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Overexpression of iNOS and down-regulation of BMPs-2, 4 and 7 in retinoic acid induced cleft palate formation

C.T. Ho[1], T.Y.H. Lau[2], Y. Jin[3], H.B. Lu[3], E. Liong[4], K.M. Leung[1] and G.L. Tipoe[1]

[1]Department of Anatomy, Faculty of Medicine, The University of Hong Kong, Faculty of Medicine Building, Hong Kong, S.A.R., P.R. China, [2]Department of Optometry & Radiography, Faculty of Health and Social Sciences, Hong Kong Polytechnic University, Hong Kong, S.A.R., P.R. China, [3]Department of Histology and Oral Pathology, Qin Du Stomatological College, Fourth Military Medical University, Xi'an, P.R. China and [4]Department of Physiology, Faculty of Medicine, The University of Hong Kong, Faculty of Medicine Building, Hong Kong, S.A.R., P.R. China

Summary. The present work studied the induction of cleft palate formation in embryos developed from pregnant BALB/c mice treated orally with retinoic acid (RA). Previous studies on mature somatic cell types showed that RA exerted inhibitory effects on inducible nitric oxide synthase (iNOS) production. For the first time, our study has shown that RA actually stimulates significant expression of iNOS at specific zones of the affected embryonic palatal tissues at three consecutive stages, from gestation day 13 (GD13) to day 16 (GD16). Enzymatically, iNOS facilitates intracellular nitric oxide (NO) synthesis from L-arginine. When NO reacts with reactive superoxides it may result in irreparable cell injury. NO was also reported to induce apoptosis in some mammalian cell systems. Based on our findings, we propose that such an increase in NO production might be associated with apoptosis in the embryonic palatal tissues in the RA-treated mice. The detrimental effects of NO resulted in a reduction in proliferating palatal cells and therefore disturbed the normal plasticity of the palatal shelves. With iNOS overexpression, our findings also showed that there was significant concomitant down-regulation in the expressions of Bone Morphogenetic Proteins (BMPs) –2, 4, and 7 with regional variations particularly in the palatal mesenchymal cells for those embryos developing cleft palate. Since specific spatial and temporal expressions of BMPs –2, 4, and 7 are critical during normal palatal morphogenesis, any deficiency in the epithelial-mesenchymal interaction may result in retarding growth at the embryonic palatal shelves. Taken together, our study has demonstrated cleft palate formation in the BALB/c embryos involved overexpression of iNOS and down-regulation of BMPs- 2, 4 and 7.

Key words: Retinoic acid, Inducible nitric oxide synthase, Bone morphogenetic proteins, Nitric oxide, Cleft palate

Offprint requests to: Thomas Y.H. Lau, MPhil., Department of Optometry & Radiography, Hong Kong Polytechnic University, Hung Hom, Hong Kong, S.A.R., P.R. China. e-mail address: orthomas@polyu.edu.hk. and George L. Tipoe, MD, PhD, Department of Anatomy, Faculty of Medicine, The University of Hong Kong Faculty of Medicine Building, 21 Sassoon Road, Hong Kong, S.A.R., P.R. China. e-mail: tgeorge@hkucc.hku.hk

Introduction

The hard palate is a common bony partition between the oral and nasal cavity. Incomplete fusion of the palatal shelves during embryonic development results in permanent cleft palate formation. Our previous study has reported induction of cleft palate formation in embryos by oral administration of retinoic acid (RA) to pregnant mice (Lu et al., 2000). However, the molecular mechanisms associated with cleft palate formation have not yet been fully understood. Nitric oxide synthase (NOS) is the enzyme that facilitates intracellular production of nitric oxide (NO) from L-arginine. Both the physiological and cytotoxic effects of NO have been documented. Much of the research works focus on the effects of NO in mature body cell types (Pfeilschifter and Vosbeck, 1991), but relatively few studies are aimed at palatal morphogenesis. There are three different isoforms of NOS expressed in different cell types such as in activated macrophages, neurons and vascular endothelial cells (Bredt et al., 1991; Pollock et al., 1991; Stuehr et al., 1991). The expression of the inducible form of NOS (iNOS) is highly tissue-specific. *In vitro* studies in normal and tumour cell types reported inhibition of iNOS activity by RA (Hirokawa et al., 1994; Umansky and Schirrmacher, 2001). However,

another *in vivo* study demonstrated that RA induced iNOS production through lipopolysaccharide (LPS)-triggered NOS-2 pathway (Devaux et al., 2000). To the best of our knowledge, there is no known study that reveals the pattern of NO expression in proliferating normal and cleft palate tissues with the induction effect of RA. The interactions between RA and NO production in embryonic tissues need further investigation.

Several bone morphogenetic protein (BMP)-encoding genes were first identified on the basis of their abilities to induce ectopic endochondral bone growth. One of the mechanisms by which BMPs act on development is to promote uncommitted multipotential stem cells to enter into chondrogenic or osteogenic rather than myogenic or adipose pathways (Yamaguchi et al., 1991; Gimble et al., 1996). In our previous study in cleft palate formation, expressions of BMP 2, 4 and 5 mRNAs were found to be down-regulated by RA during cleft palatal morphogenesis (Lu et al., 2000) but the BMP protein expressions were not determined.

Palatal morphogenesis involves delicately controlled mechanisms between adjoining palatal epithelial and mesenchymal tissues that share a specific spatial and temporal relationship. Cleft palate may be due to a disturbance in the proper signal transduction pathway in the proliferating cells at the palatal shelves. The present study aimed to determine the role of iNOS and BMP-2, 4 and 7 in cleft palate formation and to better understand the molecular mechanisms involved in palatal morphogenesis. Immunohistochemistry was utilized to provide qualitative and semi-quantitative information on the variations in the distribution and expressions of iNOS and BMPs 2, 4, & 7. Information relating to cell injury, proliferation and differentiation during palatal shelf development may be able to explain the induction effect of RA in cleft palate formation.

Materials and methods

Animals

12-week-old (~25g) BALB/c strain mice were used. The male to female mice ratio was 1:2. Overnight mating was confirmed by checking the presence of vaginal plugs in the female mice. The day with evidence of the vaginal plugs was designated as Gestation Day 0 (GD 0). On GD 10, sixteen pregnant mice were randomly divided into an experimental group (n=8) and a control group (n=8). The pregnant mice in the experimental group were fed with 80 mg/kg bw (body weight) of retinoic acid (Sigma, USA) dissolved in vegetable oil (Lu et al., 2000). The control groups were treated with the vehicle used in the experimental groups. On GD 13, 14, 15 and 16 respectively, the pregnant mice (n =2 in each group) were first deeply anaesthetized by intraperitoneal injections of Sagatal (May & Baker Ltd., England) and then killed to obtain the embryos from the uteri.

Histological tissue processing

The heads of the embryos were sampled by cranial incision and fixed in Bouin's solution for 24 hours. The specimens were then dehydrated in a series of ethanol solutions of increasing concentration followed by paraffin embedding at 56 ºC. Identification of developmental stages of the embryos were double-checked by observing the paraffin blocks with a dissecting microscope. 5 μm-thick coronal sections were obtained (Leica, Jung Biocut 2035) through the anterior region of the presumptive hard palate using the locations of vomero-nasal plates and septal cartilages as landmarks. The tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol followed by routine staining with Harris's haematoxylin and 1% aqueous eosin (H&E). Subsequently, the sections were mounted in Permount (Fisher Scientific, New Jersey, USA). The remaining sections were used for immunohistochemistry.

Immunohistochemistry for iNOS detection

iNOS expression was detected by the immuno-histochemistry staining method (DAKO Envision Plus System). 5 μm-thick paraffin-embedded sections were deparaffinized and rehydrated. Proteolytic digestion was performed by immersing sections in 0.1% trypsin (Sigma, USA) and 0.1% $CaCl_2$ (Merck) for 6 minutes at room temperature (RT) in 0.05M Tris-HCl buffer solution (pH 7.6). To quench endogenous peroxidase activity, sections were immersed in a peroxidase blocking solution for 5 minutes at RT. To reduce non-specific binding of the iNOS antibody, sections were pre-incubated with 10% corresponding normal serum for 1 hr at RT. Rabbit polyclonal iNOS primary antibody (N32030; Transduction Laboratories, USA) diluted to 1:100 in Tris-buffer with 1% bovine serum albumin (BSA) were added onto the sections which were incubated overnight at 4 ºC. A negative control section was incubated with corresponding normal serum without the iNOS antibody. After overnight incubation, sections were washed in Tris-buffer and incubated with peroxidase-labelled polymer conjugated to goat anti-rabbit immunoglobulin for 30 minutes at RT. Finally, the peroxidase was visualized by adding a buffered substrate, 3,3'-diaminobenzidine (DAB) chromogen solution, to the sections for 3-5 minutes. Positive reactivity was indicated by the development of brown-coloured precipitates at the antigen site under light microscopic observation. After colour development, sections were rinsed in distilled water and counterstained lightly with Harris's hematoxylin. Sections were processed routinely in aqueous ethanol and mounted in Permount (Fisher Scientific, New Jersey, USA).

Immunohistochemistry for BMP-2, 4, & 7 detection

BMP-2, and 7 expressions were detected by

immunohistochemistry using the Labelled Streptavidin Biotin method (DAKO LSAB Plus Kit). Paraffin-embedded sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by immersing sections in 3% hydrogen peroxide in 0.05M Tris-buffer solution for 5 minutes at RT. Proteolytic digestion was performed by immersing sections with 0.1% trypsin (Sigma, USA) and 0.1% $CaCl_2$ (Merck) in Tris-buffer for 6 minutes at RT. After incubation in 10% corresponding normal serum for 30 minutes, sections were incubated with BMP primary antibodies overnight at 4 ºC. The primary antibodies were goat anti-human BMP-2 (N-14; Santa Cruz Biotechnology, USA), BMP-4 (N-16; Santa Cruz Biotechnology, USA) and BMP-7 (N-19; Santa Cruz Biotechnology, USA) respectively. Negative control sections were incubated with corresponding normal serum without the BMP primary antibodies. After overnight incubation with antibodies, sections were washed in Tris-buffer followed by incubation in biotinylated link agent and then streptavidin peroxidase for 30 minutes respectively at RT. Following rinsing in Tris-buffer, colour development was carried out by the addition of a buffered substrate-DAB-chromogen solution onto the sections for 3-5 minutes at RT. After colour development, the sections were counterstained slightly with Harris's hematoxylin, processed and mounted (Permount, Fisher, Scientific, New Jersey, USA).

Staging and differentiation of palatal shelf development

Owing to the possibility of individual biological variations in growth and development, the embryos of the BALB/c mice in the control group were examined by light microscopy. The stage of embryonic development of each specimen was determined according to the morphological characteristics observed by the investigator (CTH). The normal development of the palatal shelves was classified into three stages according to the morphological observations of the coronal sections. In stage I, the palatal shelves on bilateral sides were vertical and in close proximity with the tongue. In stage II, the palatal shelves elevated to a more transverse position and cause to lie superior to the dorsum of the tongue. In stage III, the palatal shelves fused to gain median continuity with the formation of an epithelial seam.

In the cleft palate group, in order to overcome the difficulties in differentiating the highly heterogeneous morphological appearance of the specimens, the stages of development were arbitrarily matched to the retinoic acid treatment period i.e. Stage I = GD 13^{14}-14^{8}, Stage II = GD 14^{14}-15^{8} and Stage III = GD 15^{22}-16^{8} (the superscript represents the number of hours).

Image analysis and quantification of iNOS

The images were displayed and captured by a x100 objective lens (oil immersion) using a light microscope (Carl Zeiss Axiolab, Germany) attached to a JVC CCD camera (TK-C1380, Japan). iNOS immunostaining reactivity was determined and expressed as saturation and percentage area respectively (Leica QWIN, Cambridge, UK). Initially, the measurements were standardized for each immunostained section. The luminance setting of the incident light passing through the section at 0 was black and that at 255 was white. The data for saturation were then expressed in grey pixels with the darkest density setting at 0 and the lightest density setting at 255. The final estimates were presented as mean saturation, which estimated the relative amount of iNOS stain in the sections. The mean percentage area of iNOS expression was calculated by the areas of iNOS-positive cells divided by the reference areas of the developing palatal shelves. For both the control group and the cleft palate group, at least six animals (n=6) per each developmental stage, one block per animal, one section per block and 5 fields per section were sampled for image analysis (Gundersen and Østerby, 1981). Each of the palatal shelves of the embryo was observed and arbitrarily divided into two zones. Zone 1 was a region that commenced from the suspended tip of the palatal shelf running laterally to the side of the embryo measuring about 200 μm across (Fig. 1C). Zone 2 was an area that started from the lateral margin of zone 1 to the ossifying center of the maxillary process measuring approximately 200 μm across (Fig. 1C).

Semi-quantitative analyses of BMPs 2, 4 and 7

Based on the intensity and distribution of immunostaining on the sections, BMP-2, 4 and 7 immunostaining reactivity was scored with mutual agreement between two of the authors (CTH & GLT) who employed a semi-quantitative point scoring system ranging from 0 to 3. The slide labels were not known during the scoring process. For both the control and the cleft palate group, at least six animals (n= 6) per each developmental stage, one block per animal, one section per block and at least 7 fields per section were sampled for scoring. A zero-point score means immunostaining reactivity was negative with no brown colour stain in the cell cytoplasm. A three-point score was assigned to strong intensity of brownish stain in the cell cytoplasm when compared to cells with mild (1-point) or intermediate (2-points) intensity. The epithelial cells of the palatal shelves (EPI), the condensed mesenchymal cells near the tip of the palatal shelf (MC) and the non-condensed mesenchymal cells (MN) near to the ossifying centre of the maxilla were analysed.

Statistical analysis

PRISM® (GraphPad Prism 3.0) was used to analyse the data collected. A non-parametric Mann-Whitney U-Test was used to compare quantitative results for iNOS and BMP-2, 4 and 7 expressions. Comparisons were

performed at different stages of the same group and at similar stages between control and cleft palate groups. Additionally, results from the three different sites of cells (EPI, MC & MN) of the same stages in BMP- 2, 4 & 7 expressions were analysed. Data of each parameter was pooled to obtain a single mean and standard error of mean (SEM). A value of $p<0.05$ was considered statistically significant.

Results

Histology

The H&E-stained sections of the embryos in the control group showed a progressive bilateral increase in the size of the palatal shelves from stage I to stage III. Palatal elevation was observed at stage II and there was fusion of the palatal shelves by the end of stage III (Fig. 1A). In the cleft palate group with RA treatment, none of the obtained embryos showed fusion of the palatal shelves until the end of stage III (Fig. 1B).

iNOS staining

The iNOS stain was homogeneous and expressed in the cytoplasm of the palatal epithelial cells as well as in the mesenchymal cells. In the cleft palate group (Fig. 1D), more intense staining of iNOS was located in the mesenchymal cells of the palatal shelves in zone 1 when compared with the control group (Fig. 1C). Qualitatively, the iNOS staining in zone 1 was higher than in zone 2 in both the control and the cleft palate groups. The most intense iNOS staining was identified at stage II in the cleft palate group (Fig. 1D) when compared to other stages in both groups.

The percentage area of iNOS staining demonstrated regional variations among the control and the cleft palate groups. In the control group, there was a reduction in iNOS percentage area from stage I to stage III in both zones (Fig. 2A, B). In the cleft palate group, iNOS percentage area was the lowest at stage I in both zones when compared with stage II and III (Fig. 2A,B). However, there was actually a marked increase in iNOS expression at stage II in both zones when compared with stage I (Fig. 2A,B). This increase in iNOS expression was statistically significant due to a significant reduction of iNOS was detected at stage I in the control group. Moreover, the iNOS percentage area was also significantly increased in the cleft palate group at stage III in zone 2 (Fig. 2B).

In our quantitative expression of the mean saturation of iNOS, lower grey pixels corresponded to a higher amount of iNOS staining on the sections. In the control group, the mean saturation of iNOS staining was the lowest at stage I in both zones, whereas in the cleft palate group stage II had the lowest value (Fig. 2C,D). The mean saturation of iNOS staining in the cleft palate group was all significantly lower than the control group in both zone 1 and zone 2 when compared with similar stages of palatal development except for stage III in zone 2 (Figs. 2C,D).

Taken together, the quantitative amount of iNOS staining significantly increased in most of the palatal developmental stages of the cleft palate group when compared with the control group. The highest iNOS staining level was observed at stage II in zone 1 of the cleft palate group.

BMPs- 2, 4 and 7 immunostaining

Positive immunostaining of BMP-2, BMP-4 and BMP-7 was localized in the epithelia and the adjacent mesenchymes of the developing palatal shelves. The immunostains were more pronouncedly identified in the control groups (Fig. 1E, G, I) when compared to the cleft palate groups (Fig. 1F, H, J). Semi-quantitative scoring of the BMP staining was performed on the epithelial cells, condensed and non-condensed mesenchymal cells. When both halves of the palatal shelves fused and coalesced, no epithelial cell scoring was performed. In the control group, the intensity of BMP-2 staining increased from stage I to stage III. There was a significant four-fold increase in the intensity of BMP-2 staining in the condensed mesenchymal cells and a two-fold increase in the epithelial cells (Fig. 3A,B). Comparatively, the intensity of BMP-2 staining was significantly lower in the cleft palate group at stage III in the epithelial and condensed mesenchymal cells when compared with the corresponding controls. Meanwhile, the intensity of BMP-2 staining was relatively higher in the non-condensed mesenchymal cells than in the condensed mesenchymal cells (Fig. 3C).

There was about a 40% reduction in the intensity of BMP-4 staining at stage I in the epithelial cells between the control and the cleft palate groups (Fig. 3D). The intensity of BMP-4 staining was significantly lower in the cleft palate group than in the control group particularly at stage III in both the epithelial and condensed mesenchymal cells (Fig. 3D, E). Generally, the intensity of BMP-4 staining was higher in the non-condensed mesenchymal than in the condensed mesenchymal cells. Up to a two- to five-folds variation in the intensity of BMP-4 staining was detected between non-condensed and condensed mesenchymal cells at all stages (Fig. 3F).

The degree of BMP-7 staining at stage III was significantly reduced in the epithelial and condensed mesenchymal cells between control and cleft palate groups (Fig. 4A, B). The intensity of BMP-7 staining was higher in the non-condensed than in the condensed mesenchymal cells (Fig. 4C).

In summary, the expressions of BMPs-2, 4 and 7 were significantly down-regulated in the epithelial and condensed mesenchymal cells of the cleft palate group particularly at stage III. The non-condensed mesenchymal cells did not show a significant reduction in the expression of BMPs2, 4 and 7. On the contrary, the non-condensed mesenchymal cells expressed a

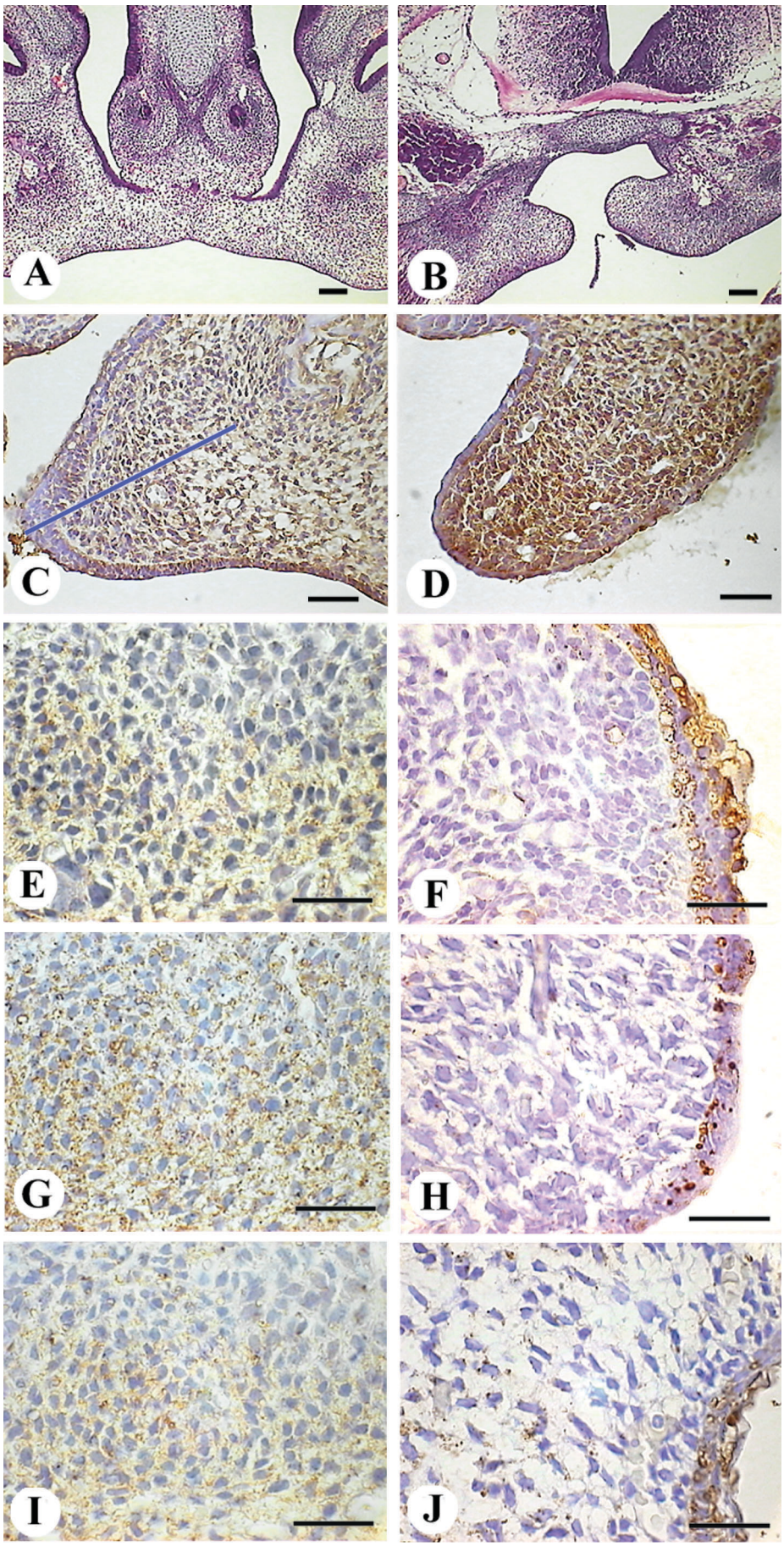

Fig. 1. Coronal sections showing palatal morphogenesis with H&E staining **(A-B)** and immunohistochemistry **(C-J)**. **A.** Complete fusion of palatal shelves by the end of stage III in the control group. Scale bar: 100 μm. **B.** Non-fusion of palatal shelves int he RA treated cleft palate group. Scale bar: 100 μm. (**C**) Control group with iNOS expression in the palatal epithelium and mesenchyme; Scale bar: 50 μm. **E, G, I.** Control group showing BMP-2 4 and 7 expression at the palatal shelf respectively. Scale bar: 50 μm. **F, H, J.** Down regulation of BMP-2 4 and 7 at the condensed mesenchyme in the cleft palate group respectively. Scale bar: 50 μm.

higher intensity of BMPs2, 4 and 7 at all stages of the palatal development than those of the condensed mesenchymal cells.

Discussion

Overexpression of iNOS

The present study has shown that RA induced cleft palate formation in the embryos of BALB/c mice with significant iNOS overexpression at stage II of the palatal development. Previous studies of iNOS were directed to normal and tumour cell types (Pfeilschifter and Vosbeck, 1991) with very little investigation in the growing palatal tissues. iNOS expression can be induced in a variety of cell types such as neutrophils, epithelial cells, endothelial cells, vascular smooth muscles, hepatocytes, pancreatic islet cells and chondrocytes. Studies have shown that RA alone does not cause overexpression of iNOS and that cellular interaction with lipopolysaccharides (LPS) via the LPS-triggered NOS-2 pathway may be necessary (Datta and Liano, 1999; Devaux et al., 2000). Paradoxically, *in vitro* studies showed that iNOS production might be inhibited by RA in vascular smooth muscles (Hirokawa et al., 1994; Umansky and Schirrmacher, 2001). For the first time, our results revealed that RA induced overexpression of iNOS in developing palatal tissues in mice without involving LPS. The iNOS induction mechanism may be due to the fact that the embryonic differentiating cells at the palatal shelves were relatively vulnerable and susceptible to external stress when compared to other mature body cell types. High levels (nmoles) of iNOS can be produced intracellularly and sustained for a long period of time. The detrimental effects associated with NO production may result in decreased interaction of the epithelial cells with the regional mesenchymal cells in the palatal shelves.

NO production facilitated by iNOS is a critical component of host defence against bacterial, viral and parasitic infections (Moncada, 1992; Nathan and Xie, 1994). In the presence of inflammation, NO can be produced one thousand times more via iNOS facilitation where many cytokines and macrophages are also activated. In contrast to other constitutive isoforms of NOS that are active only when intracellular calcium concentrations are elevated. iNOS can be activated when the defensive mechanism is at work. The regulation of

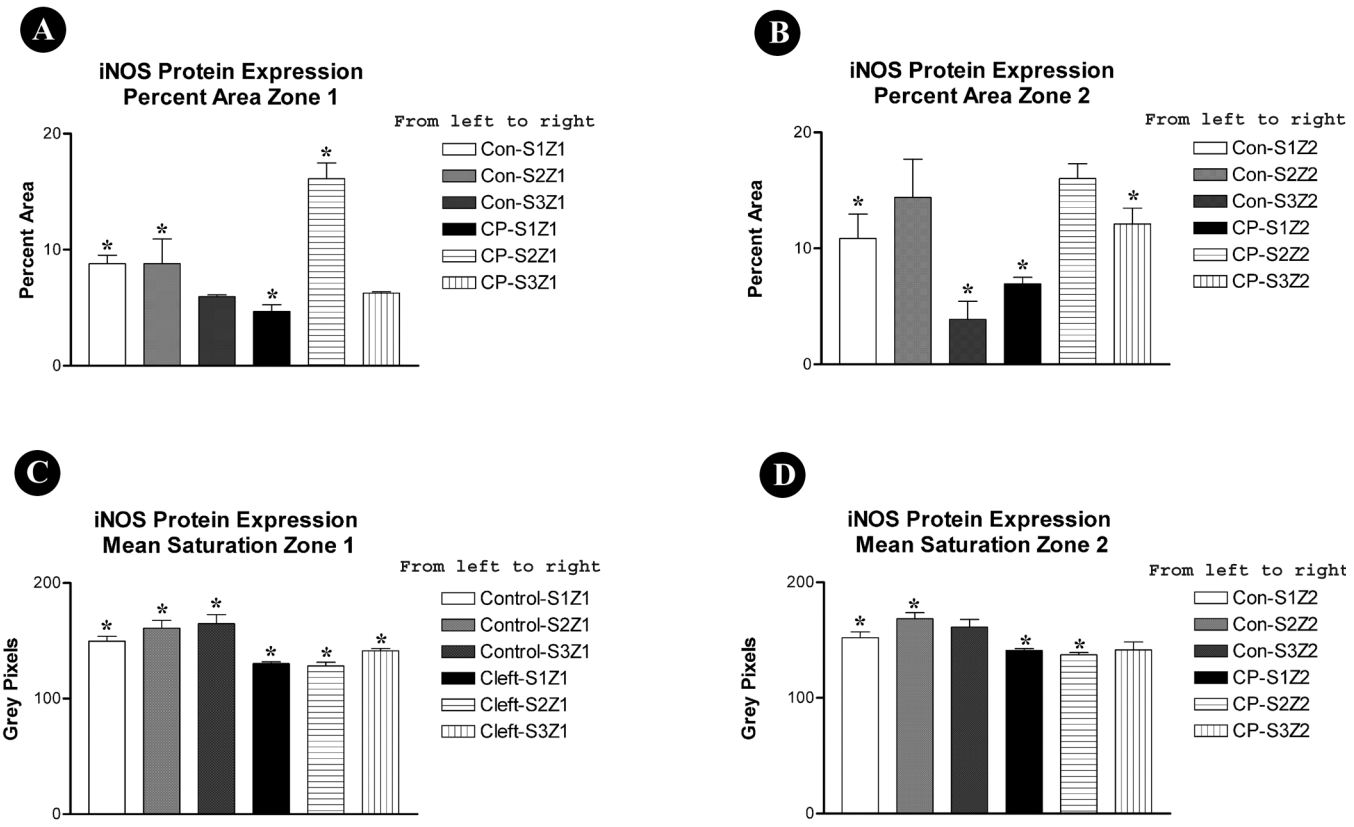

Fig. 2. iNOS expression expressed as mean percentage area **(A, B)** and mean saturation **(C, D)** in zone (Z) 1 and zone 2 using image analysis. Each column represents the mean ± SEM; statistically significant ($p<0.05$) differences between control (Con) and cleft palate (CP) groups at the same stage (S) are indicated by asterisks (*).

iNOS is primarily at the level of transcription. Transcription of iNOS can be induced by a variety of stimuli, including lipopolysaccharide, cytokines, and bacterial cell wall products. Large amounts of NO produced via iNOS are harmful to cells because they form peroxynitrite, which is a reaction product between NO and intracellular superoxide. Therefore, increased NO production may impute extensive cell damage

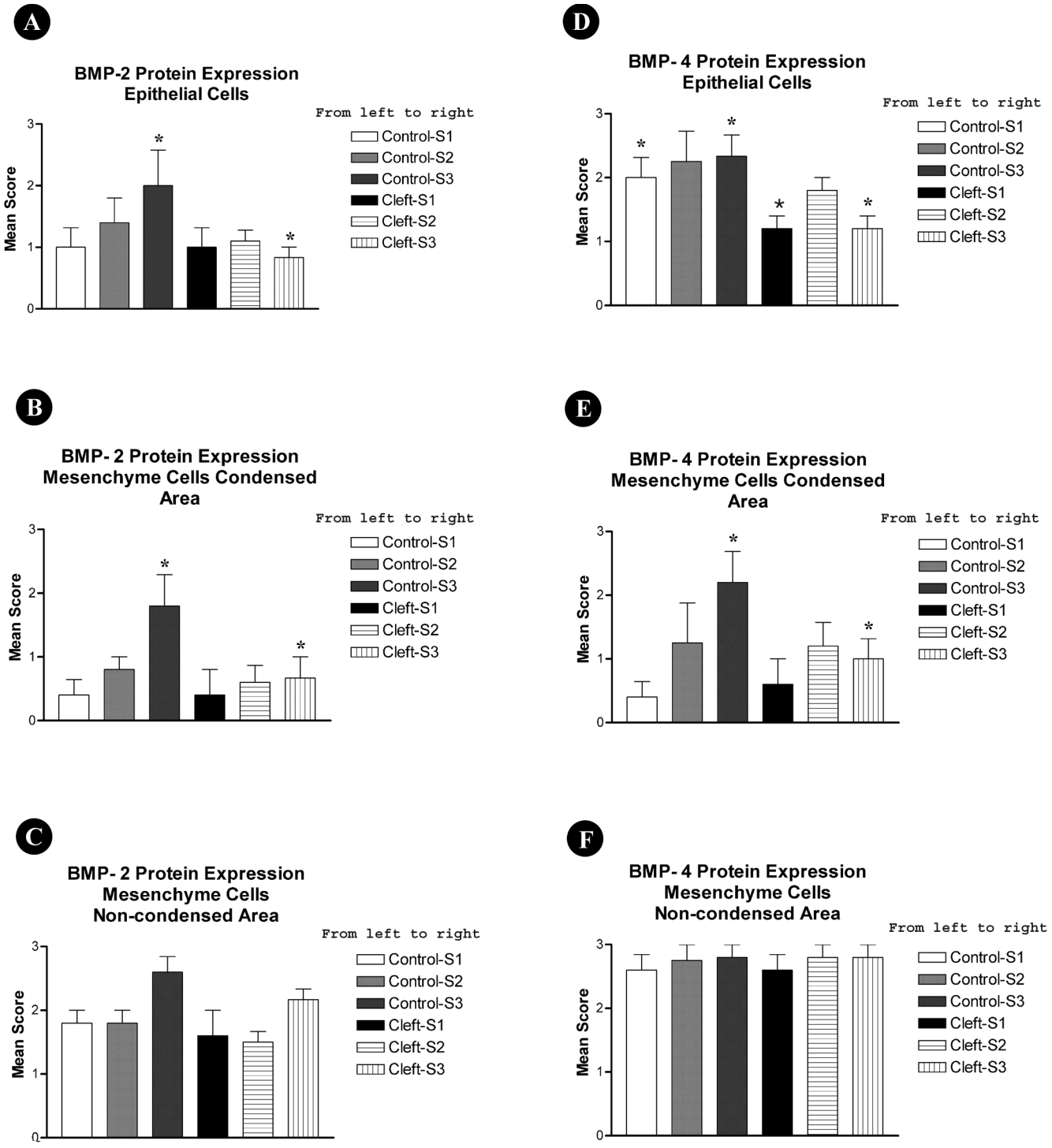

Fig. 3. Scoring of BMP-2 **(A-C)** and BMP-4 **(D-F)** expression in the epithelium, condensed and non-condensed mesenchyme. Each column represents the mean ± SEM; statistically signficant ($p<0.05$) differences between control and cleft palate group at the same stage (S) are indicated by asterisks (*).

including palatal mesenchymal cell injury. However, in normal developmental growth of the palatal shelves, NO is basally produced, either by the epithelial or neuronal-derived cells in a limited amount. In our study, we suggest that the basal production of iNOS in the normal palatal shelves, though still in a much lower amount than the cleft palatal shelves, could be related to a natural defence mechanism of the epithelial and mesenchymal cells against incidental microbial infection (Änngard, 1994). Without real-time quantitative measurement of NO, we have taken a cautious interpretation of our experimental data since overexpression of iNOS may not necessarily lead to a greater amount of NO production.

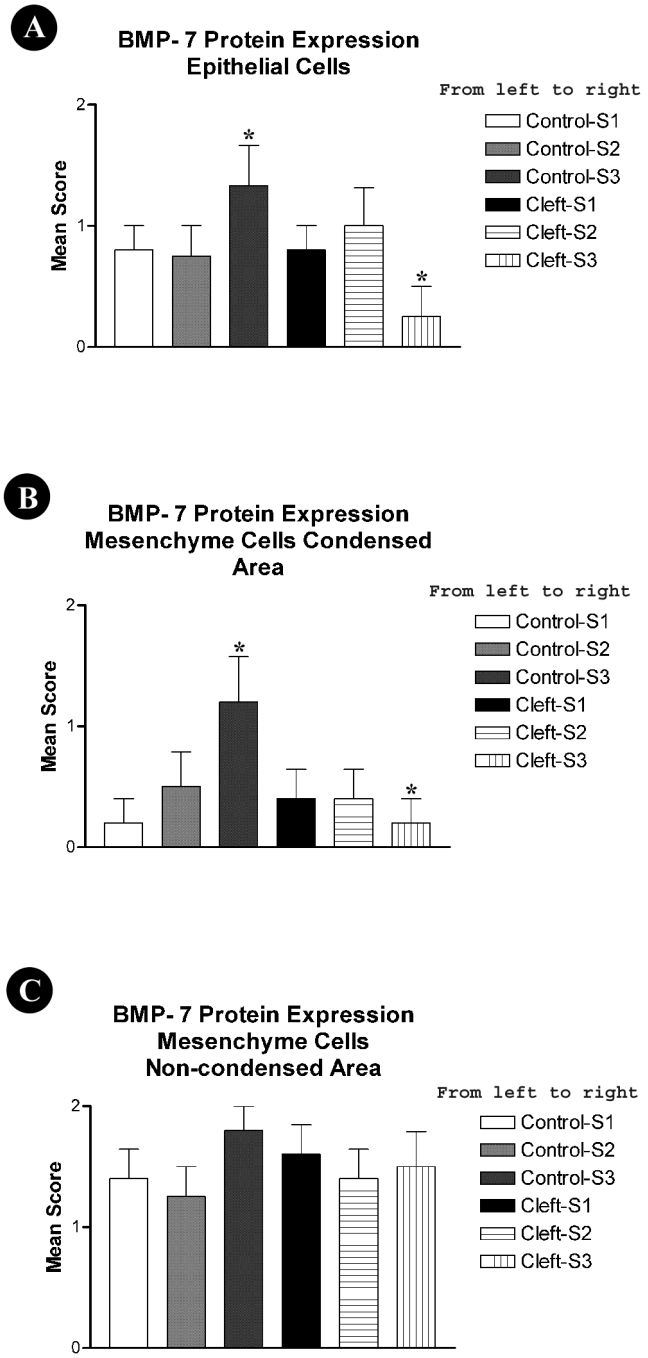

Fig. 4. Semi-quantitative analysis of BMP-7 (A-C) expression in the epithelium, condensed and non-condensed mesenchyme. Each column represents the mean ± SEM; statistically significant ($p<0.05$) differences between control and cleft palate group at the same stage (S) are indicated bty asterisks (*).

Current evidence shows that an increased level of oxidative DNA damage is related to increased incidence of apoptosis with overexpression of iNOS. Evidence of apoptosis in the ectomesenchymal stem cells during RA-induced cleft palate formation has also been reported. (Suwa et al., 2001). The possible mechanism of NO-mediated apoptosis involves accumulation of tumour suppressor protein p53, damage to mitochondrial functions, alterations in the expression of the Bcl-family members, activation of the caspase cascade and DNA fragmentation (Umansky and Schirrmacher, 2001). The exact mechanism as to how NO leads to DNA damage in cells undergoing apoptosis has not been completely clarified. One possible explanation is that the reaction of NO with oxygen radicals resulting in the production of highly toxic nitrous radical peroxynitrite. Peroxynitrite may attack aromatic amines such as pyrimidine and purine, finally leading to DNA strand breaks (Lindahl and Andersson, 1972). The effect of NO on nucleic acids has been determined for naked DNA in normal cells (Nguyen et al., 1992; Fehsel et al., 1995). In addition, NO produced by iNOS may react with ferrous-sulphate-containing proteins to form nitrosyl complexes, which lead to cellular damage (McDaniel et al., 1996).

The iNOS gene has Nuclear Factor-κB (NF-κB)-binding sites in the promoter regions. NF-κB activation is induced by oxygen radicals such as hydrogen peroxide and is repressed by antioxidants (Schenk et al., 1994). The expression of iNOS is regulated via NF-κB since the promoter of the human iNOS gene contains a regulatory DNA sequence to which NF-κB binds (Chartrain et al., 1994; Nunokawa et al., 1996). Studies have also shown that NF-κB activation may be associated with apoptosis. In particular, NF-κB stimulates the tumour suppressor gene p53 and the NF-κB subunit p65 (Velasco et al., 1997; Mahr et al., 2000), which interact with the cell-cycle inhibitor $p21^{WAF1}$ (Wu and Lozano, 1994; Bash et al., 1997). Grilli reported that blocking NF-κB activity could protect neurons against neurotoxicity (Grilli et al., 1996). Studies have shown that NF-κB activation can either be detrimental or protective, depending on the cell type in which it is expressed and the nature of the insult. Further study is necessary to correlate the activity of NK-κB and iNOS overexpression in cleft palate formation.

Down-regulation of bone morphogenetic protein

The effects of retinoids on gene expression are

mediated by mechanisms involving binding of the RA to nuclear retinoic acid receptors (RARs) α, ß and γ (Mangelsdorf et al., 1994) to form transcription factors. Investigations on tissue distribution of RAR mRNAs have demonstrated that RAR-α and RXR-ß, RAR-γ, RXR-α and RXR-γ mRNA are expressed in a selective manner (Mangelsdorf et al., 1994). The RAR type expressed in the palatal cells has not been determined. We propose that RA binds to its receptors followed by inactivation of downstream molecules such as bone morphogenetic proteins (BMPs). Therefore when the biological effects of BMPs are diminished across the epithelium to the mesenchyme, this will eventually affect the normal palatal shelf development both in its growth rate and its spatial morphology. In the present study, there was a significant reduction in BMP-2, 4 and 7 expressions at stage III of palatal development where the epithelial and the condensed mesenchymal cells were mostly affected. The relatively higher expressions of BMP-2, 4 and 7 in the non-condensed mesenchymal cells when compared to the condensed mesenchymal and the epithelial cells may be due to yet unidentified sources of paracrine stimulation from adjacent growth centers.

Transforming growth factors (TGF-ß), interleukin-4 (IL-4), IL-10 and corticosteroids have all been shown to suppress the induction of iNOS, but it is not clear whether these act at the level of the gene. TGF-ß is known to destabilize iNOS mRNA and decrease its translation (Moncada, 1992; Nathan et al., 1994). Results of our study has shown down-regulation of BMP- 2, 4 and 7 proteins in the cleft palate group at stage III with an equilibrium shift towards growth retardation. These findings concur with our previous data that showed that BMP-2 and BMP-4 mRNA expressions were both reduced (Lu et al., 2000). TGF-ß and related cytokines such as activins and BMPs regulate cell fate by controlling proliferation, differentiation and apoptosis and are therefore important for the development and maintenance of mammalian tissues. Previous studies of different isoforms of TGF-ß have pinpointed their importance spatially and temporally during palatal morphogenesis (Fitzpatrick et al., 1990). Both TGF-ß and BMPs are members of the TGF-ß superfamily. Recent reports from experiments in TGF-ß, especially the TGF-ß3 knock-out mice, have implicated the lack of TGF-ß as inducer of cleft palate of varying penetrance (Sanford et al., 1997; Koo et al., 2001). Other human studies also confirmed that TGF-b3 as one of the candidate genes (Scapoli et al., 2002). Cross-talk between pathways in embryonic palatal cells has been determined (Nugent and Greene, 1994; Nugent et al., 1998). Recently, convergence of the cAMP, TGF-ß and retinoic acid signaling pathways in palatal cells is found (Nugent et al., 2001).

In our animal model, BMP-2 and BMP-4 stimulated the undifferentiated mesenchymal cells in the palatal shelves to differentiate into osteoblast and chondrocytes to form the bony palate. It is possible that the observed BMPs reduction is the direct cross-talk by retinoic acid to the TGF-ß pathway. Alternatively, other mechanisms may be involved in a feedback towards either the iNOS or the BMP genes. The effects of these factors are brought about by regulating the expression of genes encoding key determinants of cell phenotype, such as cell-cycle regulators, differentiation factors and cell-adhesion molecules. There is an intriguing relationship between the epithelial and mesenchymal cells that may be interrupted in some ways where feedback stimulation is absent. Therefore, after exposure to retinoic acid, the amount of BMP-2, 4 and 7 proteins was reduced in the epithelial and condensed mesenchymal cells. When the mesenchymal cells failed to release growth factors to promote proliferation and differentiation of the epithelial cells, elevation of the developing palatal shelves failed to occur.

The present study strongly supports the hypothesis that there are molecular mechanisms involving regulations of iNOS and BMPs-2, 4 and 7 in the development of the cleft palate in BALB/c mice. Subject to the effects of RA, there was significant overexpression of iNOS, particularly at stage II of the palatal shelf development. There were also down-regulations of BMP-2, 4 and 7 expressions in the cleft palate group, particularly in the epithelial and condensed mesenchymal cells. The high iNOS expression possibly led to an increased production of NO. NO reacting with superoxides may induce irreparable cell damage that eventually triggers specific regulatory genes responsible for the apoptotic pathway. The reduction of the BMP-2, 4 and 7 expressions in the cleft palate group could also affect the normal cellular proliferation and differentiation during palatal morphogenesis.

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