shown in several models, tongue withdrawal is a prerequisite for proper palatogenesis.49 RA prevents tongue withdrawal through down-regulation of Tbx1, a candidate gene for DiGeorge syndrome, which includes CP.37,48 Furthermore, a recent study showed that Wnt1Cre;Tgfbr2 conditional knockout mice develop microglossia and CP by downregulation of fibroblast growth factor 10 (FGF10).50 Interestingly, RA is a physiological regulator of FGF10 expression during the development of various organs.51 In general, FGFs coordinate epithelial-mesenchymal interactions. Impaired FGF signaling also contributes to about 3% of the non-syndromic CLP cases.52 In FGF10 knockout mice, the elevation of the palatal shelves is physically prevented by adhesion and fusion of the shelves to the tongue and mandible.53 The unusual fusion pattern probably occurs

**Table 1 In vitro effects of retinoic acid.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>RA form and concentration</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mesenchymal cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC-3T3-El</td>
<td>ATRA 1 × 10⁻⁶ mol/L</td>
<td>↑ Apoptosis</td>
<td>Guo et al. (2008)⁶⁶</td>
</tr>
<tr>
<td>MEMP</td>
<td>ATRA 5 × 10⁻⁶ and 10 × 10⁻⁶ mol/L</td>
<td>↓ Proliferation, BMP-7 expression</td>
<td>Han et al. (2006)⁹⁹</td>
</tr>
<tr>
<td>ATRA 0.33 × 10⁻⁶ and 3.3 × 10⁻⁶ mol/L</td>
<td></td>
<td>↓ TGF-β1</td>
<td>Nugent et al. (1998)¹⁰⁵</td>
</tr>
<tr>
<td>ATRA 0.03 × 10⁻⁵, 0.33 × 10⁻⁶, and 3.3 × 10⁻⁶ mol/L</td>
<td></td>
<td>↑ TGF-β2 and TGF-β3</td>
<td>Nugent et al. (1998)¹⁰⁵</td>
</tr>
<tr>
<td>13-cis RA (isotretinoin) 1 × 10⁻⁵, 1 × 10⁻⁴, and 4 × 10⁻⁴ mol/L</td>
<td></td>
<td>↓ Mesenchymal proliferation</td>
<td>Watanabe et al. (1988)³⁵</td>
</tr>
<tr>
<td>ATRA 1 × 10⁻⁶ to 1 × 10⁻⁸ mol/L</td>
<td></td>
<td>↓ Mesenchymal proliferation, and HA synthesis</td>
<td>Yoshikawa et al. (1987)³⁴</td>
</tr>
<tr>
<td>ATRA 1 × 10⁻⁶ to 1 × 10⁻¹⁰ mol/L</td>
<td></td>
<td>↓ TGF-β3 through stimulation of the expression of TGF-interacting factor</td>
<td>Zhang et al. (2009)⁴⁶</td>
</tr>
<tr>
<td>ATRA 1 × 10⁻⁶ mol/L</td>
<td></td>
<td>↓ TGF-β receptors</td>
<td></td>
</tr>
<tr>
<td><strong>Epithelial cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEE</td>
<td>ATRA 1 × 10⁻⁵, 1 × 10⁻⁷, and 1 × 10⁻⁹ mol/L</td>
<td>↑ EGF and MEE proliferation, altered differentiation of MEE cells</td>
<td>Abbott et al. (1988)¹⁰⁸</td>
</tr>
<tr>
<td>ATRA 1 × 10⁻⁶ mol/L</td>
<td></td>
<td>Altered differentiation of MEE cells</td>
<td>Abbott and Pratt (1987)⁷⁸</td>
</tr>
<tr>
<td>PANGE</td>
<td>ATRA 10 × 10⁻⁶ mol/L</td>
<td>↓ GAGs, collagen, and collagen crosslinks</td>
<td>Hatakeyama et al. (2004)⁷²</td>
</tr>
<tr>
<td>PPK</td>
<td>1 × 10⁻⁶ mol/L, form unknown, most probably ATRA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT2</td>
<td>ATRA 10 × 10⁻⁶ mol/L</td>
<td>↓ WNT3A, WNT8A, WNT8B, WNT10B, and WNT11</td>
<td>Katoh (2002)¹⁰⁹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ WNT2, WNT7B, and WNT14B</td>
<td></td>
</tr>
</tbody>
</table>

**Abbrreviations and symbols:** ↑, Stimulation; ↓, inhibition; –, no effect; ATRA, all-trans retinoic acid; BMP, bone morphogenetic protein; EGF, epidermal growth factor; GAGs, glycosaminoglycans; HA, hyaluronan; MC-3T3-El, fibroblast-like mouse cell line; MEE, medial edge epithelial cell; MEMP, mouse embryonic mesenchymal palatal cell; MMF, multipotent mesenchymal fibroblast; NT2, human teratocarcinoma cell line; PDGF, platelet-derived growth factor; PMS, pluripotent mesenchymal stem cell; PANGE, postnatal gingival epithelial cell; PNVHOF, postnatal human oral fibroblast; PNK, postnatal keratinocytes; RECP, rat embryonic mesenchymal palatal cell; TGF, transforming growth factor.

**SHELF ELEVATION: PUTATIVE EFFECTS OF RETINOIC ACID**

The shelves elevate to a horizontal position above the dorsum of the tongue, while the mandible elongates and the tongue moves downward (Figures 3A,B). Excess RA was found to increase apoptosis in the base of the fetal tongue, which might physically impair the horizontal elevation of the palatal shelves.37 Furthermore, excess RA prevents tongue withdrawal by disturbance of tongue muscle development.48 As shown in several models, tongue withdrawal is a prerequisite for proper palatogenesis.49 RA prevents tongue withdrawal through down-regulation of Tbx1, a candidate gene for DiGeorge syndrome, which includes CP.37,48 Furthermore, a recent study showed that Wnt1Cre;Tgfbr2 conditional knockout mice develop microglossia and CP by downregulation of fibroblast growth factor 10 (FGF10).50 Interestingly, RA is a physiological regulator of FGF10 expression during the development of various organs.51 In general, FGFs coordinate epithelial-mesenchymal interactions. Impaired FGF signaling also contributes to about 3% of the non-syndromic CLP cases.52 In FGF10 knockout mice, the elevation of the palatal shelves is physically prevented by adhesion and fusion of the shelves to the tongue and mandible.53 The unusual fusion pattern probably occurs...
as a result of ectopic expression of TGF-β3, a downstream target of FGF10 that is essential for the adhesion and fusion of the palatal shelves. Disturbance of the intrinsic mechanisms that cause shelf elevation may cause CP as well. ECM production and hydration are the key factors in the elevation of the palatal shelves. The main component of the palatal ECM is collagen. Collagen fibers form a network to provide tensile strength to the palatal shelves. In addition, the stiffness of the collagen network is determined by the number of intermolecular cross-links. Glycosaminoglycans (GAGs) and proteoglycans are also essential components of the ECM. They form aggregates that bind large amounts of water, and the resulting swelling pressure is the driving force behind shelf elevation.

Prior to elevation, excess RA may directly suppress collagen synthesis through binding to retinoic acid response element sites in the collagen promoter region. In addition, the formation of collagen crosslinks may be reduced. Hypothetically, these weakened palatal shelves are not able to elevate to a horizontal position. The mesenchymal cells that secrete the palatal ECM also produce matrix metalloproteinases (MMPs) that are responsible for ECM remodeling. The level of active MMPs increases during palatal shelf elevation, while the expression of tissue inhibitors of matrix metalloproteinases (TIMPs) is constant. RA inhibits MMP synthesis and stimulates the expression of TIMPs. The latter might be mediated by nuclear receptors similar to the action of glucocorticoids and other hormones. This might lead to reduced remodeling of the ECM and impaired elevation of the palatal shelves. Another mechanism involves RA-induced decorin, which binds to collagen and thereby stimulates collagen fiber assembly. This might enhance the stiffness of collagen fibers and prevent elevation. In human as well as murine mesenchymal cells, RA inhibits the synthesis of GAGs, possibly by interference with the p38 mitogen-activated protein kinase pathway in TGF-β1 and -2 signaling. This may result in a reduced swelling pressure and impaired shelf elevation.

In conclusion, RA may prevent palatal shelf elevation by impeding tongue withdrawal and by disrupting the collagen network. Furthermore, RA inhibits the synthesis of GAGs, which decreases the hydration of the ECM and might thus reduce the driving force for shelf elevation.

**SHELF FUSION: PUTATIVE EFFECTS OF RETINOIC ACID**

After the palatal shelves have reached a horizontal position, the MEE approach and adhere to each other in order to form the MES. Prior to the contact of the bilateral palatal shelves, the MEE consists of two cell layers; the basal MEE cells attached to the basement membrane, and the superficial MEE cells, also called the periderm. The first contact takes place in the middle third of the palate and proceeds in anterior and posterior directions. In order to make contact, the peridermal cells develop protrusions, mainly filopodia. These filopodia not only increase the surface area of the MEE available for fusion but also possess large numbers of cell adhesion molecules. In this section, the effects of RA on the expression of adhesion molecules and the formation of filopodia will be discussed. Subsequently, the focus will be on the actual fusion of the palatal shelves.

If the shelves are able to make contact, excess RA may also impair subsequent differentiation of MEE cells into MES cells. RA exposure of palatal shelves prolongs EGFR expression in the MEE. This induces an aberrant ciliated secretory cell phenotype in the MEE, which prevents normal cell-to-cell adherence. The normal expression of filopodia and chondroitin sulfate proteoglycans that mediate adhesion is regulated by growth factors like PDGF and TGF-β3. Altered growth factor signaling by RA may thus interfere with palatal shelf adherence by reducing the number of filopodia and the expression of chondroitin sulfate proteoglycans in peridermal cells. In contrast, RA does not affect the expression of E-cadherin, which is needed for epithelial adhesion. In oral and dermal postnatal keratinocytes, excess RA also leads to a loss of desmosomes and hemidesmosomes by blocking the synthesis of desmosomal proteins, but the effect of RA on desmosomes in the MEE is not clear. Upon adhesion of the palatal shelves, the peridermal cells migrate to the oral and nasal sides and contribute to the formation of the epithelial triangles. Subsequently, most peridermal cells undergo apoptosis. Then, the basal MEE cells of both shelves intercalate, resulting in a single epithelial layer that is delimited on both sides by a basement membrane. With the formation of this MES, the actual palatal fusion starts.

A critical step in palatal fusion is the disappearance of the MES, by which a continuous mesenchymal tissue is formed. Recent findings from animal studies indicate that persistence of the MES can induce a secondary separation of the adhered palatal shelves at later fetal stages or in newborns. The adhering but unfused shelves are pulled apart by lateral growth of the head. This might correspond with submucous palatal clefts in humans. The exact fate of the MES cells is still controversial. Currently, most authors believe that the MES cells disappear mainly by apoptosis, but migration and epithelial-to-mesenchymal transition (EMT) may also occur. In the migration theory, it is hypothesized that MES cells migrate out of the seam and incorporate into the nasal and oral epithelia. EMT refers to the
phenotypical change of epithelial cells into mesenchymal cells. EMT occurs in embryonic development but is also thought to be involved in tumor progression and fibrotic disorders. The EMT theory suggests that MES cells undergo EMT and become part of the palatal mesenchyme. Since the evidence for MES migration and EMT is relatively weak, only the effect of RA on apoptosis will be discussed.

RA mediates apoptosis during normal palatogenesis and is synthesized by MES cells soon after shelf contact. There is no evidence that excess RA directly affects apoptosis in the MES, but it may interfere with signaling pathways that regulate apoptosis, such as the WNT and TGF-β pathways. Twelve of 19 members of the WNT family are expressed in the developing palate. WNT11 is expressed in the MES and induces apoptosis by inhibition of fibroblast growth factor receptor 1b. A single nucleotide polymorphism in the WNT11 gene is also associated with clinical nonsyndromic CP and CLP. Since RA was shown to repress the expression of several

Table 2  In vivo effects of retinoic acid.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Exposure day</th>
<th>RA form and concentration</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>E9</td>
<td>Retinyl acetate 118 mg/kg for 3 consecutive days</td>
<td>↓ Mesenchymal proliferation</td>
<td>Kochhar and Johnson (1965)</td>
</tr>
<tr>
<td>Mouse</td>
<td>E10</td>
<td>ATRA 100 mg/kg</td>
<td>↑ Apoptosis in cell populations that derived from the first branchial arch</td>
<td>Sulik et al. (1987)</td>
</tr>
<tr>
<td>Mouse</td>
<td>E11,5</td>
<td>ATRA 100 mg/kg</td>
<td>↑ Apoptosis in the tongue primordium; ↓ Tongue withdrawal, elevation of palatal shelves, mesenchymal proliferation, filopodia on the MEE cells, altered differentiation of MEE cells</td>
<td>Okano et al. (2007)</td>
</tr>
<tr>
<td>Mouse</td>
<td>E12</td>
<td>ATRA 100 mg/kg</td>
<td>↑ TGF-α, TGF-β1 in mesenchymal cells, TGF-β2 in nasal epithelial cells, altered differentiation of MEE cells</td>
<td>Okano et al. (2007)</td>
</tr>
<tr>
<td>Mouse</td>
<td>E12</td>
<td>ATRA 70 mg/kg</td>
<td>↓ E-cadherin in the MEE cells</td>
<td>Luning et al. (1994)</td>
</tr>
<tr>
<td>Mouse</td>
<td>E12</td>
<td>ATRA 75 mg/kg</td>
<td>↑ TGF-α, TGF-β1 in mesenchymal cells</td>
<td>Abbott et al. (1988)</td>
</tr>
<tr>
<td>Mouse</td>
<td>E12</td>
<td>ATRA 70 mg/kg</td>
<td>↓ TGF-α, TGF-β1 in nasal epithelial cells, altered differentiation of MEE cells</td>
<td>Degitz et al. (1998)</td>
</tr>
<tr>
<td>Mouse</td>
<td>E12</td>
<td>ATRA 70 mg/kg</td>
<td>↓ TGF-β1 in mesenchymal cells, hydration</td>
<td>Degitz et al. (1998)</td>
</tr>
<tr>
<td>Mouse</td>
<td>E12</td>
<td>ATRA 75 mg/kg</td>
<td>↓ BMP-2,4,5</td>
<td>Lu et al. (2000)</td>
</tr>
<tr>
<td>Mouse</td>
<td>E12</td>
<td>ATRA 70 mg/kg</td>
<td>↑ EGF signaling</td>
<td>Zhang et al. (2003)</td>
</tr>
</tbody>
</table>

Abbreviations and symbols: ↑, Stimulation; ↓, inhibition; –, no effect; ATRA, all-trans retinoic acid; BMP, bone morphogenetic protein; ECM, extracellular matrix; EGF, epidermal growth factor; MEE, medial edge epithelial; TGF, transforming growth factor.
WNT genes during early body axis extension in the embryo, it might also affect WNT signaling during palatogenesis. RA-induced downregulation of WNT11 signaling may inhibit MES apoptosis, but the exact interaction between these factors during palatal fusion remains to be elucidated. Another interesting regulatory protein in the RA-mediated apoptosis during palatogenesis is p63. The p63 protein is a master regulator of ectodermal development. Mutations in p63 give rise to at least seven ectoderm-related developmental disorders, of which CP is one of the cardinal features. RA treatment of differentiated human primary keratinocytes results in an elevated level of ΔNp63α, the most abundant isoform expressed in skin keratinocytes as well as in oral and nasal palatal epithelia. Normally, expression of p63 is reduced in MEE during palatal shelf fusion, and downregulation of p63 is suggested to be a prerequisite for proper periderm development and palatal fusion. Recently, an enzyme involved in RA metabolism, the retinal dehydrogenase/reductase retSDR1/DHRS3, has been identified as a direct transcriptional target of p63. This finding raises the possibility that p63 regulates RA levels and that the interplay between RA and p63 affects palatogenesis. Apoptosis of the MES cells subsequently activates the proteolytic breakdown of the basement membrane by MMPs. RA reduces synthesis of MMPs and stimulates the transcription of their inhibitors, the TIMPs, possibly through interference with EGFR and TGF-β signaling pathways. This may lead to persistence of the basement membrane and impaired fusion of the palatal shelves.

In summary, RA may prevent initial shelf contact by inducing apoptosis within the MEE. In addition, it may prevent shelf adherence by disrupting normal epithelial differentiation. After adhesion, RA may impair the disappearance of the MES, which precludes the formation of a continuous palate.

**HIGH VERSUS LOW DOSES OF VITAMIN A**

The previously discussed research primarily involved high doses of vitamin A in animal models. These experiments showed clear effects on the different stages of palatogenesis that might lead to clefting. According to the 2001 report of the US Food and Nutrition Board, the recommended RDA for vitamin A is 770 μg during pregnancy. For an average person, the RDA means a dosage of 10.5 μg/kg. The dosages tested in animal experiments lie in the range of 10.5-10 μg/kg to 21-10 μg/kg, which is 1,000 to 20,000 times the RDA.

Epidemiological studies on nutritional exposure to vitamin A are scarce. Only a few studies clearly describe the dosage used and have an acceptable statistical power. Extreme vitamin A deficiency is lethal for the embryo, while minor deficiencies, below half the RDA, mainly induce ocular abnormalities. Doses slightly above the RDA (1,500 μg) were found to protect against CLP and CP in the offspring. On the other hand, other studies did not find such an effect. A study on high vitamin A intake (4,500 μg) shows that defects related to cranial neural-crest-derived structures, such as craniofacial, cardiac, and thymic defects, are 3.5 times more prevalent than after low intake (≤1,500 μg). In summary, these studies suggest harmful effects for vitamin A deficiency (≤375 μg), possible protective effects in the range of the RDA, and harmful effects at high doses (≥4,500 μg), consistent with a U-shaped dose-effect curve. Thus far, no safe upper limit for vitamin A intake has been established, but the evidence suggests an intake of up to 4 times the RDA (3,000 μg) is harmless.

The same amount is suggested by the US Food and Nutrition Board as the tolerable upper level of intake for vitamin A. In the light of the limited number of available studies, the question remains whether slight nutritional overexposure to vitamin A contributes to clefting in humans. Theoretically, this might increase the risk of clefting in genetically predisposed individuals with specific polymorphisms of enzymes or other proteins involved in vitamin A metabolism. However, as for folate, such interactions have not yet been definitely established.

**CONCLUSION**

Nutritional factors such as vitamin intake are important in the etiology of CP. Therefore, the development of preventive nutritional strategies is of great interest. Overexposure to vitamin A can cause congenital malformations such as CP. The putative biological mechanisms underlying RA-induced CP are summarized in Figure 4. Depending on the time of exposure, excess RA might disturb all three stages of palatogenesis: 1) during shelf outgrowth, it may decrease mesenchymal proliferation and thus prevent tissue expansion; 2) in the next stage, RA may prevent shelf elevation by affecting the ECM composition and hydration; and 3) during the actual fusion of the shelves, it may affect epithelial differentiation and apoptosis, which precludes the formation of a continuous palate.

In general, RA seems to act through interference with growth factor signaling in all stages of palatogenesis. The exact interactions of RA with relevant signaling pathways in palatogenesis need to be investigated in more depth. Future research should focus specifically on the effects of slight nutritional overexposure of vitamin A to improve the dietary recommendations to pregnant women or
those who want to become pregnant, thereby reducing the risk of CP in offspring.

Acknowledgments

Funding. No special funding was received for this project.

Declaration of interest. The authors have no relevant interests to declare.

REFERENCES


