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IMMUNOHISTOCHEMICAL ANALYSIS OF MORPHOGENS AND DEATH PROTEINS IN PALATAL FUSION

CHINH XUAN NGUYEN BDSc (WA) FRACDS

A thesis submitted in partial fulfilment of the requirements for the degree of

MASTER OF DENTAL SCIENCE (PAEDIATRIC DENTISTRY)

Discipline of Paediatric Dentistry
University of Sydney
February 1998
STATEMENT OF AUTHORSHIP

The experiments described in this thesis were performed at Westmead Hospital Dental Clinical School and the Institute for Clinical Pathology and Medical Research (ICPMR), between January 1995 and December 1997. Some work was carried out by Dr Neil Hunter and Dr John Gibbins and support staff in the Institute of Dental Research, Sydney. The work described is that of the author unless otherwise stated and has not been submitted in whole or in part for any other degree.

This research was presented at:
The Colgate Award Postgraduate Student Presentations
The Australasian Academy of Paediatric Dentistry
Sydney, Australia
August 1997

[Signature]

12 January 1998
ACKNOWLEDGEMENTS

To my research supervisor, Dr Neil Hunter, I would like to express my respect, admiration and gratitude. He has brought to the Department of Paediatric Dentistry and to me, in particular, an astonishing scientific knowledge, especially in the field of developmental biology. His tireless work, expert guidance, powerful vision and dedication throughout the experimental and documentary stages of this study are invaluable and acknowledged and I give my greatest appreciation.

I would also like to express my special thanks to Dr John Gibbins, whose extensive understanding of the cell biology and development was a wonderful reference point. He has given me a bank of scientific knowledge and a great deal of encouragement.

To my clinical supervisor, Associate Professor Richard Widmer, who has made Paediatric Dentistry become a vital part of my life. His constant support, dedication, and above all his unique sense of humour have given me the most enjoyable and special quality training in Paediatric Dentistry. Also my special thanks to Dr Angus Cameron, to whom I am indebted for shaping my education and assistance with some graphical aspects of this thesis. And lastly but not least, it is my tribute to Dr Nicky Kilpatrick, who gave me significant support during the course of my training.
The support given to me by the technical staff at the Dental Research Support Unit and the Institute for Clinical Pathology and Medical Research, Westmead Hospital, has not gone unnoticed and they deserve my sincere thanks.

This work is dedicated to my family, especially to my mother, who has supported me over the years in all my endeavours and shares with me every moment of pride and happiness in my achievements. Without my family’s constant support and encouragement, this work would not have been completed and I present this to them as a token of my heartfelt thanks and respect.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma/leukaemia-2 gene</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CG-rich 5'-UTR</td>
<td>Cytosine guanine-rich 5'-untranslated region</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DVR</td>
<td>Decapentaplegic-Vg-related</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transformation</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycoaminoglycan</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>H_{2}O_{2}</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin converting enzyme</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL3</td>
<td>Interleukin 3</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>M phase</td>
<td>Mitotic phase</td>
</tr>
<tr>
<td>MART</td>
<td>Microwave antigen retrieval technique</td>
</tr>
<tr>
<td>MEE</td>
<td>Medial edge epithelium</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotides</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>Platelet-derived growth factor-A</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>S phase</td>
<td>Synthetic phase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TGF-β1,2&amp;3</td>
<td>Transforming growth factor-beta1,2&amp;3</td>
</tr>
<tr>
<td>TGF-βR</td>
<td>Transforming growth factor-beta receptor</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor-alpha</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP-biotin nick end-labelling</td>
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ABSTRACT

Immunohistochemical Analysis of Morphogens and Death Proteins in Palatal Fusion.

Palatal fusion is a critical aspect of oro-facial development which remains poorly understood. The secondary palate is formed by fusion of the apposing shelves, mediated by the dispersion of the mid-line epithelial seam to allow mesenchymal confluence. Three theories have been proposed to account for the dispersal of the medial edge epithelium (MEE). These are:

(1) Programmed cell death (PCD or Apoptosis)
(2) Epithelial-mesenchymal transformation (EMT)
(3) Migration of epithelial cells from the seam to nasal and oral epithelial surfaces.

Proteins of the transforming growth factor-beta (TGF-β) family have the potential to regulate all three aspects of epithelial seam response. On this basis, utilising a focal rat model, specific antibodies were used to probe for the presence of the morphogens of interest and the corresponding receptors.

Another protein family Bcl-2 and Bax is reported to influence cellular susceptibility to death. Bax is known to accelerate apoptotic cell death induced by cytokine deprivation,
whereas Bcl-2 blocks apoptosis and confers a death sparing effect.

The results obtained indicate a strong expression of TGF-β family and Bcl-2/Bax in the epithelial seam, especially in the presence of dead seam cells, and that this precedes the lateral spread of reactivity to the oral and nasal aspects of the palatal epithelium. Furthermore, a fine tuning in the expression of Bax/Bcl-2 in coordinating cellular activities, rather than purely antagonistic in nature, was observed. The expression of Bcl-2 and Bax appeared to correlate with the sequence of the palatal fusion process rather than with the death of specific cells. In addition, the expression of TGF-β1,2,3 and Bcl-2/Bax was intense during the fusion stage of palatal development. In the pre-fusion and post-fusion stages, the expression of these proteins was much weaker and more scattered.

The results also showed that TGF-β1,2,3 are expressed in a coordinated manner and that TGF-β3 was not the dominant protein in the fusion process. Of note, also TGF-β RI&II had similar patterns of expression as to those of the TGF-βs. This suggests that TGF-βs might have either autocrine or paracrine action or both in driving the three possible processes in palatogenesis.

The data obtained are compatible with an important role for the morphogens in the sequence of palatal fusion. The known functions and mechanisms of action of these proteins provide a solid foundation for understanding this aspect of cranio-facial development.
AIMS

• To investigate the presence of the morphogenic proteins TGF-β1,2,3 and the corresponding receptors and Bax/Bcl-2 in relation to the palatal fusion process.

• To determine whether the TGF-β family has the potential to regulate all three proposed mechanisms for dispersion of the mid-line seam.

• To evaluate the relative importance of individual TGF-βs at different stages of palatal development.

• To evaluate the relative importance of Bcl-2 and Bax at different stages of palatal development.
INTRODUCTION

Palatogenesis in mammalian embryos involves a complex series of events including tissue movements, epithelial-mesenchymal interactions, cell differentiation and death, migration and transformation {Ferguson MWL 1988}. Soluble growth factors, extracellular matrix molecules and the cellular receptors for both play an interactive role during these processes {Sharpe PM, et al. 1988}. Perhaps the most critical stage in mammalian palatal development is the formation of a mid-line seam between medial edge epithelial (MEE) cells of the apposing palatal shelves. Subsequent disruption of this epithelial seam leads to mesenchymal continuity and an intact, fused palate. The expression of many growth factors in the region of the medial edge suggests that they may play an important role in palatal fusion. These factors include epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) {Abbott & Birnbaum 1990} {Dixon MJ, et al. 1991}, transforming growth factor-β (TGF-β) {Heine UI, et al. 1987} {Gehris, D'Angelo, et al. 1991}, fibroblast growth factors-1 and 2 {Sharpe PM, et al. 1993}, insulin-like growth factor II {Ferguson MWJ, et al. 1992} and platelet-derived growth factor-A {Qui CX, et al. 1995}. All are present in the medial edge epithelial seam, or underlying mesenchyme, at the time of palatal fusion. However, the evidence for a physiological function of any one of these growth factors has been largely circumstantial, relying on immunohistochemical and in situ hybridization data which establish only that a given factor is expressed at the right time and in the right place. Further definition of the developmental roles of palatal growth factors came from organ
culture experiments in which a given growth factor was added to palatal tissues and the
effect on subsequent development observed. This approach revealed that exogenous
TGF-β1,2,3 accelerated palatal fusion {Dixon MJ, et al. 1992} {Gehris & Greene
1992} {Brunet CL, et al. 1993}, exogenous EGF (or TGF-α) blocked fusion {Brunet CL,
et al. 1993}, while many growth factors altered the extra-cellular matrix composition of
cultured palatal tissue {Foreman, Sharpe, et al. 1991}. While such findings are
interesting, they may represent pharmacological actions of exogenous growth factors
rather than physiological functions of growth factors endogenous to the developing
palate.

The three main TGF-β isoforms (TGF-β1,2,3) are expressed in a temporally and spatially
regulated manner throughout palatogenesis. In general, the expression patterns are non-
overlapping suggesting that each isoform may have a specific function {FitzPatrick,
Denhez, et al. 1990} {Gehris, D'Angelo, et al. 1991}. It has previously been demonstrated
that the application of specific antisense oligodeoxynucleotides (ODN), to prevent
mRNA expression, and neutralizing antibodies to block the activity of the active peptide
of TGF-β3, but not TGF-β1 or TGF-β2, prevented the epithelial-mesenchymal
transformation of the medial seam and inhibited fusion. This inhibition could be rescued
by exogenous TGF-β3 {Brunet, Sharpe, et al. 1995} {Potts JD, et al. 1991}.

All three TGF-βs are expressed in epithelial cells at stages of embryonic development,
and all are potent growth inhibitors of epithelial cells in vitro, although TGF-β2 shows
the most widespread epithelial expression. Furthermore, some epithelial cells are induced
to differentiate in the presence of TGF-β {Grayar JL, et al. 1989}. Whether epithelially-derived TGF-βs are mainly involved in autocrine regulation of growth and differentiation and/or in paracrine interactions between the epithelium and the underlying mesenchyme, remains to be determined.

TGF-β1 has been demonstrated to be synthesised by epithelium and is predominantly localised in the adjacent mesenchyme. In general, it has been shown that the embryological localisations of RNA transcripts and protein product for TGF-β1 show a strong correlation indicative of both autocrine and paracrine mechanisms. Furthermore, where epithelial TGF-β1 mRNA expression is seen, this mRNA is co-expressed with either TGF-β2 and/or TGF-β3 mRNA. Therefore, TGF-β2 and β3 might similarly be involved in the general control of morphogenesis {Lehnert SA, et al. 1988}.

Unlike tgf-β1 and β3, epithelial expression of the tgf-β2 gene is not only limited to morphogenetically active tissue, but is also seen in epithelial cells of established structures that are in the process of differentiation {FitzPatrick, Denhez, et al. 1990}. Glick showed that TGF-β2 levels are elevated in vitro and in vivo in growth-inhibited keratinocytes or those induced to differentiate by retinoic acid or by calcium ions, respectively. Further, the growth-inhibitory effects of retinoic acid in vitro can be blocked by antibodies to TGF-β2 {Glick AB, et al. 1989}. It was speculated that the endogenous in vivo function of TGF-β2 in some epithelia is in autocrine modulation of growth and/or differentiation {Pelton RW, et al. 1989}. 
The distribution of Bcl-2 in human foetal tissues suggests an important potential role of this morphogenic protein in general development and morphogenesis (Lebrun DP, et al. 1993). The presence of Bcl-2 at several sites characterised by inductive interactions between epithelial and mesenchymal structures may be critical in the embryological formation of some structures by decreasing the focal rate of cell death. This implication for Bcl-2 in regulating active cell death, such as during the developmental process is considered important in biological research.
CHAPTER 1

LITERATURE REVIEW

A. DEVELOPMENT OF THE PALATE

1. Introduction

This is a complex series of events determined by both genetic signals and environmental influences. Any disturbance to this multifactorial process can lead to deformities of lip and palate, the incidence of which is approximately 1:1000 live births depending on racial differences and whether foetal deaths are counted {Vanderas AP 1987}.

The formation of lip and palate extends over 15 days in humans. During this long period developing systems may be simultaneously affected by teratogenic influences. The current concept of the failure which leads to facial clefting is that this occurs when the developing facial prominences fail to obliterate the developmental grooves. This may be due to a lack of merging of the processes, or failure of mesodermal migration across the merged boundaries with abnormal persistence of the groove.
Extra-oral photograph of an infant showing maxillary bilateral cleft lip and palate.
2. Cleft Lip/Palate

Cleft lip and cleft lip and palate are aetiologically different from isolated cleft palate. In general, males have more lip clefts (cleft lip and alveolus; 61%) and clefts of lip and palate (67%) than females. Females, on the other hand, have more palatal clefts (54%) than males. Left-sided clefts are more common than the right-sided ones for lip (75%) and for lip and palate (66%). When clefts of lip and palate occur bilaterally, the left side is more severely affected than the right side in 74% of cases {Hall RK 1994}.

Refer to Figure 5.

The incidence of clefts:
- Cleft lip and alveolus: 16%
- Cleft lip and palate: 45%
- Cleft palate alone: 39%
{Hall RK 1994}

(a) Classification

(i) Clefts of the lip:

This occurs due to failure of both maxillary and nasolateral prominences to merge with the nasomedial prominence, although the nasolateral prominences unite successfully with the maxillary prominences.

Lip clefts may vary in extent from a notch in the vermillion border to a partial or complete cleft of the lip and alveolus, with the involvement of the anterior nasal floor and
distortion of the alar cartilage and nostril. Often, disturbance of the dental lamina within the alveolus is seen as a fusion, germination, a supernumerary tooth, or abnormal crown form or congenitally missing teeth. Such disturbances are present in both deciduous and permanent dentitions.

(ii)  Clefts of the lip and palate:

This is due to more extensive failure of fusion, of palatal processes and of nasolateral and nasomedial processes. The bony palatal shelf in the unilateral cleft is usually attached to the vomer on the non-cleft side. The vomer is unattached in bilateral cases, ending anteriorly at the pre-maxilla which, being unrestrained by the normal functional matrix of intact bony arch and lip musculature, shows excessive growth.

Presurgical orthopaedic treatment is carried out when the lateral palatal shelves are severely distorted and there is unrestrained, excessive growth of the maxilla and vomer. This is done by guiding future growth of the maxilla in a normal direction, and by restraining further excessive growth of the premaxillary/vomer complex.

(iii)  Clefts of the palate:

Disturbances occurring between the 5th and 12th week of gestation will result in clefting of soft and hard palates. Three palatal processes develop to separate nasal and oral cavities. The primary palate (median palatine process) develops during the 5th week from fusion of the two lateral nasal processes (frontonasal prominence), and the secondary palate develops from the two lateral palatal processes of the maxillary processes.
Membrane bone forms the premaxilla and maxillary and palatine bone extends into the lateral palatine processes to form the hard palate.

(iv) **Submucous cleft palate:**

This defect occurs when there is incomplete muscle union in the mid-line of the soft palate due to failure of the palatal mesenchyme to merge at the time of the ectodermal embryonic palatal fusion. As a result, the intrinsic and extrinsic palatal musculature is attached only to the posterior border of the hard palate. The oropharyngeal and nasopharyngeal mucosa may be intact and appear normal. The uvula may be partially or completely bifid (overt submucous cleft) or grooved (occult submucous cleft). A bluish or translucent ‘line’ may be seen in the mid-line of the soft palate, and the depression as well as a bony notch can be palpated in the mid-line of the posterior border of the hard palate. Nasal escape of air due to velopharyngeal incompetence can be heard during speech. Pharyngeal incompetence can also occur in the absence of a submucous cleft, if the soft palate is relatively immobile or short or if the nasopharynx is unusually deep {Hall RK 1994}.

It is clear that disruptions to any one or more of the critical mechanisms of normal palatal development may result in the birth defect of cleft palate. In cleft lip and palate, the cleft in the palate is largely a result of the preceding cleft in the lip. The tongue becomes trapped above the primary cleft, which increases the resistance to shelf elevation and so a cleft in the secondary palate results.
(b) **Pathogenesis**

Clefting can result from any one of the following disruptions in normal developmental mechanisms:

(i) **Inhibition of cell division and migration:**
  - Palatal shelves too small to meet.

(ii) **Failure of shelf elevation at the correct time**
  - Altered synthesis of glycosaminoglycans and collagen
  - Inhibition of palatal cell contraction
  - Interference with neurotransmitter synthesis or receptors
  - Vascular insufficiency or haemorrhage
  - Excessive resistance factors, e.g. tongue resistance

Postural moulding defects like Pierre Robin syndrome are caused by contraction of the foetal membranes due to the insufficiency of amniotic fluid. This traps the mandible beneath the sternum and consequently leads to increase in tongue resistance.

(iii) **Excessive head width**

Asynchrony of the growth plateau in head width and the timing of shelf elevation can lead to failure of fusion as the palatal shelves are pulled laterally by growth in head width. Both racial and sex differences have been reported to be associated with this
phenomenon. The occurrence of cleft palate is rare in African-Americans, common in Caucasians and frequent in Mongoloid races. This correlates with facial, shape, particularly facial width {Shaw WC 1993}.

(iv) **Failure of shelf fusion.**

This is associated with:

- Defective mesenchymal signalling of critical epithelial-mesenchymal interactions
- Failure of medial-edge epithelial cell adhesiveness (surface coat and desmosomes)
- Failure of medial-edge epithelial cell migration
- Failure of differential gene expression
- Mechanical disruption or excessive shelf movement

{Shaw WC 1993}.

(v) **Post-fusion rupture**

- Causes yet to be identified

{Shaw WC 1993}. 
(c) Cleft Lip/Palate genes and teratogens

(i) Introduction

Little is known about the molecular genetics of cleft palate although there are some indications that genes positionally close to the major histocompatibility locus may be involved. Also there are associations between facial clefting and mutations in the tgf-α locus. The gene involved in X-linked cleft palate has recently been mapped to the XQ13-XQ21 regions of the human X chromosome. Genes coding for various extra-cellular matrix molecules such as collagen types I, III, V, XII, glycoaminoglycans (e.g. hyaluronic acid), proteoglycans, tenascin, and regulatory molecules including epidermal and fibroblast growth factors, TGF-β3 and corresponding receptors are important in palatal development. Because many intracellular signalling pathways used by growth factors can be subverted by oncogene products, it seems likely that certain proto-oncogenes may be important in palatal development, e.g. erb-B and c-sis. Further, it also seems likely that cytokeratin genes may be important for regional palatal epithelial differentiation. Recently, a family of genes called homeobox genes (master genes) has been implicated in the regulation of development of cartilage, muscle, and bone {Shaw WC 1993}.

Numerous teratogens such as retinoids, corticosteroids, aspirin and diazepam can cause clefting in animals and man. Due to the multifactorial pathogenesis, a genetically susceptible mother will not produce a cleft palate child unless she is exposed to certain
noxious environmental factors in correct dose, possibly in the correct combinations, and at the correct time during pregnancy (6-10 weeks of gestation) {Shaw WC 1993}.

(ii) Incidence

(a) Cleft lip with/without cleft palate

If both parents are normal, the risk for another child to have a cleft is 4%. However, if one parent and one child are affected, there is 12% risk to subsequent sibs. Clefting, however, also occurs in association with other abnormalities, in some cases chromosomal aberrations, and in others a mutant gene may be responsible.

(b) Cleft palate

If both parents are normal, the risk to subsequent siblings is 1 in 50. However, when both parents are normal but a relative is affected, the risk is 1 in 10 for subsequent siblings. The risk is 1 in 6 when one parent is affected {Shaw WC 1993}.

An understanding of normal palatal formation is essential in understanding the mechanisms of clefting.
3. **Embryology**

During craniofacial development, the nasal pits, bounded by the lateral and nasal processes, deepen forming two blind-ended sacs bounded by an epithelial wall. The epithelial wall of the nasal pit forms a contact with the epithelium of the roof of the stomodeum forming the oronasal membrane. This membrane ruptures creating a communication of the nasal cavity and the stomodeum through the primitive choana {Ten Cate 1987}.

The palate develops from primary and secondary components, which makes the distinction between oral and nasal cavities possible. The frontonasal process forms the primary palate, which is a triangular, anterior portion of the palate, extending from the alveolus anteriorly to the upper right and left canines, and posteriorly to the incisive foramen. Further, the frontonasal process also forms the primary nasal septum as a ‘sculpturing’ process by the invagination of the nasal pits. The final form of the primary palate and nasal septum is due to subsequent differential growth. Recent morphological observations on fresh human embryos and specific labelling of cells in the medial, lateral, and maxillary processes have shown that the maxillary process completely overgrows the medial nasal process to join the contralateral counterpart in the mid-line and thereby excludes the frontonasal process from forming any part of the definitive upper lip {Ten Cate 1987}.
The formation of the secondary palate occurs during the sixth and eighth weeks of human development (15\textsuperscript{th} day in rat) behind the primary palate, from the maxillary processes. As the head extends from the thoracic cage, the tongue descends between parallel palatal processes which swing upwards and medially apposing each other in mid-line (human: 7\textsuperscript{th} week, rat: 16\textsuperscript{th} day). The free edges of the palatal processes fuse firstly with the posterior margin of the primary palate and then with each other in an anterio-posterior direction \{Yin XM, \textit{et al.} 1987\}.

Anteriorly, along the mid-line, the superior surface of the palatal processes attain fusion with the free inferior border of the nasal septum. At the junction of the primary and the secondary palates, the incisive foramen is formed. With the formation of the nasal septum and the palate, the original stomodeum becomes subdivided into nasal and oral cavities. The completion of palatogenesis is achieved by the 12\textsuperscript{th} week in human (rat:18\textsuperscript{th} day) \{Yin XM, \textit{et al.} 1987\} \{Mjor I 1986\}.

Refer to Figures 1, 2&3.
STAGES OF PALATAL DEVELOPMENT

Figure 1: Pre-fusion stage of palatal development corresponds to developmental sequence A&B.
Figure 2: Fusion stage of palatal development corresponds to developmental sequence C&D.
Figure 3: Post-fusion stage of palatal development corresponds to developmental sequence E&F.
a. Palatal shelf elevation

The mechanism is not fully understood, but it appears to involve a combination of mechanical and intrinsic forces. Initially, the palatal processes are vertically adjacent to the tongue. As the cranium rotates away from the cardiac prominence and the mandible enlarges, the tongue descends from between the palatal shelves. Other mechanical factors thought to contribute to shelf elevation are continued growth in cranial height and size of the oronasal cavity (Dievert VM 1978), and myoneural maturation of the tongue (Wragg LE, et al. 1972).

The intrinsic forces involved in palatal shelf elevation are multifactorial. The glycosaminoglycans (GAG’s) within the extra-cellular matrix of the palatal shelf mesenchyme are predominantly hyaluronic acid which is highly electrostatic and causes osmotic changes. The hydration and expansion of the hyaluronic acid may result in an erectile force directed by bundles of type I collagen which run down the center of the vertical palatal shelves (Ferguson MWJ, et al. 1987) (Foreman, Sharpe, et al. 1991).

The other intrinsic factor that may play an important role in palatal fusion is the contraction of individual mesenchymal cells within the palatal shelves, which can exert an elevating force. These cells contract under the control of neurotransmitters, including serotonin and acetylcholine, which are released by nerves and palatal mesenchyme (Zimmerman EF, et al. 1984).
Moreover, palatal shelf elevation occurs in a conductive milieu of craniofacial growth. At the time of elevation, there is constant growth in head height but almost no growth in head width. Shelf elevation therefore occurs on the growth plateau in head width. Cleft palate may occur when the palatal processes are incapable of physically meeting one another. This phenomenon may help to explain the occurrence of an isolated cleft palate, which is more common in females than in males. In general, female embryos elevate their palatal shelves approximately 7 days later than their male counterparts. This places the female embryos closer to a threshold in head width beyond which cleft palate will result. Consequently there will be more spontaneous cases of cleft palate in female embryos {Shaw WC 1993} {Mjor I 1986}.

b. Palatal shelf fusion

The palatal shelves consist of a mesenchymal tissue core covered by epithelium. As the shelves appose in horizontal position, the outermost layer of epithelium lining, the periderm, is shed, exposing the medial edge epithelium (MEE) for fusion {Waterman RE, et al. 1974}. Upon the shedding of periderm, the MEE of each shelf becomes apposed in the mid-line resulting in the formation of a mid-line epithelial seam. The MEE exhibits surface glycoprotein coating which imparts specific adherence properties {Greene RM, et al. 1976}. Desmoplakin, a specific adhesion glycoprotein is assembled within the MEE just prior to and upon shelf contact {Ferguson MWJ, et al. 1984}. The apposing MEEs are tightly bound by desmosomes following the initial contact of the shelves {Chaudhry AP, et al. 1973}. To enable fusion of the palatal shelves, it is necessary that the epithelial
seam break down and become discontinuous in order for the mesenchymal core of the palatal shelves to become confluent. Even though the epithelial cells of the seam divide, growth of the seam fails to keep pace with palatal growth so that the seam first thins to a single layer of cells and then breaks up into discrete islands of epithelial cells. The basal lamina surrounding these cells is then lost, and the epithelial cells lose epithelial characteristics and assume fibroblast-like features. Microscopically, not all the epithelial cells break up, rather, a few of them remain embedded (epithelial rests), and it is possible that these can multiply and form cysts. The epithelium on the oral aspect of the palate differentiates into stratified, squamous cells and those on the nasal aspect of the palate into pseudostratified, ciliated columnar cells with associated goblet, mucus-secreting cells \{Ten Cate 1987\} \{Brand RW, et al. 1990\}.

Refer to Figures 4&5.
Figure 4: Development of the human palate, ventral and coronal views at 6.5, 7, 9 weeks respectively. A, palatine processes in vertical position on either side of the tongue. B, palatine processes in horizontal position prior to fusion. C, palatine processes fused with each other and with the nasal septum, thus establishing the definitive nasal and oral cavities. (Adapted from Ten Cate, 87)
Figure 5: Palatal and lip clefts seen in ventral view. A, normal; B, cleft lip and alveolus; C, cleft lip and primary palate; D, unilateral cleft lip and palate; E, bilateral cleft lip and primary palate; F, bilateral cleft lip and palate; G, cleft palate only; H, bifid uvula. (Adapted from Ten Cate, 87)
B. THEORIES PROPOSED FOR THE FATE OF THE MIDLINE EPITHELIAL SEAM:

(1a) Apoptosis

There are two unique types of cell death which are distinct in their applications, morphological appearances and biological processes. These are necrosis and apoptosis (sometimes regarded as synonymous with programmed cell death).

Necrosis can be viewed as a pathological form of cell death that occurs as a result of severe and spontaneous injury. Due to the nature of this process, it is common to see groups of necrotic cells within specific areas of interest. Cells undergoing necrosis show early changes in mitochondrial shape and function associated with the loss of plasma membrane integrity and cellular homeostasis. The end stage of necrosis is the swelling of necrotic cells which then burst and either undergo autolysis or are removed by phagocytes. The process of phagocytosis often involves the release of proteolytic enzymes or the generation of reactive oxygen metabolites with the discharge of cellular contents into the surrounding tissue spaces causing damage to adjacent cells and evoking an inflammatory response. The degradation of DNA in necrotic cells occurs as a result of chromatin digestion by lysosomal proteases and endonucleases. The proteases destroy the histones and expose the entire length of DNA to nucleases and this can be shown as a smear pattern on gel electrophoresis {Wyllie AH 1993}. 
In contrast to necrosis, in apoptosis the mitochondria continue to function normally but early changes are seen within the nuclear compartment. The affected cells later shrink in volume and lose microvilli and cell-cell junctions. The chromatin rapidly forms dense crescent-shaped aggregates and complex invaginations develop in the nuclear membrane, resulting in a segmented nucleus. The plasma membrane further becomes convoluted so that the cell becomes separated into a cluster of membrane bound “apoptotic bodies” which often contain morphologically normal mitochondria and other cellular organelles {Kerr JFR, et al. 1972}. The altered surface characteristics of the apoptotic bodies act as signals to phagocytes and adjacent viable cells to phagocytose the dying cells. Interestingly, the lysosomal enzymes of the apoptotic cells do not participate in the cell death process, although lysosomal activation occurs within the phagocytic cells. Further, as there is little leakage of cellular contents into the surrounding tissue space during the removal of apoptotic cells, no inflammatory response is evoked {Morris RG, et al. 1984} {Wyllie AH 1993}.

(i) Physiological and pathological roles of apoptosis

Apoptosis occurs in a wide range of physiological and pathological stages of development. There has been extensive research in the area of clonal selection of thymocytes to study the roles of apoptosis. During thymic development, pre-T cells enter the thymus and re-arrange the genes for the antigen receptor. At the completion of this process, cells can differentiate into mature T cells possessing unique receptor characteristics and are then selected for further maturation (positive selection). When the
coupling between antigen receptor and antigen is inappropriate, the T cells undergo apoptosis and are deleted (negative selection) {Sentman DL, et al. 1991}.

Apoptosis has been implicated in cell-mediated immunity, response to viral infection, development of autoimmunity and regression of neoplasms. Cells bearing foreign antigen can be killed through apoptosis by effector T cells, via cell-mediated immunity. It is important to note that cytotoxic T lymphocytes can eliminate target cells without sustaining damage. On the other hand, T cells infected with virus can limit viral replication by undergoing self-death mediated by apoptosis. Furthermore, failure to deplete self-reactive T lymphocytes by apoptosis has been suggested to be responsible for autoimmune disease {Cohen JJ, et al. 1992}.

It is possible that in tumour development apoptosis may eliminate cells carrying cancerous genes and repress the development of neoplasms; failure of apoptosis can result in progression of neoplasia. Further, apoptosis may trigger progression of neoplasms by affecting cell cycle progression through other signals {Ueda N, et al. 1994}.

(ii) Metabolic changes during apoptosis:

The processes involved in the DNA fragmentation of apoptosis have not yet been identified. The mechanisms that have been proposed include:

- Rise in cytosolic calcium
- Altered expression of mRNA
- Alteration in protein synthesis.

A variety of stimuli has been shown to cause an uncontrolled rise in cytosolic calcium prior to DNA cleavage and cell death \{Ueda N, et al. 1994\}. Cohen and Duke proposed that the rise in intracellular calcium concentration may activate calcium-dependent endonucleases \{Cohen JJ, et al. 1992\}. Mixed findings are observed in cells depleted of extra-cellular calcium or following chelation of intracellular calcium, i.e. apoptosis does occur in some cells \{Batistatou A, et al. 1993\}.

The biochemical and morphological changes caused by ATP are preceded by a rapid increase in the cytoplasmic calcium of the susceptible cell. Calcium fluxes alone, however, are not sufficient to cause apoptosis, as the pore-forming protein, perforin, causes cell lysis without DNA fragmentation or the morphological changes associated with apoptosis. Therefore, ATP can cause cell death through two independent mechanisms, one of which, requiring an active participation on the part of the cell takes place through apoptosis \{Zheng, Zychlinsky, et al. 1991\}.

Apoptosis appears to be dependent on synthesis of mRNA and protein, because it can be arrested by application of inhibitors shortly after the stimulus is applied. However, the same inhibitors of mRNA and protein synthesis do not affect or stimulate apoptosis in other cell types. It is important to stress that there is no common biological sequence of metabolic events for cells during apoptosis, and that different cell types have distinct
regulatory systems in response to a variety of stimuli that can determine whether or not the cell undergoes apoptotic death {Cohen JJ, et al. 1984}.

(iii) **DNA fragmentation in apoptosis:**

During apoptosis, the nuclear chromatin is cleaved by the action of endogenous Ca^{2+}/Mg^{2+}-dependent endonucleases that are inhibited by zinc {Cohen JJ, et al. 1984}. Characteristically, DNA is cleaved into oligonucleosomal fragments of length 180-200 base pairs, which can be used as a biochemical marker for apoptosis. Further, the fragmentation results from double stranded breaks in the linker regions between the nucleosomal cores of the DNA {Wyllie AH 1993}. Due to the nature of the nucleosomes, which are regularly spaced along the double helix, DNA fragments can be detected as a ladder pattern on agarose gel electrophoresis. Cohen, on the basis of ultrastructural and biochemical criteria, showed an intermediate stage of large fragments of DNA which preceded endonuclease cleavage of DNA into the oligonucleosomal fragments. This finding is of importance in the recognition and identification of apoptotic cells at an early stage of death. Although the initial steps in the induction of apoptosis may vary in different cell types, it appears that there is convergence to a small number of common pathways in the later stage of apoptosis {Cohen GM, et al. 1994}. More recently, end-labelling techniques have been developed to mark apoptotic cells in situ {Gavrieli Y, et al. 1992} {Gold R, et al. 1993}.
The original evidence for apoptosis of mid-line palatal epithelial seam cells came from three independent observations. Firstly, it was reported that DNA synthesis ceased 24-36 hours prior to palatal fusion leading to the loss of mitotic potential {Hudson CD, et al. 1973} {Pratt RM, et al. 1975} {Greene RM, et al. 1976}. Secondly, the ultrastructural changes observed in MEE were consistent with cell death {Farbman AI 1968} {Shapiro BL, et al. 1969} {Hudson CD, et al. 1973} {Chaudhry AP, et al. 1973} {Pratt RM, et al. 1984}. Finally, evidence for increased lysosomal enzyme activity in the MEE was presented {Hayward AF 1969} {Smiley GR 1970} {Pratt RM, et al. 1975}.

Ferguson’s work on mouse, chick, and alligator revealed that terminal differentiation of the MEE occurred in single palatal shelf and independent of shelf contact. Regional differentiation of the nasal epithelia into pseudostratified ciliated columnar cells and the oral epithelia into stratified squamous cells also occurred in single palatal shelf models as well. Further, nasal, medial edge and oral palatal differentiation is specified by the mesenchyme in a species-specific fashion. Thus, MEE cell death is induced by the underlying mesenchyme and thus the epithelium appears to play only a passive role {Ferguson MWJ, et al. 1984} {Gehris & Greene 1992}.

Despite the important role of apoptosis in the disruption of MEE, Ferguson also found that 50% of MEE cells were involved in an epithelial-mesenchymal transformation (EMT). These cells failed to express cytokeratins, but instead expressed the intermediate filament vimentin and thus became indistinguishable from the underlying mesenchymal cells {Ferguson MWL 1988}.
Recently, cytochemical investigation of foetal mouse palate using specific labelling of DNA fragmentation, by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) to identify apoptosis and immunohistochemical staining of keratin to identify cells of epithelial phenotype was used to study the disappearance of the MEE (Mori C, et al. 1994). The results indicated that during shelf elevation and prior to contact, few cells showed TUNEL-positive staining. These cells were thought to be sloughing peridermal cells. During the initial contact of the palatal shelves, the MEE remained negative for TUNEL staining. However, once the mid-line seam began to attenuate and break up into a discontinuous seam, TUNEL-positive staining was found in nuclei of cells within epithelial islands of the disintegrated palatal seam. It was interesting that some TUNEL-positive, keratin-negative cells just outside the epithelial islands were detected.

As the islands disappeared the TUNEL staining became less intense and was restricted to the oral and nasal "epithelial triangles" adjacent to the oral and nasal surfaces of the palatal shelves. A similar pattern of TUNEL-positive, keratin-positive staining was seen in the fusion line of nasal septum and the dorsal palate. These results suggest that DNA fragmentation occurs in some cells along the palatal mid-line when the epithelial seam becomes discontinuous. It seems that apoptosis is closely associated with the disruption of MEE but may not be required for initial contact or epithelial fusion of apposing palatal shelves.

Refer to Table 1 and Figures 6 to 12.
Table 1: Comparing characteristics of necrosis and apoptosis

<table>
<thead>
<tr>
<th>NECROSIS</th>
<th>APOPTOSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affects contiguous cell masses</td>
<td>Affects scattered individual cells</td>
</tr>
<tr>
<td>Chromatin marginates into small aggregates</td>
<td>Chromatin marginates as large crescents</td>
</tr>
<tr>
<td>Increase in cytoplasm and cell volume</td>
<td>Decrease in cytoplasm and cell volume</td>
</tr>
<tr>
<td>Organelles swell (Mitochondria and ER)</td>
<td>Organelles retain integrity</td>
</tr>
<tr>
<td>Cell ruptures</td>
<td>Cell fragments into “apoptotic bodies”</td>
</tr>
<tr>
<td>Cell contents are released</td>
<td>Phagocytosis of apoptotic bodies</td>
</tr>
<tr>
<td>Extensive inflammatory response</td>
<td>No inflammatory response</td>
</tr>
<tr>
<td>Smear pattern of DNA</td>
<td>Ladder of DNA fragmentation</td>
</tr>
</tbody>
</table>

Figure 6: Morphological characteristics of Necrosis and Apoptosis

(Adapted from Ueda and Shah, 94)

Figure 7: Apoptosis and necrosis stimuli. Physiological stimuli result in apoptosis whereas pathological stimuli result in necrosis. However, mild injury could result in apoptosis. (Rotello et al, 91)
Figure 8: The proposed model correlating DNA fractionation and fragmentation of apoptosis. Intact DNA of normal thymocytes (F1&F2) is cleaved into large fragments in preapoptotic population (F3) containing condensed chromatin. Apoptotic thymocytes are then formed with characteristic ultrastructure and biochemistry (F4). (Adapted from Cohen et al, 94)

Figure 9: Sequence of events in cells undergoing apoptosis. (Rotello et al, 91)
Figure 10: Regulation of apoptosis. Hypothetical model proposing the existence of apoptosis inducers and/or repressors in cells. (Adapted from Fang et al, 95)

<table>
<thead>
<tr>
<th>REGULATORY ROLE</th>
<th>END RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN DEVELOPMENT</td>
<td>PROGRAMMED DELETION of CELL POPULATIONS</td>
</tr>
<tr>
<td>IN ADULT TISSUES</td>
<td>GROWTH MODULATION = CELL PROLIFERATION minus APOPTOSIS</td>
</tr>
</tbody>
</table>

Figure 11: Roles of apoptosis. Two main functions of apoptosis in development and growth regulation. (Adapted from Fang et al, 95)

Figure 12: Model for apoptosis mediated by oxidants. (Adapted from Fang et al, 95)
(1b) **Programmed Cell Death (PCD)**

**(i) Introduction**

The term apoptosis is often used interchangeably with programmed cell death. However, these two terms should be used in the correct context and with caution. Apoptosis is a morphologic definition that describes a form of death characterised by specific cellular changes including shrinkage, nuclear condensation and cell fragmentation resulting in the formation of apoptotic bodies. Programmed cell death is, on the other hand, a functional definition, describing cell death that occurs at a precisely predicted time and place and is truly genetically determined. Therefore, not all cell death through apoptosis is programmed.

**(ii) Cell death genes**

*Caenorhabditis elegans* shares conserved cell death genes with mammals. Horvitz *et al*; who pioneered the genetic studies of the nematode *C. elegans*, have contributed significantly to the understanding of programmed cell death. In the development of the hemaphrodite worm, 131 of the 1,090 somatic cells undergo programmed cell death in a genetic pathway defined by 14 genes. The divisions and deaths of individual cells can be observed in the live organism because *C. elegans* is transparent. These cell deaths are precisely programmed in every animal and each event occurs at individual characteristic times in development. Progress has been made in recent times in understanding the

Morphological and genetic studies have indicated that essentially all programmed cell deaths in *C. elegans* probably involve the same mechanism. First, nearly all dying cells undergo the same sequence of morphological changes {Sulston JE, *et al.* 1977} {Sulston JE, *et al.* 1983}. Second, a number of mutations affecting the process of cell death have been isolated. These mutations, which have defined the genes *ced-1* (for cell death abnormal), *ced-2*, and *nuc-1* (nuclease deficient), affect all programmed cell deaths {Sulston JE, *et al.* 1983}.

Programmed cell deaths can be identified in living nematodes using Nomarski differential interference contrast microscopy {Sulston JE, *et al.* 1977}. The first sign of the impending death of a cell is a slight increase in its refractility. The nucleus of the dying cell becomes increasingly refractile. Subsequently, the nucleus of the dying cell decreases in refractility, begins to appear crumpled, and then gradually disappears. In *ced-1* and *ced-2* mutants, dying cells remain in the highly refractile stage {Ellis RE, *et al.* 1986}.

In *C. elegans* two genes, *ced-3* and *ced-4*, have been identified that must function for cells to undergo programmed death. *Ced-3* encodes a cysteine protease and *ced-4* encodes a calcium-binding protein. Mutations of either of these genes results in the survival of almost all cells that normally die {Ellis RE, *et al.* 1986}. In *ced-3* or *ced-4*
mutants, all 131 cells that are normally destined to die, live. A third gene, ced-9, a homolog of bcl-2, represses the death pathway and protects cells that are destined to live. {Hengartner MO, et al. 1994}.

Ced-9 shows significant structural and functional homology to bcl-2. Ced-9 and Bcl-2 amino acid sequences share 24% identity and 49% similarity. They have similar hydrophobicity profiles, including the carboxy terminal signal anchor sequence. Ced-9 has the highly conserved amino acids of BH1 and BH2 domains {Hengartner MO, et al. 1994}. Functionally, transgenic bcl-2 can block some cell deaths in C. elegans and can partially substitute for ced-9 by preventing ectopic deaths in ced-9 mutants{Hengartner MO, et al. 1994} {Vaux DL, et al. 1992}. Further, the same mutation of Gly145Glu in Bcl-2 results in loss of function, but is a gain-of-function mutation in Ced-9. The sequence and functional conservation between ced-9 and bcl-2 has suggested that in its basic tenets, the genetic pathway of cell death may be common to all multicellular organisms {Yang E, et al. 1996}.

In C. elegans, the phenotype of ced-3, ced-9 double mutants is the same as ced-3 single mutants, i.e cells live. This indicates that ced-9 is not downstream of ced-3, but ced-9 could be an upstream negative regulator of ced-3 and ced-4. Epistasis mapping has also established that ced-4 is upstream of ced-3. Ced-9 is a regulator of cell death, whereas ced-3, and perhaps ced-4, encode effector molecules of cell death. In a gain-of-function ced-9 mutant, all 131 cells live, whereas loss-of-function mutations cause cells that normally should live to die {Hengartner MO, et al. 1992} {Hengartner MO, et al. 1994}. 

40
Ced-3 is homologous to the gene for mammalian interleukin-1β (IL-1β)-converting enzyme (ice). Cloning of the C. elegans ced-3 gene shows that the protein is homologous to the mammalian enzyme ICE {Yuan J, et al. 1993}. ICE is cysteine protease that cleaves the 33kDa pro-IL-1β at an aspartic acid residue into the biologically active 17.5kDa IL-1β. The homology between Ced-3 and ice suggested that ice may function as a mammalian cell death gene. Indeed, over-expression of ice causes Rat-1 fibroblasts to undergo apoptosis {Miura M, et al. 1993}. Ice itself has not proven to be directly affected by bcl-2, and many cells that undergo apoptosis do not express IL-1β. Mice deficient in ice can not synthesize mature IL-1β, but their thymocytes are able to undergo apoptosis induced by dexamethasone and gamma-irradiation, suggesting that ice is not essential for these cell death processes {Li P, et al. 1995} {Kuida K, et al. 1995}.

A mammalian ice/ ced-3 family of programmed cell death genes are being identified whose products function as effectors of cell death. These ced-3/ice effector molecules and those yet to be cloned are likely to interact with the bcl-2 pathway directly or indirectly to execute apoptosis. The existence of multiple ICE proteases may also reflect different lineage or substrate specificities {Steller H 1995}.

Refer to Figure 13.
Figure 13: C. elegans and mammals share a common genetic cell death pathway. CED-9 represses cell death and CED-4 and CED-3 are required for the execution of cell death. Similarly, Bcl-2 protects cells from apoptosis, whereas the ICE-like proteases (mammalian homologs of CED-3) are cell death effectors.

(Adapted from Yang and Korsmeyer, 96)
2. Epithelial-Mesenchymal Transformation:

It was speculated that such transformation allowed for both conservation of embryonic cell populations and a mechanism for mesodermal confluence within the palatal processes. The disappearance of palatal medial edge epithelium after fusion of secondary palatal shelves is often cited as a classical example of embryonic remodelling by programmed cell death. The periderm of the two-layered MEE begins to slough after the shelves assume the horizontal positions, allowing junctions to form between apposing basal epithelial cells. The midline seam so formed consists of two layers of basal cells. Even though the epithelial seam cells are dividing, the growth fails to keep pace with palatal growth and the epithelial seam thins to one layer of cells, and then breaks up into small islands. The basal lamina later disappears and the elongating MEE cells extend filopodia into the adjacent connective tissue. Further, electron micrographs reveal a transitional step in which the epithelial cells lose epithelial characteristics and gain fibroblast-like features by transforming. These MEE cells were observed, through immunofluorescence technique, to express the mesenchymal cytoskeletal protein, vimentin. It is thought that MEE is an ectodermal structure that retains the ability to transform into mesenchymal cells. Epithelial-mesenchymal transformation may also be expressed in other embryonic remodelling, resulting in conservation of selective embryonic cell populations {Fitchett & Hay 1989} {Hay ED, et al. 1995}. 
(i) **Fate of the outer layer (peridermal cells):**

As the name implies, these cells are derived from the outer layer of flattened covering cells equivalent to the periderm of the ectoderm. In some areas of the posterior portion of the palate, full desquamation of the MEE has not yet taken place even in the most advanced precontact shelves. In these areas, the dying peridermal cells may be trapped in the seam. In the anterior part of the palate at the time of shelf fusion, however, most of the peridermal cells are shed and the mid-line seam consists of the two adjoined basal cell layers. The peridermal cells contain lysosomes and appear to die and be removed by phagocytes {Waterman RE, et al. 1974} {Fitchett & Hay 1989}.

(ii) **Changes in the basal lamina (basement membrane):**

Trelstad et al (82); found that the dissolution of the basal lamina was an early and important finding in regressing Mullerian duct. The resolution of the basal lamina of the MEE seemed a likely indication that the palatal epithelium gives rise to mesenchymal cells. Prominent filopodia seem to be involved in the displacement and disintegration of basal lamina {Fitchett & Hay 1989}.

(iii) **Changes in the epithelial morphology:**

The basal epithelial cells undergo marked changes in cell shape as the mid-line epithelial seam disappears. Prior to sloughing of the periderm, the basal MEE cells are cuboidal in
shape with a centrally placed nucleus and a flat basal surface. Once the mid-line seam starts to form, the basal cells elongate in the plane of the seam and the basal surface becomes irregular. As the seam stretches, the cells slide past each other to become a monolayer, and the basal lamina begins to break up. Filopodial processes begin as small protuberances under the basal lamina, and with time they probe into the mesenchymal space.

Desmosomes are a prominent feature of the palatal epithelium, found not only between cells of the same basal layer, but also anchoring the apposed layers to each other. Presumably, desmosomes regress and reform during seam thinning, which accounts for various sizes of the desmosomes {Fitchett & Hay 1989}.

The cytoplasm of the basal epithelial cells contains abundant glycogen, rough endoplasmic reticulum, and free ribosomes, but Golgi complexes are not well developed. Abundant glycogen is also observed in pre-existing mesenchymal cells of the palatal shelves {Fitchett & Hay 1989}.

Vital cell labelling techniques and phenotypic makers were utilised to trace MEE cells during palatal fusion in vivo {Shuler CF, et al. 1992}. Immunohistochemical markers for keratin, vimentin and laminin were employed as phenotypic markers of epithelial cells, mesenchymal cells and the basal lamina, respectively. In the early stages of palatal fusion, MEE cells were attached to an intact laminin-containing basement membrane and contained keratin intermediate filaments. At later stages of palatal development, laminin-
containing basement membrane disintegrated, and MEE cells showed no keratins. By the completion of palatal fusion, labelled cells remained vital but showed mesenchymal morphology and staining for vimentin intermediate filaments, characteristic of mesenchymal cells. When epithelial cells transform into mesenchyme in the embryo, or when they are induced to do this in vitro, they switch from the keratin intermediate filament profile to one rich in vimentin, and the effect of cell matrix interactions on cell shape is profoundly altered (Shuler CF, et al. 1992). Vimentin-actin interactions with the extra-cellular matrix may be a major factor in the ability of a cell to become mesenchymal (Hay 1989). During epithelial-mesenchymal transformation, the presumptive mesenchymal cell seems to turn on the new front end mechanism as a way of emigrating from the epithelium into the underlying matrix with which it makes 'fixed' contacts. Master genes may exist that regulate the expression of epithelial genes on the one hand, and mesenchymal genes on the other (Hay 1990).

(iv) Epithelial-mesenchymal interactions:

Shipley showed TGF-β1 to be a growth inhibitor for most cell types in culture (Shipley GD, et al. 1986). Several epithelial cell types undergoing growth, differentiation and morphogenesis express high levels of TGF-β1 mRNA. These include thyroid, parathyroid, thymus, submandibular salivary glands, hair follicles, teeth, and secondary palate. In situ hybridisation and immunohistochemical techniques have been utilised to demonstrate TGF-β1 mRNA in regions where epithelial-mesenchymal interactions are
taking place with periods of intense epithelial growth, cell migration and mesenchymal condensation {FitzPatrick, Denhez, et al. 1990}.

Ignotz reported that the role of epithelially-derived TGF-β in organogenesis is most likely in the elaboration of extra-cellular matrix produced by mesenchymal cells and in the induction of cell surface receptor synthesis for the extra-cellular matrix components {Ignotz RA, et al. 1986}. The composition of this extra-cellular matrix can influence cellular phenotype by affecting cellular growth, differentiation and migration of mesenchymal cells as well as the overlying epithelium {Rizzino A 1988}. It is also possible that TGF-β synthesized by epithelium is a negative regulator of the epithelium by acting in an autocrine fashion to control epithelial homeostasis {Akhurst RJ, et al. 1988}.

TGF-β is responsive to controls by various post-transcriptional processes in certain tissues {Roberts AB, et al. 1990} {Kim SJ, et al. 1992}. Thus, it is important to consider rates of secretion, activation of the latent form, stability of the protein and the capacity of target cells to respond to the signal.

Acting in concert, growth factors could regulate events critical to formation of the secondary palate, including cessation of medial epithelial cell proliferation, synthesis of extra-cellular matrix proteins in the mesenchyme, programmed cell death of medial epithelial peridermal cells, and transformation of basal epithelial medial cells to mesenchymal cells {Abbott & Birnbaum 1990} {Gehris, D'Angelo, et al. 1991}.

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Refer to Figures 14 & 15.
Figure 14: Depicting the major characteristics of epithelium and mesenchyme. (Adapted from Hay and Zuk, 95)

Figure 15: Depicting epithelial-mesenchymal transformation (A) and mesenchymal-epithelial transformation (B). Epithelial cells transforming to mesenchyme elongate and break through the basal lamina. Mesenchymal cells aggregate and synthesize new basal lamina. (Adapted from Hay and Zuk, 95)
3. Migration of MEE

Carette reported that MEE cells may survive and retain epithelial phenotype but migrate out of the seam and merge with the oral and nasal epithelium of the palatal shelves {Carette MJM, et al. 1992}. Schuler found similar results using confocal laser scanning microscopy which offered increased resolution, enhanced contrast as well as thin optical sectioning {Shuler, Guo, et al. 1991}.

While several other factors may be involved in the thinning and disruption of the MEE, including expansion in palatal height, it appears that apoptosis is involved in the final breakdown once the seam has been disrupted {Mori C, et al. 1994}. Mori concluded that all three processes are involved at various stages of palatal fusion and that their occurrence is spatially and sequentially regulated.
C. GROWTH FACTORS/ MORPHOGENIC PROTEINS:

TGF-βs

1. Introduction:

The transforming growth factor beta (TGF-β) superfamily is one of the largest groups of highly conserved intercellular signaling molecules regulating embryonic growth and differentiation. Currently, the TGF-β superfamily includes at least 24 members and several subgroups. The main groups are TGF-βs, decapentaplegic-Vg-related (DVR) proteins (including the bone morphogenetic proteins, BMP), and activins {Wall NA, et al. 1994}.

TGF-βs are multifunctional growth factors that regulate many aspects of cellular activities, including cell growth and differentiation, steroidogenesis, wound healing, bone remodelling, haemopoiesis and specific morphogenetic and histogenetic events in embryonic development {Roberts AB, et al. 1990}. They are also potent chemoattractant factors for macrophages, and neutrophils and stimulate the incorporation of fibronectin and collagen into the extra-cellular matrix {Barnard JA, et al. 1990}{Reibman J, et al. 1991}{DeLarco JE, et al. 1978}{Ignotz RA, et al. 1987}. The effects of TGF-β include control of the biological activity of TGF-βs mediated through specific cell surface

Genetic, biochemical, and functional studies have shown that TGF-β-related proteins mediate inductive interactions during development. These interactions range from a simple switch in the fate of the responding cells to those as morphogens acting over relatively large distances in a graded fashion to differentially specify cell fate {Lyons RM, et al. 1991}.

2. Structure and Function:

TGF-β is a two-chain polypeptide of 25 kDa isolated from many tissues, including bovine kidney, human placenta, human platelets, liver, heart, brain, muscle, gut, and uterus {Roberts AB, et al. 1983}. It has been characterized by the ability to stimulate reversible transformation of non-neoplastic murine fibroblasts. The response of cells to TGF-β is bifunctional, i.e TGF-β inhibits the anchorage-dependent growth of fibroblasts and tumour cells by increasing cell cycle time. Moreover, the anchorage-independent growth of many carcinoma cell lines is inhibited by TGF-β at concentrations in the same range as those that stimulate colony formation of fibroblasts. Whereas epidermal growth factor and TGF-β synergize to induce anchorage-independent growth of fibroblasts, the effects on the growth of carcinoma cells are antagonistic. Interestingly, in fibroblasts transfected with a cellular myc gene, TGF-β synergizes with platelet-derived growth
factor to stimulate colony formation but inhibits the colony formation induced by epidermal growth factor. The effects of TGF-β on cells have been shown to be not a function of the peptide itself, but rather of the total set of growth factors and their receptors that is operant in the cell at a given time {Roberts AB, et al. 1985}.

Three isoforms TGF-β1, 2, & 3 are known, and although only 70% homologous with each other, there is exceptional inter-species conservation of amino acid sequence within different isoforms. There is a complicated regulatory network whereby each of the TGF-β isoforms either upregulate or downregulate the expression of other isoforms which are represented by distinct genes on separate chromosomes. TGF-β can act by both autocrine and paracrine mechanisms to regulate the behaviour of almost every cell in the mammalian organism. TGF-β is now well recognised as the prototypical multifunctional peptide growth factor {Akhurst, FitzPatrick, et al. 1990}.

TGF-βs elevate intracellular cAMP levels and are potent inhibitors of B lymphocyte activation. TGF-β has been shown to arrest stimulated B cells in the G1 phase of the cell cycle. Furthermore, TGF-β and cAMP may inhibit B cell responses not only by blocking cell cycle progression in activated cells, but also by inducing apoptosis in resting cells {Lomo J, et al. 1994}.

Although a number of transduction mechanisms may be available to TGF-β superfamily members, evidence gathered through the use of specific kinase and G-protein inhibitors and through assays measuring activation and levels of signalling intermediates, suggests
that at least one signalling pathway interacts with Ras and Raf proteins via a G-protein intermediate. Raf begins the cytoplasmic kinase cascade that leads to gene regulation. The myriad of responses regulated by TGF-β superfamily members makes the understanding of signal transduction mechanisms utilized by these proteins of great interest to a wide range of biological disciplines {Kolodziejczyk & Hall 1996}.

TGF-β’s have multiple effects on bone cells depending on the phenotype and/or stage of differentiation. Osteoblasts, the cells responsible for formation of new bone and perhaps cellular control of bone remodelling, are directly affected by TGF-β’s, which can induce differentiation or proliferation, depending on the osteoblastic cell type examined. TGF-β’s inhibit the formation of osteoclast precursors and bone resorption and, in greater concentrations, have inhibitory effects on isolated osteoclasts, the cells responsible for bone resorption. TGF-β’s may act as bone-coupling factors linking bone resorption to bone formation {Bouncild & Mundy 1990}. TGF-β1&2 have been shown to stimulate the production of not only collagenous extra-cellular matrix components, but also dramatically increase the synthesis in vivo of hyaluronate and chondroitin sulfate {Ogawa, Sawamura, et al. 1990} {Roberts, Flanders, et al. 1990}.

Refer to Table 2.
3. Synthesis:

The latent forms of TGF-β’s are synthesised by various normal and malignant cells and are secreted as latent high molecular weight complexes {Pircher R, et al. 1984}, although they can be released in active form following transient acidification at unphysiologically low pH values. Miyazono investigated the activation of the latent form of TGF-β purified from human platelets by incubating at various pH values. TGF-β activity was unmasked at pH values below 3.5 and over 12.5. These results suggest that TGF-β is already cleaved from the NH₂-terminal remnant of the precursor but resides noncovalently associated with the 210-kDa component. Miyazono further demonstrated that the carbohydrate structures in the precursor remnant were involved in rendering TGF-β inactive in the latent TGF-β complex. Various monosaccharides were investigated and it was found that the sialic acid and/or mannose-6-phosphate present in the carbohydrate complexes of the TGF-β precursor might be important for TGF-β latency {Miyazono K, et al. 1990}. Whether the interaction with carbohydrate structures is important for the latency of TGF-β’s remains to be investigated {Brown, Wakefield, et al. 1990}.

Platelets, macrophages and lymphocytes are all important sources of TGF-β and almost all TGF-β activity produced by these cells is latent. Very high concentrations of the latent form of TGF-β exert some TGF-β activity but the active TGF-β molecule is 200-fold more effective compared to the latent form {Miyazono K, et al. 1989}. Since TGF-β is a potent growth regulator, the activation of TGF-β from its latent complex must be carefully controlled {Miyazono K, et al. 1990}. Fibronectin and α₂-macroglobulin in
serum can bind the free form of TGF-β. TGF-β bound to fibronectin was reported to be active, whereas the TGF-α2-macroglobulin complex was inactive. It is possible that α2-macroglobulin can act as a scavenger that inactivates released TGF-β, and thus has another functional role than the latent complex containing the precursor remnant and the binding protein {Miyazono K, et al. 1990}.

Recently, osteoclast-derived cells were found to be able to activate TGF-β in an in vitro culture system. The precise mechanism of the activation of TGF-β by this cell line remains to be elucidated {Oreffo RO, et al. 1989}.

It has been reported that latent TGF-β produced by fibroblasts could be partially activated by proteases such as plasmin and cathepsin-D {Oreffo RO, et al. 1989}. If interaction with carbohydrate structures is important for the latency of TGF-β, the following mechanisms are possible in vivo:

(i) Enzymes could remove or modify sialic acid or mannose-6-phosphate. For example, certain phosphomonoesterases, such as alkaline phosphatase and acid phosphatase have been found to activate the latent form of TGF-β. These enzymes may act on mannose-6-phosphate, remove the phosphate residues from the carbohydrate structures, and unmask the TGF-β activity.

(ii) The low molecular weight substances could displace TGF-β from the carbohydrate structures in the latent complex.
(iii) The interaction between the mannose-6-phosphate residues of the latent form of TGF-β and mannose-6-phosphate receptors could activate TGF-β.

Further studies will need to aim at elucidating whether any of these mechanisms are involved in the regulation of TGF-β activity {Miyazono K, et al. 1990}.

4. Mechanisms of Action:

The functional properties of TGF-β receptors and the chain of molecular events which lead to the diversity of responses need to be identified and characterised to appreciate the complex mechanism of action of these pluripotent peptides. Attempts to determine whether TGF-β acts through any known signalling pathways have been inconclusive and the possibility that the receptors for the members of TGF-β family signal via a novel mechanism has been proposed {Massague J, et al. 1990}. This process is distinct from those involving tyrosine kinases, ion channels or activation of phospholipase/C kinase pathways. Downstream regulatory events include changes in the expression of gene products involved in cell cycle and gene expression control {Coffey RJ, et al. 1988} and the control of cell adhesion apparatus {Ignatz RA, et al. 1986}. 
5. **Receptors for TGF-β:**

TGF-β receptor types I and II are glycoproteins which bind TGF-β1 with higher affinity than TGF-β2. The high molecular weight type III receptor or betaglycan is an integral membrane proteoglycan that displays similar affinity for all tested forms of TGF-β {Cheifetz S, *et al.* 1988}. Most cell lines display all three receptor types, although the relative proportions vary. The expression of receptors is consistent across most species and tissues of origin, with no apparent correlation with the derivation of the cell lines, i.e whether they are from normal, neoplastic or transformed tissues. Type IV receptors have been detected on a pituitary tumour cell line only and have been implicated in regulating the release of follicle-stimulating hormone. Current literature supports the hypothesis that one receptor mediates multiple TGF-β responses, rather than multiple receptors mediating plural responses.

Two types of single transmembrane serine/threonine kinase receptors, types I and II, have been found to mediate the cellular effects of the TGF-β family ligands. Molecular characterization of the type I and II receptors of the prototypical TGF-β revealed that the two types of receptors play different roles in mediating downstream signalling. The type II receptor is an active serine/threonine kinase receptor that can bind TGF-β independently, but cannot signal without the type I receptor {Wrana J, *et al.* 1992}. The type I receptor can only bind to TGF-β in the presence of the type II receptor {Ebner R, *et al.* 1993} {Wrana JL, *et al.* 1994}.
Functional analysis of these molecules has clarified the basis for the collaborative involvement in the initiation, propagation, and diversification of TGF-β signals. The type II receptors act upstream of type I receptors. One difference between the two is in the ligand binding properties. The type II receptors for TGF-β and activin recognize these ligands free in the medium, whereas the type I receptors do not. The type I receptors recognize ligand-bound type II receptors, forming oligomeric complexes {Massague J 1996}. The type II receptor complex as well as the type I-II receptor complex can co-exist in cells that express all three TGF-β receptors {Massague J 1992}, whereas the type III receptor binds and presents TGF-βs to type II receptors.

A central event in the generation of signals by these complexes is phosphorylation of the type I receptor. This is likely to be catalyzed by the type II receptor kinase, that does not seem to be augmented by ligand binding. In essence, the ligand may be acting as an adaptor that brings a substrate, the type I receptor, to the primary receptor kinase. The evidence to date argues that the type II receptors are unable to generate responses independently of the type I receptors. However, over-expression of an inactive TGF-β RII has been reported to block TGF-β growth inhibitory effect but not the effect on extra-cellular matrix protein synthesis. It has been proposed that different TGF-β responses may require different levels of signalling and thus be inhibited to varying degrees by a dominant-negative receptor {Moustakas, Lin, et al. 1993}. 

(a) **Type I receptor:**

This receptor is involved in signal transduction and is characterised by the ability to discriminate between three forms of TGF-βs. These proteins show decreasing affinity for the receptor in the following order: TGF-β₁>TGF-β₂>TGF-β₃ {Cheifetz & Massague 1989}. It can mediate a diverse series of cellular responses including growth suppression, expression of cell adhesion genes and marked changes in cell morphology {Boyd, Cheifetz, et al. 1990}.

(b) **Type II receptor:**

This receptor shares some similarity with the characteristics of the type I receptor, however, the binding domains of this receptor are quite distinct and show a great variability in size. This glycoprotein contains a complex type N-linked carbohydrate receptor that is not required for cell surface expression or for ligand binding. A functional role is yet to be identified but it may form part of a multi sub-unit TGF-β receptor complex {Bhushan, Lin, et al. 1994}.

(c) **Type III receptor:**

This receptor is the most abundant and is the highest molecular weight receptor for TGF-β in most cell lines. It is heterogeneous in nature and has similar affinity for TGF-β₁ and
TGF-β2. This membrane proteoglycan consists of varying classes of glycosaminoglycan chains (GAGs) which varies in proportion in different cell types. It has been suggested that type III receptor has a mixed population with different affinity for TGF-βs, possibly as a result of clonal cell variation or differences in radio-ligand preparations {Segarini PR, et al. 1984}. It has been shown that the binding site for TGF-β resides in the core protein, and that GAGs are not required for functional expression of the receptor on the cell surface {Cheifetz & Massague 1989}. It is not known whether the soluble form of betaglycan is derived from the membrane-bound form by a hydrolytic process, or if it is the product of alternative mRNA splicing on a separate gene. Betaglycan may be involved in the presentation of TGF-β to the type I signal-transduction receptor, and it may also be able to accumulate in extra-cellular matrices and act as a reservoir or clearance system for bioactive TGF-β {Massague J 1992} {Boyd, Cheifetz, et al. 1990}.

**Type IV receptor:**

Unlike the other TGF-β receptor types, the type IV receptor does not have endoglycosidase-sensitive N-linked carbohydrate. The biological significance of this receptor remains to be determined. It is important to note, however, that the relative affinity of TGF-β1 and activin matches the receptor’s relative potency in inducing the expression and release of follicle-stimulating hormone in the pituitary. It is possible that ligands that contain two β type chains like TGF-β or activin may bind to the receptor and activate it, whereas ligands that are dimers of one β chain and one α chain, like inhibin,
may bind but not activate the receptor. Activins and inhibins constitute a complex set of factors with diverse biological activities \(\{\text{Yin XM, et al. 1994}\}\).

Refer to Figures 16\&17.
**Figure 16:** Schematic presentation of the four TGF-β binding proteins, and their affinity for multiple members of TGF-β family. (Massague et al, 90)

**Figure 17:** Summary of TGF-β receptors and responses. (Adapted from Mjor and Fejerskov, 86)
6. **TGF-β and Retinoblastoma Gene (rb):**

The retinoblastoma gene encodes protein pRb, which has growth suppressor activity. pRb is expressed throughout cell cycle but exits in multiple phosphorylated forms specific for certain phases of the cycle. The more highly phosphorylated forms are found during S and G2-M, whereas the underphosphorylated form prevails during G1 and in the growth-arrested state. TGF-β interrupts the cell cycle by inhibiting late G1 events when phosphorylation of \( rb \) gene product is due to take place, and by preventing phosphorylation arrests cells in late G1 \{Laiho M, \textit{et al.} 1990\}. These effects of TGF-β suggest that mammalian G1 cyclin-dependent kinases may constitute general targets for negative regulators of the cell cycle \{Koff A, \textit{et al.} 1993\}. It has been suggested that TGF-β1 inhibits T cell proliferation by down-regulating predominantly IL-2-mediated proliferative signals \{Ahuja, Paliogianni, \textit{et al.} 1993\}. TGF-β1 and pRb appear to function in a common growth-inhibitory pathway in which TGF-β1 acts to retain pRb in the underphosphorylated, growth-suppressive state \{Laiho M, \textit{et al.} 1990\}. 


Table 2: A review of the immunoregulatory effects of TGF-β

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>INHIBITION</th>
<th>STIMULATION</th>
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<tr>
<td>Cell surface expression</td>
<td>Fc receptor</td>
<td>Fc receptor III</td>
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<td></td>
<td>MHC class II</td>
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<td></td>
<td>IL-2 p55 receptor</td>
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<tr>
<td>Cytokine production</td>
<td>TNF-α, IFN-γ</td>
<td>TNF-α, IL-1α,β mRNA</td>
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<td>Proliferation</td>
<td>Early haemopoietic-progenitor cells.</td>
<td>Differentiated Haemopoietic progenitor Cells</td>
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<td>Thymocytes, T/B cells</td>
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<tr>
<td>Effector cell generation</td>
<td>Natural killer and Lymphocyte-activated-killer cell.</td>
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<td>Cytotoxic T cells.</td>
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<td></td>
<td>Macrophages.</td>
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<td>Others</td>
<td>IgM/IgG synthesis</td>
<td>IgA synthesis</td>
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<td></td>
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<td>Chemotaxis</td>
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(Adapted from Lucas et al, 91)
7. Multifunctional Roles for TGF-β:

The range of biological activities of TGF-βs are extremely broad and versatile. They surpass that of other known groups of peptide regulatory factors, in both normal and pathological conditions. TGF-βs can be produced by most cell types and exert a wide range of effects in a context-dependent autocrine, paracrine or endocrine fashion via interactions with distinct receptors on the cell surface (Cox 1995).

Currently, monoclonal antibodies to TGF-β and cell lines engineered to produce latent TGF-β have allowed a more detailed investigation of the immuno-regulatory effects of endogenously produced TGF-β (Lucas C, et al. 1991).

Although the general understanding of the immunoregulatory properties of TGF-β is incomplete, recent data suggest that they may be an important new class of immunosuppressive molecules. TGF-β secreted by lymphocytes is in a stable, biologically inactive or latent complex, unable to bind to specific TGF-β receptors and is not immunosuppressive until activated. Since most cells produce latent TGF-β, physiological mechanisms must exist to activate TGF-β before it can affect immune functions in an autocrine or paracrine manner. TGF-β has been shown to have an autocrine growth inhibitory effect on B and T cell proliferation and differentiation, immunoglobulin production as well as impairing natural killer cell function, although
how lymphoid cells activate TGF-β is as yet unidentified {Cox 1995}. Palladino et al (1990) have postulated that the immunosuppressive effects of cyclosporin A may be mediated through the induction of endogenous TGF-β secretion. Current interest is now focused on the possible role of latent TGF-β produced by tumour cells in the circumvention of immune surveillance {Palladino MA, et al. 1990}.

The ability of TGF-β to regulate the expression of gene products involved in cell cycle and gene expression control, together with its interactions with other regulatory peptides and hormones, makes TGF-β a potentially important modulator of apoptosis {Rotello RJ, et al. 1991}.

TGF-β has several down-regulatory functions on the immune system such as inhibition of interleukin-2 receptor induction, decrease of interferon-γ-induced class II major histocompatibility antigen expression, inhibition of macrophage activation, as well as cytotoxic and lymphokine-activated killer cell generation {Kasid A, et al. 1988}. The inhibitory action of TGF-β on the growth and functional activities of haemopoietic and T cells is associated with a reduction in the cell surface receptor expression for IL-2. Exogenous TGF-β added to macrophage cultures is able to reverse the effect of interferon-γ {Dubois, Ruscetti, et al. 1990}.

On the other hand, tumour necrosis factor-α at high concentrations (100 IU/ml) totally blunts the suppressive effect of TGF-β. In neoplasia, the presence of TGF-β in lesions
has been correlated with the advancing edge of primary tumours or depth of tumour invasion, also indicating the role of TGF-β in lesion progression {Barral A, et al. 1995}.

8. Relevant TGF-β Family:

(a) TGF-β1

TGF-β1, a highly conserved 25-kDa disulfide-linked homodimer, can stimulate or inhibit growth and differentiation of cells of multiple lineages. The net effect of TGF-β1 action is determined by its interaction with other cytokines and the nature of the target cell. TGF-β1 stimulates fibroblasts to proliferate and synthesize collagen, attracts and activates monocytes {Wahl SM, et al. 1987}, and is chemotactic for human neutrophils {Reibman J, et al. 1991}. Conversely, TGF-β1 inhibits proliferation and function of T and B lymphocytes, and suppresses the growth and lytic activities of cytotoxic T cells, NK cells, and lymphokine-activated killer cells {Kasid A, et al. 1988}. It also regulates cytokine production by immune cells and modulates macrophage function {Kulkarni AB, et al. 1993}. Furthermore, TGF-β1 has been shown to induce apoptosis in gastric cancer cells through TGF-β RI&II and a p53- independent pathway {Yamamoto, Maehara, et al. 1996}.

TGF-β1 elicits an epithelial-mesenchymal transition in vivo. The action of TGF-β1 in enhancing malignant progression may mimic its proposed function in modulating
epithelial cell plasticity during embryonic development (Cui W, *et al.* 1996). It has been suggested that TGF-β1 may play an important role in bone formation at the active site of the mid-palatal suture in response to rapid palatal expansion and that application of TGF-β1 during the early stages may induce rapid bone formation (Sawada & Shimizu 1996).

Conformation or structure of TGF-β1 and its localization vary in keratinocytes with distinct differentiation phenotypes suggesting that TGF-β1 is a potential modulator of keratinocyte differentiation *in vivo*. Selective association of TGF-β1 with nonproliferating keratinocytes in the suprabasal layers of the epidermis and its exclusion from the proliferating keratinocytes in the basal layer suggest that it may be a physiological regulator of keratinocyte proliferation. In addition, the intracellular localization of TGF-β1 peptide in both normal and psoriatic keratinocytes suggests that it is synthesized by epidermal keratinocytes *in vivo* (Kane, Knapp, *et al.* 1990).

TGF-β1, alone and in conjunction with epidermal growth factor (EGF), is capable of stimulating increases in both phosphoinositide metabolism and calcium influx, leading to significant increases in intracellular levels of Ca$^{2+}$ and inositol trisphosphate (IP3). It has been shown that Ca$^{2+}$ influx and inositol phosphate release are coupled in Rat-1 cells, and suggested that influx of Ca$^{2+}$ from the extra-cellular medium is required for the change in IP3 accumulation observed in response to both EGF and TGF-β1 (Rodland, Muldoon, *et al.* 1990).
(b) **TGF-β2**

TGF-β2 has been shown to be critically involved in the regulation of cell proliferation, migration and/or condensation and differentiation during both development and tissue repair {Hock, Canalis, *et al.* 1990} {Sporn & Roberts 1990} {Millan, Denhez, *et al.* 1991} such as stimulating bone and cartilage formation in the fronto-nasal mesenchyme and secondary palate {Berghuis HM, *et al.* 1994}. On the other hand, TGF-β2 has been shown to be a potent regulator of osteoclastic bone resorption, by modulating both osteoclast migration and osteoclast differentiation. Further, no expression of TGF-β2 mRNA is seen in mature cartilage or in areas of intramembranous or endochondral ossification. The TGF-β2 probe also hybridizes with invaginating dental epithelium and the medial edge epithelium of the secondary palate co-expresses TGF-β1 and β3 {FitzPatrick, Denhez, *et al.* 1990}.

(c) **TGF-β3**

TGF-β3 may play a crucial role during palatogenesis {Brunet, Sharpe, *et al.* 1995}, the formation of Meckel’s cartilage, mammary gland development and wound healing {Shah M 1995}. In general, TGF-β3 mRNA does not show widespread epithelial expression. It is limited only to the early columnar, bronchial epithelium, and the medial edge epithelium of the secondary palate {FitzPatrick, Denhez, *et al.* 1990}.
Mice lacking TGF-β3 exhibit an incompletely penetrant failure of the palatal shelves to fuse. Instead, the medial edge epithelial cells survive at the mid-line position, and the basement membrane resists degradation, leading to the development of cleft palate. This is demonstrated by positive staining for laminin and collagen IV and the presence of desmosomal components in the persistent mid-line epithelial seam. The defect apparently results from impaired adhesion of the apposing epithelial surfaces which appear to migrate rather than adhere following contact. It is evident that the process of touching or contact of the apposing MEE alone is not sufficient to initiate the breakdown of the midline seam. Supplementation of the culture medium with the mature form of TGF-β3 was able to fully correct the defective fusion in the null mutant specimens. This highlights the specific role for TGF-β3 in the events that control transdifferentiation of the medial edge epithelial cells including degradation of the underlying basement membrane. These results demonstrate that TGF-β3 affects palatal shelf fusion by an intrinsic, primary mechanism rather than by effects secondary to craniofacial morphometrics {Kaartinen, Cui, et al. 1997} {Proetzel, Pawlowski, et al. 1995}. Organ culture studies indicate that TGF-β1 and TGF-β2 accelerate palatal shelf fusion and that antisense oligodeoxynucleotides or neutralizing antibodies to TGF-β3, but not to TGF-β1 or TGF-β2, block the fusion process. The isoform-specific role for TGF-β3 and other TGF-βs allows greater flexibility and regulation of function especially in the palatal fusion process {Brunet, Sharpe, et al. 1995}.

Analysis of the fused region in the TGF-β3-null embryos indicated that the lesion primarily affects the MEE rather than the underlying mesenchyme. Further, the initiating
cause of the defect may lie in aberrant palatal shelf fusion rather than in a deficiency in proliferation or migration of the palatal shelf mesenchyme {Proetzel, Pawlowski, et al. 1995}.

TGF-β3 may stimulate the migration of MEE cells away from the midline {Carette MJM 1992}. This may be via its effects on the cells (e.g. acquisition of a migratory "mesenchymal" phenotype), or on cell adhesion molecules, or on the composition of the underlying extra-cellular matrix {Foreman, Sharpe, et al. 1991}.

The inhibition of TGF-β3 activity may have more indirect effects, because of the interplay between several different growth factors and their receptors. Removing one of the elements in the signalling cascade may disrupt the whole system, for example, TGF-β1 has been shown to down-regulate EGF receptors in palatal mesenchyme cells {Sharpe PM 1992} and palatal epithelia {Brunet CL 1993}. Such down-regulation may be necessary to prevent EGF or TGF-α activity. Both EGF and TGF-α prevent normal palatal fusion {Abbott BD 1987}. Removal of TGF-β3 activity by anti-sense or antibody may prevent palatal fusion by allowing EGF or TGF-α to be active in the medial edge region at inappropriate times.

Although the patent sutures are less immunoreactive for TGF-β isoforms than fused sutures, there is a distinct pattern of the TGF-β3 isoform that is immunolocalized to the margin of the normal patent sutures. This suggests a possible role for TGF-β3 in maintaining cranial suture patency. The increased immunoreactivity of TGF-β3 in the
actively fusing sutures compared with the patent control sutures indicates that these
growth factors may play a role in the biology underlying premature suture closure {Roth,

Structural comparison of TGF-β3 to that of TGF-β2, reveals a virtually identical central
core. Differences exist in the conformations of the N-terminal alpha-helix and in the beta-
sheet loops. If these differences are recognized by the TGF-beta receptors, they might
account for the individual cellular responses {Mittl, Priestle, et al. 1996}.

In contrast to the TGF-β1&2 isoforms, TGF-β3 has shown the ability to downregulate
scarring and fibrosis in vivo, under certain experimental conditions {Murata, Zhou, et al.
1997}. It is concluded that stimulation of collagen synthesis by TGF-β3 occurs through
TGF-β1-dependent and independent pathways. By downregulating the response to TGF-
β1 and by shifting from one pathway to the other, TGF-β3 can dampen and provide fine-
tuning to the overall collagen deposition program induced by TGF-βs {Murata, Zhou, et
al. 1997}.

9. TGF-β and Embryogenesis:

Although the causal mechanisms underlying growth and differentiation that occur during
palatal morphogenesis are not clearly understood, it is apparent that any disruption in the
coordination of developmental events, at any level, could result in the malformation of the palate.

TGF-β is present in tissues derived from neural crest mesenchyme, such as the palate, larynx, facial mesenchyme, nasal sinuses, meninges and teeth {Heine UI, et al. 1987}. Immunohistochemical staining of these tissues during periods of morphogenesis is greatest when remodelling of mesenchyme or mesoderm occurs, as during formation of the palate. Studies in vitro have shown that TGF-β controls the formation of connective tissue proteins such as collagen and fibronectin. It does this by increasing synthesis of connective tissue proteins and by blocking degradation of matrix constituents, resulting in a net accumulation within the tissues {Nath RK, et al. 1994}. There is a remarkable conservation of TGF-β sequences between species and given the ability to modulate cellular differentiation, matrix formation and angiogenesis, it is not surprising that this peptide is believed to play a fundamental role in embryogenesis {Nath RK, et al. 1994}. Immunostaining shows greater expression of TGF-β2 isoform compared with TGF-β1 by all cells in foetal skin, with predominance in epithelium and hair follicles. TGF-β2 is found in extracellular space and both TGF-β1&2 may have a role in foetal wound repair {Nath RK, et al. 1994}.

The epithelial lining of palatal shelves is regionally heterogeneous and undergoes different cell fates. Regional specification of palatal epithelium is controlled by the underlying mesenchyme through the induction process {Ferguson MWJ, et al. 1984}. The autocrine and paracrine mechanisms of action of TGF-β influence the epithelial-
mesenchymal interactions, for example mRNA for TGF-β is detected in epithelia overlying mesenchymal tissues containing the TGF-β protein. The spatial distribution of TGF-β3, followed by TGF-β1 and TGF-β2, may be critical in the induction of one member of TGF-β family by another, in order to amplify specific morphogenic effects {Ferguson MWJ, et al. 1984}.

10. TGF-β and Apoptosis:

TGF-β causes not only a decrease in cell proliferation but also an increase in apoptosis. Given that apoptosis and proliferation are growth regulatory components in normal epithelia {Rotello RJ, et al. 1991}, it has been proposed that stem cell proliferation is regulated by cell-proliferation-factors, whereas daughter cells are the target of cell-death-factors that induce apoptosis {Bryant PJ 1988}.

Lin and Chou proposed that apoptosis induced by TGF-β1 in a human hepatoma cell line did not involve de novo protein synthesis but rather an endogenous endonuclease was activated which cleaved DNA and resulted in cell death {Lin JK, et al. 1992}. This suggests that TGF-β-induced apoptosis, could represent another type of programmed cell death. Injection of TGF-β1 into rats was found to trigger apoptosis in the liver; suggesting that the effect of TGF-β1 may be due to a priming action on the hepatic cells {Oberhammer FA, et al. 1992}.
11. Transcriptional Control of Expression of TGF-βs:

(i) Alternate splicing tgf-β1 gene

In porcine tissues there is evidence for alternate splicing of TGF-β1 mRNA with the omission of exons 4 and 5; translation of the resulting mRNA would produce a novel peptide having TGF-β1 precursor sequences at the N-terminus, but having a different 45 amino acid long sequence in place of the mature TGF-β1 at the C-terminus, due to a frameshift in the translation of exons 6 and 7 {Kondaiah P, et al. 1988}. Porcine tissues also express a novel 3.5kb TGF-β1 mRNA which utilizes an alternate polyadenylation signal. The significance of the alternate splicing or the use of an alternate polyadenylation signal in control of tgf-β1 transcription or translation, or the extent of the occurrences of these alterations in other species is not known {Kondaiah P, et al. 1988}.

(ii) Control of tgf-β transcription in vitro

This has been demonstrated in several systems including viral transformation {Jakowlew SB, et al. 1988}, activation of lymphocytes {Derynck R, et al. 1985}, and autoinduction {Van Obbergen Schilling E, et al. 1988}. The increased levels of TGF-β1 mRNA can be correlated with increased amounts of TGF-β1 secreted into the medium. Data with v-ras-transformed keratinocytes demonstrate selective control not only of transcription, but also of translation. The mRNA levels of v-ras-transformed cells respond to changes in
Ca\textsuperscript{2+} concentrations in the same manner as for primary keratinocytes, but the cells secrete exclusively TGF-\(\beta\)1 at both low and high Ca\textsuperscript{2+} concentrations {Glick AB, et al. 1989}. Glick demonstrated that the transcription of TGF-\(\beta\)1 and TGF-\(\beta\)2 is not independently controlled, but also that viral transformation can alter the type of TGF-\(\beta\) secreted by a cell, and that translational control can be independent of transcriptional control {Glick AB, et al. 1989}. They further emphasized the need for mechanistic analysis of the promoters of these tgf-\(\beta\) genes. Studies with the tgf-\(\beta\)1 promoter suggest that these effects are principally transcriptional {Kim SJ 1989}.

The observations that expression of the different tgf-\(\beta\)s is controlled independently suggest that molecular cloning and analysis of the respective promoter regions of these genes will implicate different sets of transcription factors in activation of their expression. Furthermore, some of these transcription factors may be novel proteins unique to the TGF-\(\beta\) family of promoters, and some may be specifically modified, post-translationally. The inter-relations between the autoinduction of growth factors such as TGF-\(\beta\), and nuclear proto-oncogenes/transcription factors, suggest that the effects of each in control of cell growth, differentiation, and development might depend, in part, on the ability to cross-regulate expression. The aberrant expression of either could lead to excessive amplification and might thus contribute to the process of oncogenesis {Roberts AB, et al. 1990}. 
12. **Post-Transcriptional Regulation of the tgf-β1 Gene:**

Long GC-rich 5'-UTRs have been observed for several genes that are involved in regulation of cellular proliferation. *Tgf-β1,2,3* have profound effects on cellular proliferation and share the property of having unusually long 5'-UTRs, all of which appear to play a role in post-transcriptional regulation {Kim SJ, *et al*. 1989}. Kim *et al.*, have identified a new regulatory control affecting *tgf-β1* expression and have demonstrated that the 5'-UTR of the human TGF-β1 mRNA exerts a strong inhibitory effect on growth hormone production. The inhibition is specific for certain cell types and the results correlate well with observed ratios of TGF-β1 mRNA levels compared with levels of the secreted peptide in these cells {Kim SJ, *et al*. 1992}.

Computer analysis identified two highly stable stem-loop structures in the 5'-UTR of the human TGF-β1 mRNA. Intramolecular duplex structures that are positioned close to the 5'-end of an mRNA have been shown to inhibit the initiation of translation of several eukaryotic genes, presumably by preventing binding of a 40S ribosomal subunit. The GC-rich region immediately downstream from the transcriptional start site of the *tgf-β1* gene inhibited growth hormone production with a high degree of specificity. Moreover, it is also likely that the proposed secondary structures present in the 5'-UTR of the TGF-β1 mRNA may serve as binding sites for cytoplasmic factors that are cell type-specific {Kim SJ, *et al*. 1992}.
D. DEATH PROTEINS AND THEIR ANTAGONISTS:

BCL-2/BAX

1. Introduction:

*Bcl-2* is the acronym for the B-cell lymphoma/leukaemia-2 gene. It was first discovered because of involvement in B-cell malignancies, where chromosomal translocations activate the gene in the majority of follicular non-Hodgkin’s B-cell lymphomas. In these translocations, the *bcl-2* gene is moved from the normal chromosomal location at 18q21 into juxtaposition with powerful enhancer elements in the immunoglobulin heavy chain IgH locus at 14q32. This results in de-regulation of the translocated *bcl-2* gene and over-production of Bcl-2 mRNAs and the encoded protein. Bcl-2 functions primarily in preventing cell death instead of promoting proliferation {Vaux DL, *et al*. 1988} {Nunez G, *et al*. 1990} {Korsmeyer 1992}.

2. Functional Characteristics of Bcl-2:

(i) Bcl-2 prolongs cell survival: over-expression of Bcl-2 increases the viability of certain cytokine-dependent cells after cytokine withdrawal, e.g IL-3 and maintains the cells in Go. Bcl-2 expression vectors permitted prolonged cell survival in the absence of IL-3, but without concomitant cell proliferation {Vaux
DL, et al. 1988}. It is known that IL-3 and several other colony stimulating factors help to maintain haemopoietic cell survival in vitro by preventing apoptosis. Antisense-mediated reductions in bcl-2 gene expression have been shown to accelerate the rate of cell death in the setting of growth factor withdrawal. Furthermore, marked reductions in Bcl-2 protein levels, although rendering cells more prone to apoptosis, are by themselves often insufficient to cause cell death {Reed J, et al. 1990}. Bcl-2 is also capable of protecting T cells against a variety of apoptotic signals, including glucocorticoids, γ-irradiation, phorbol esters, ionomycin, and cross-linking of cell surface molecules by anti-CD3 antibody {Vaux DL, et al. 1988} {Nunez G, et al. 1990} {Borner 1996}.

(ii) Over-expression of Bcl-2 alters lymphoid development and leads to neoplasia: a long latency and progression from polyclonal hyperplasia to monoclonal malignancy are consistent with the hypothesis that oncogenic events in addition to Bcl-2 over-expression are necessary for tumour formation. The progression to lymphoma in these bcl-2 transgenic mice constitutes evidence in vivo that the t(14;18) and bcl-2 over-expression play a primary role in oncogenesis {McDonnell TJ, et al. 1989} {Marin MC, et al. 1995}.

(iii) Bcl-2 protects against neuronal cell deaths induced by various apoptotic stimuli. Bcl-2 is an important regulator of sympathetic neuron survival during the period of naturally occurring programmed neuronal death. In addition to delaying cell death in the setting of growth factor or neurotrophic factor withdrawal, over-
expression of Bcl-2 can prevent or markedly reduce cell killing induced by a wide variety of stimuli. Bcl-2 appears to block a relatively early event associated with apoptotic cell death in that none of the characteristic morphological changes such as cell shrinkage, chromatin condensation, and nuclear fragmentation occurred, and DNA degradation into oligonucleosomal length fragments was markedly reduced or prevented. Gamma-irradiation and the wide variety of drugs to which Bcl-2 confers resistance have diverse mechanisms of action, but all induce DNA damage either directly or indirectly. The protection afforded by Bcl-2 does not involve reductions in drug-induced damage to DNA, increased rates of DNA repair, inhibition of drug-induced alterations in nucleotide pools, or changes in cell cycle kinetics \{Walton WI, et al. 1993\} \{Fisher TC, et al. 1993\}.

(iv) Bcl-2 can in some cases block or delay the apoptotic death of virus-infected cells and convert a lytic viral infection into a non-lytic persistent infection, which suggests the possibility that virally encoded Bcl-2 homologues could perhaps contribute to viral latency or allow for persistent infections in the absence of cell lysis \{Levine B, et al. 1993\} \{Alnemri ES, et al. 1992\}. Bcl-2 homology extends to DNA viruses. The gene of the EBV expressed early in lytic and some latent infections is homologous to bcl-2 in the BH1 and BH2 domains \{Cleary MI, et al. 1986\}.

(v) Bcl-2 functions in multiple cell death systems: For example, apoptosis due to external toxic stimuli can be rescued by Bcl-2. This protein is also involved in

(vi) Bcl-2 is centrally involved in the suppression of apoptosis, however, overexpression fails to protect cells in some circumstances from what appears to be apoptotic death. Bcl-2 does not have a substantial effect on negative selection of thymocytes and does not easily prevent apoptosis in targets of cytotoxic T-cell killing {Oberhammer FA, et al. 1992}. Moreover, bcl-2 gene prevents radiation-induced apoptosis and blocks glucocorticoid and calcium ionophore-induced apoptosis. This suggests either that there are multiple independent intracellular mechanisms of apoptosis, some of which can be prevented by Bcl-2, or that there are additional pathways involving proteins that differentially regulate Bcl-2 functions. Because Bcl-2 is able to inhibit apoptosis resulting from so many different signals and intracellular pathways, it must act after the convergence of many signals in the apoptotic pathway {Vaux DL, et al. 1992} {Yang E, et al. 1996}.

The over-expression of bcl-2 does not protect against every example of cell death. Consequently, it is theoretically possible that more than one distal pathway of cell death exists. Given that some tissues such as the liver lack Bcl-2 altogether, and yet are subject to regulation by apoptosis, Bcl-2-independent mechanisms clearly do exist {Oberhammer FA, et al. 1992} {Sentman DL, et al. 1991} {Vaux DL, et
The recent discovery of proteins that share sequence homology with Bcl-2 and that can repress its apoptosis-blocking function suggests a possible explanation for why the over-expression of Bcl-2 is ineffective in preventing apoptosis in some types of cells and circumstances {Boise LH, et al. 1993}.

**Bcl-2** appears to act downstream, perhaps preventing the presence of damaged DNA from being translated into a signal for activation of the genes involved in apoptosis, or by blocking the action of the products of those genes once induced.

**Bcl-2** blocks apoptosis induced by *c-myc*. Since *c-myc* stimulates both mitogenesis and apoptosis, concomitant activation of *bcl-2* can nullify the apoptotic influence of *c-myc*, thus unleashing the proliferative effects of *c-myc* and leading to a further selective growth advantage {Bissonnette RP, et al. 1992} {Vaux DL, et al. 1993} {Fanidi A, et al. 1992}. Accumulation of mitogenic mutations alone, may result in cell death when paracrine factors are deleted, but simultaneous or additional acquisition of events suppressing cell death, such as upregulation of *bcl-2*, will lead to carcinogenesis. Synergy between these oncogenes of two different classes could result in more potent transformation than by either oncogene alone {Marin MC, et al. 1995} {Yang E, et al. 1996}.

**Recent demonstration** that Bcl-2 can interact with R-Ras in the yeast two-hybrid system and evidence that the two proteins co-immunoprecipitate in mammalian cells raises interesting possibilities. The effector domain of R-Ras is predicted to
be capable of interacting with the Raf kinase, and gene transfer experiments have shown that bcl-2 and raf oncogenes can act synergistically to suppress apoptosis. Thus, Bcl-2 may control a signal transduction pathway, which could target signalling molecules to critical intracellular locations, such as the mitochondrial junction and nuclear pore complex (Fernandez MJ, et al. 1993).

Tumor suppressor p53 has been implicated in genomic surveillance. It is known to undergo increases in protein levels and activity in response to drug-induced DNA damage, which can induce apoptosis under a variety of conditions (Sachs L, et al. 1993). Perhaps a functional connection between p53 and Bcl-2 may exist. It has been shown that gene transfer-mediated over-production of Bcl-2 protein can partially suppress p53-induced apoptosis (Wang Y, et al. 1993). Bcl-2 can inhibit p53-dependent and p53-independent cell death pathways. In M1 myeloid leukaemia cells, expression of p53 induces apoptosis. This is correlated with upregulation of Bax, resulting in increased Bax/Bcl-2 ratio. However, there is no evidence that Bax is required for p53-induced deaths (Selvakumaran M, et al. 1994) (Miyashita T, et al. 1994).
3. **Structure of Bcl-2 and Related Proteins:**

(i) **Bcl-2**

The amino acid sequence of the 25-26 kDa, Bcl-2 protein from cDNA cloning, suggests a biochemical function for this regulator of apoptosis. It has 19 hydrophobic amino acids near the COOH terminus followed by just two charged residues that presumably serve to anchor the protein in membranes {Cazals-hatem D, *et al.* 1992}. Mutagenesis studies have confirmed that the hydrophobic COOH terminal region allows post-translational insertion into membranes, such that the amino component of Bcl-2 protein should be orientated towards the cytosol {Chen-levy S, *et al.* 1990}. The importance of membrane insertion for Bcl-2’s function as a blocker of apoptosis has been previously demonstrated {Hockenbery D, *et al.* 1990}. A second shorter form of Bcl-2 protein that lacks a hydrophobic tail can potentially be produced through alternative splicing {Tsujimoto Y, *et al.* 1986}.

(ii) **Bcl-x**

Bcl-x displays 44% amino acid homology to Bcl-2 but shows different lineage specificity. The gene product exists in two forms, Bcl-xl which encodes 233 amino acids and contains the highly conserved BH1 and BH2 domains, and an alternative spliced form Bcl-xs which lacks a 63 amino acid stretch encompassing BH1 and BH2. Bcl-xl, similar to Bcl-2, inhibits apoptosis in many assay systems. Bcl-xs, on the other hand,
counters the protective effect of Bcl-2 and Bcl-xl. Over-expression of Bcl-xl protects cells from apoptosis upon IL-3 withdrawal, similar to Bcl-2. Bcl-xl can heterodimerize with Bax in mammalian cells, and single amino acid substitutions in BH1 abolished binding to Bax and abrogated the death-repressor effect. Like Bcl-2, Bcl-x has a hydrophobic carboxy-terminal transmembrane domain and its sub-cellular distribution is similar to Bcl-2 {Boise LH, et al. 1993} {Chao DT, et al. 1995} {Hsu & Youle 1997}. Bcl-xs does not form dimers with Bcl-2 but acts as a dominant negative mutant form of Bcl-2 by competing for substrates or regulators to prevent Bcl-2 from suppressing apoptosis. This explains why some cells can undergo apoptosis by either bypass or being regulated independently of Bcl-2 {Sentman DL, et al. 1991} {Fang W, et al. 1995}.

Despite the similarities, functional differences do exist between Bcl-2 and Bcl-x. B-lymphocytes undergoing apoptosis, as a consequence of cross-linking of surface IgM or as a result of exposure to immunosuppressants, can be rescued by Bcl-xl but not by Bcl-2. As well, activation of peripheral T cells leads to rapid induction of Bcl-xl but not Bcl-2. The patterns of expression suggest that Bcl-2 is the most important factor in maintaining the homeostasis of resting T cells, whereas Bcl-xl may be more important in post-activation survival decisions {Gonzalez GM, et al. 1994}. 
(iii) Bax

Bax is a 21-kDa molecular weight protein (192 amino acids) which contains a transmembrane domain. It has a 21% homology (43% similarity) with Bcl-2, and appears to form heterodimers with Bcl-2 protein as well as homodimers {Hunter & Parslow 1996}. Site-directed mutagenesis of BH1 and BH2 in bcl-2 showed that these two conserved domains were important for binding to Bax. When binding was disrupted, Bcl-2's protective function was also eliminated, suggesting that Bcl-2 must bind Bax to exert its effect. A novel, short "suicide domain" in Bax has been identified. Inserting this domain in place of the corresponding, divergent sequence in Bcl-2, converts Bcl-2 from an inhibitor into an activator of cell death {Hunter & Parslow 1996}.

Neither the BH1 nor the BH2 domain of Bax was required for binding to the wild-type Bcl-2 and Bax proteins. Moreover, Bax (delta BH1 and delta BH2) mutant proteins demonstrated efficient homo-and heterodimer formation, further confirming the lack of requirement for BH1 and BH2 for Bax/Bax homodimerization. Bax/Bax homodimerization was not dependent on the inclusion of the NH$_2$-terminal 58 amino acids of the Bax protein in each dimerization partner {Zha, Aimé-Sempé, et al. 1996}. This is different to Bcl-2/Bcl-2 homodimers which involve head-to-tail interactions between the region of Bcl-2 where BH1 and BH2 resides, and an NH$_2$-terminal domain in Bcl-2 that contains another domain BH4 which is conserved amongst anti-apoptotic members of the Bcl-2 family. Similarly, heterodimerization with Bcl-2 occurred without the NH$_2$-terminal domain of either Bax or Bcl-2, suggesting a tail-to-tail interaction {Zha,
Aimé-Sempé, et al. 1996). The essential region in Bax required for both homodimerization with Bax and heterodimerization with Bcl-2 was mapped to residues 59-101. This region in Bax contains a stretch of 15 amino acids that is highly homologous in several members of the Bcl-2 protein family, suggesting the existence of a novel functional domain termed BH3. Deletion of this 15-amino acid region abolished the ability of Bax to homodimerize and to heterodimerize with Bcl-2 (Zha, Aimé-Sempé, et al. 1996).

Furthermore, the ability of Bcl-2 to bind Bax alone can be insufficient for an anti-cell death function (Hanada, Aimé-Sempé, et al. 1995). The existence of Bcl-2/Bax heterodimers implies that Bcl-2 functions as a homodimer or oligodimer. Overexpression of Bax accelerates apoptosis induced by cytokine deprivation in an IL-3-dependent cell line and counters the repressor effect of Bcl-2 on apoptosis. Thus the ratio of Bcl-2 to Bax determines the amount of Bcl-2/Bax heterodimers versus Bax/Bax homodimers and is important in determining susceptibility to apoptosis. Bax protein contains a hydrophobic carboxy-terminus like Bcl-2 and has been co-localized to mitochondria with Bcl-2. Bax is widely expressed in tissues, including a number of sites in which cells die during normal maturation (Oltvai ZN, et al. 1993) (Yin XM, et al. 1994) (Krajewski S, et al. 1994) (Gratiot DJ, et al. 1993).
(iv) Bad

This protein is a newly described heterodimerizing partner of Bcl-2 and Bcl-xl, (Bcl-2/Bcl-xl associated death promoter). It differs from other family members with homology limited to only the most conserved amino acids in the BH1 and BH2 domains. Bad also lacks the typical carboxy-terminal transmembrane domain, suggesting that it is not an integral membrane protein. Bad efficiently counters the death-repressor effect of Bcl-xl, but is less effective against Bcl-2. The strong interaction between Bad and Bcl-xl, sequesters Bcl-xl, resulting in the availability of free Bax, and thus cell death is restored. Bad displaces Bax from Bcl-xl/Bax or Bcl-2/Bax heterodimers in a concentration-dependent manner. It negatively regulates cell death by modulating the amount of Bax in homodimers versus heterodimers. The discovery of Bad shows that the cell death regulators Bcl-2 and Bcl-xl are regulated by protein-protein interactions {Yang E, et al. 1995} {Gajewski TF, et al. 1996}.

(v) Bak

This protein is a Bcl-2 family member with BH1 and BH2 domains and is functionally similar to Bax, i.e it antagonizes Bcl-2 activity. Bak interacts with Bcl-2 and Bcl-xl and opposes the death-repressor activity. Bak also has the capacity to activate a cell death pathway when induced in Rat-1 fibroblasts. Thus there are multiple death repressors (Bcl-2, Bcl-xl) and multiple death promoter partners (Bax, Bcl-xs, Bad and Bak). Bak

4. Yeast Two-Hybrid Assays

This demonstrates the specificity of heterodimer formation among Bcl-2 family members. Bax is found to strongly heterodimerize with Bcl-xl and Bcl-2, suggesting that it may be a common partner in the regulation of cell death. In contrast, Bcl-xs, which opposes Bcl-xl and Bcl-2, only heterodimerizes with Bcl-xl and Bcl-2, suggesting that it is an alternately spliced form that reverses the protection provided by Bcl-xl and Bcl-2 by sequestering these molecules. Similarly, Bak heterodimerizes more strongly with Bcl-xl than with Bcl-2. Homodimers of Bax and Bcl-2 are also recapitulated in this system. The results from yeast two-hybrid assays show that there is selectivity in heterodimer formation within the Bcl-2 family of proteins and that there is a hierarchy in the strength of binding between the various partners. Although BH1 and BH2 domains in Bcl-2 are essential for heterodimer formation, deletion mapping in the yeast two hybrid system indicates that other regions of the molecules regulate dimer formation \{Farrow SN, et al. 1995\}.

Bax as well as peptides derived from the BH3 domains of Bax and Bak block both Bcl-2/Bax binding and Bcl-2/Bcl-2 binding. Similar assays demonstrate that Bcl-xl can form both homodimers and heterodimers and that these interactions are also inhibited by Bax.

Only Bax and Bak killed yeast via a process that did not require IL-1β converting enzyme-like proteases. Bax/Bak lethality was suppressed by co-expression of Bcl-2 family members that are anti-apoptotic in vertebrates, namely Bcl-xl, Bcl-2, Mcl-1, and A1. Furthermore, Bcl-xl and Bcl-2 suppressed Bax toxicity in yeast by distinct mechanisms. Bad, Bcl-xs, and Ced-9 lacked suppressor activity. These inactive proteins bound to anti-apoptotic members of the Bcl-2 family but not to Bax or Bak. In contrast, most Bcl-2 family proteins that attenuated death bound to Bax and Bak. However, two mutants of Bcl-xl suppressed Bax-induced cell death while having no Bax binding activity. Therefore, Bcl-xl functions independently of Bax binding, perhaps by interacting with a common target or promoting a pathway that antagonizes Bax {Tao, Kurschner, et al. 1997}.

5. Biochemical and Cell Biology of Bcl-2 Activity:

Bcl-2's full activity requires an integral membrane position. The carboxy-terminus of Bcl-2 contains a hydrophobic 19-amino acid stretch resembling a membrane spanning domain. Sub-cellular fractionation, immunofluorescence, and confocal microscopy
studies using anti-Bcl-2 antibodies indicated that Bcl-2 is an intracellular membrane protein whose distribution varies somewhat depending on cell type. Bcl-2 has been most convincingly localized to the mitochondria, the predominant site in haemopoietic cells, as well as the smooth endoplasmic reticulum and the peri-nuclear membrane. Targeting studies using purified mitochondria and in vitro-translated Bcl-2 protein showed that the carboxy-terminus functions as a signal anchor sequence responsible for targeting and insertion into the mitochondrial outer membrane. This exposes most of the polypeptide to the cytosol, in which it remains sensitive to protease digestion. Bcl-2 devoid of the signal anchor sequence is only partially functional in protection against apoptosis. However, a portion of the truncated Bcl-2 is still bound to its membrane-associated heterodimerizing partner, Bax. It has been argued that Bcl-2’s full function depends on its subcellular membrane localization. Most of the amino portion of Bcl-2 is exposed, in which case it may interact with proteins in the cytosol or other Bcl-2-like molecules similarly anchored in the mitochondria. Bcl-2’s function is not dependent on an intact electron transport/oxidative phosphorylation chain, as shown by the ability of Bcl-2 to block apoptosis in cells lacking mitochondrial DNA and unable to carry out electron transport


Recent electron microscopic data suggest that Bcl-2 protein is not randomly distributed in the membranes of the nuclear envelope but rather is concentrated in patches compatible
with nuclear pore complexes (NPC). These findings raise the possibility of a role for Bcl-2 in nuclear transport, NPC formation, or nuclear envelope assembly and maintenance (Krajewski S, et al. 1993).

Bcl-2 can inhibit oxidant-induced apoptosis: This is shown by the fact that Bcl-2 can protect cells against H$_2$O$_2$ and t-butyl hydroperoxide, which generate reactive oxygen species. At low concentrations, these oxidant stresses kill cells by an apoptotic process. Furthermore, Bcl-2 can protect against death induced by agents that decrease intracellular glutathione. This suggests that reactive oxygen species may be involved in apoptotic pathways susceptible to rescue by Bcl-2. Bcl-2 also does not have a significant effect on the generation of superoxide anion (O$_2^-$). It does not inhibit lipid peroxidation, a downstream event in oxidative damage and a frequent accompaniment of apoptosis. Thus, Bcl-2’s death repressor function does not solely depend on the protection of cellular constituents from oxidative damage. Although Bcl-2 can block oxidant-induced apoptosis, in the absence of a proven biochemical activity, it remains an open question whether Bcl-2 has a direct or indirect effect on the oxidant pathway (Hockenbery DM, et al. 1993) {Kane DJ, et al. 1993} {Shimizu S, et al. 1995} {Jacobson MD, et al. 1995}.

Ca$^{2+}$ plays an important role in apoptosis, particularly through the activation of Ca$^{2+}$-dependent endonucleases that may be involved in the internucleosomal DNA digestion, typical of apoptotic cells. Bcl-2 has been shown to block apoptosis induced by Ca$^{2+}$-ionophores in thymocytes. Over-production of Bcl-2 does not prevent rises in intracellular Ca$^{2+}$, suggesting that Bcl-2 blocks an apoptotic signal downstream of this
event {Zhong LT, et al. 1993} {Baffy G, et al. 1993}. Interestingly, IL-3-dependent cells deprived of the cytokine experience a gradual loss of Ca\(^{2+}\) from the ER and a rise in the relative amounts of Ca\(^{2+}\) in the mitochondria. Gene transfer-mediated elevations in Bcl-2 protein prevented the alterations in Ca\(^{2+}\) and suppressed apoptotic cell death. This suggests that Bcl-2 either directly or indirectly, can influence Ca\(^{2+}\) partitioning, but whether these alterations in Ca\(^{2+}\) contribute functionally to the apoptotic process remains unknown.

Another hypothesis concerns the role of oxidative injury in the induction of cell death and the finding that Bcl-2 blocks the accumulation of lipid peroxides, as well as possibly other reactive oxygen species in at least some settings {Kane DJ, et al. 1993} {Hockenbery DM, et al. 1993} {Lam M, et al. 1994} {Baffy G, et al. 1993}.

6. Clinical Aspects of Bcl-2:

(i) Translocations involving bcl-2 alone are not sufficient to cause cancer, i.e additional events are necessary for malignant transformation to occur {Liu Y, et al. 1994}.

(ii) Bcl-2 expression is found in tumours of some hormonally responsive epithelium. It allows cells to live longer and accumulate genetic alterations. Loss of Bcl-2 is likely to be a late event accompanied by additional genetic changes. In multi-
variate analysis, it appears that the prognostic role of Bcl-2 is related to p53 status, which in itself has independent prognostic significance {Miyashita T, *et al.* 1994}.

(iii) The ability of Bcl-2 to inhibit cell death induced by many agents with different mechanisms of action is consistent with Bcl-2 being a downstream molecule in the apoptotic pathway. In Acute Myeloblastic Leukaemia and Acute Lymphoblastic Leukaemia, the intensity of Bcl-2 staining and the number of positive cells are lower in cases that respond to chemotherapy than in non-responders; therefore, high Bcl-2 expression is an indicator of poor response in acute leukemia. Induction of Bcl-xl may play a role in the aetiology of chemotherapy and radiation-resistant tumours and may prove to have prognostic significance as well {Datta R, *et al.* 1995}.

(iv) Given that inappropriate survival can be a primary event in neoplasia and that cells undergo apoptosis in response to chemotherapy, the outcome of cancer may be affected by changing the setpoint at which cells undergo apoptosis in response to a signal. In cancers that over-express Bcl-2, decreasing Bcl-2 expression may allow a cell that contains otherwise intolerable genetic alterations to die. Altering the threshold for cell death, may render the cancer cell more sensitive to chemotherapeutic agents. This might be approached by downregulating Bcl-2 expression in cancer cells, either by targeting Bcl-2 directly or indirectly through an upstream regulator of Bcl-2. Because the susceptibility to cell death can be
determined by competing positive versus negative regulators in the Bcl-2 family, the threshold of death could be altered by changing the ratio of these members.

(v) Bcl-2 is widely expressed early in mouse foetal development in tissues derived from all three germ layers and this expression becomes restricted with maturation. During organ differentiation, Bcl-2 expression becomes increasingly restricted to certain cell types within each tissue. For example, Bcl-2 is expressed throughout the undifferentiated intestinal epithelium but is restricted to the zones of progenitors as cells within the villi matured. The wide distribution of Bcl-2 in the developing mouse suggests that many immature cells require a death repressor molecule or that Bcl-2 may have roles beyond regulating developmental cell death. This may reflect a common need for immature cells to express survival factors in order to overcome a stage-specific vulnerability to cell death. There is supportive evidence for the importance of inducible cell survival as a regulatory process in normal homeostasis and morphogenesis in many foetal tissues and structures.

Bcl-2 has been demonstrated in cells in the basal or degenerative regions of several complex epithelia, suggesting that Bcl-2 may serve to maintain the stem-cell pool by allowing a program of post-mitotic differentiation with eventual senescence and death. Bcl-2 also appears to be expressed in a selected group of terminally differentiated cells which are responsive to hormonal stimulation {Lebrun DP, et al. 1993}. 96
Inductive interactions between tissues are common during normal morphogenesis, and the initial morphological change in such events is often the formation of focal condensations of the induced cells. The presence of Bcl-2 protein at several sites characterized by inductive interactions between epithelial and mesenchymal structures suggests that Bcl-2 might play a role in the "commitment" process undergone by groups of cells destined to participate in the formation of new structures. In this context, the expression of Bcl-2, possibly under the direct or indirect influence of soluble factors from nearby tissues, could contribute to the formation of the cellular condensations that are associated with the embryological formation of some structures by decreasing the focal rate of cell death relative to mitosis {Veis-Novack, et al. 1994}.

Bcl-2 is not expressed in the majority of normal follicular centre cells which undergo programmed cell death unless they are rescued and transformed into long-term memory cells by antigen-derived selection. Although the expression of Bcl-2 appears to be associated with cell development, maturation, and differentiation, the exact biological function of the protein is unknown. This implication for Bcl-2 in regulating the developmental process, such as active cell death during the fusion process of palatal shelves is considered important in biological research {Miyazono K, et al. 1989}.

Refer to Figures 18 to 24.
**Figure 18:** Schematic diagram of the cell death pathway. Various stimuli generate a cell death signal(s), the ratio of heterodimers of the cell death regulators determine the susceptibility to death, and cell death effectors execute PCD. The precise immediate steps and the critical protease substrates are not known. (Adapted from Yang and Korsmeyer, 96)
Figure 19: The relative ratios of Bcl-2 and Bax heterodimers to homodimers determine the susceptibility to PCD. (Adapted from Yang and Korsmeyer, 96)

Figure 20: Bad is a negative regulator of apoptosis. It displaces Bax from Bcl-2/Bax or Bcl-xL/Bax heterodimers, allowing more Bax/Bax homodimer formation, which promotes death. (Adapted from Yang and Korsmeyer, 96)
Figure 21: Sequence and domain comparisons of Bcl-2 family proteins. (Zha et al, 96)

Figure 22: Schematic representations of dimerizations between Bax, Bcl-2, and Bcl-Xl.

(Hsu and Youle, 97)
Figure 23: Model for the functional relevance of Serine Phosphorylation of Bcl-2. 
(Adapted from Gajewski and Thompson, 96)

Figure 24: Model for phosphorylation of Bad. A, Kinase activation prevents Bad interacting with Bcl-xl, results anti-apoptotic function. B, Raf-1 translocated to Bcl-2 resulting in serine phosphorylation of Bad. (Adapted from Gajewski and Thompson, 96)
CHAPTER 2

MATERIALS & METHODS

The study was approved by the Central Sydney Area Health Services Animal Ethics Committee.

Animals

The animals used in this study were an outbred line of Sprague-Dawley rats, bred at the Institute of Dental Research (IDR). The rats were kept in the Animal House of the IDR, Sydney, in cages with wood chip litter and fed with commercial pellets. Timed, pregnant rats were produced in-house by breeding the rats overnight. The morning-after was counted as 0.5 day gestation. The rats used were between 70 to 120 days old. The procedure included two male rats added to cages containing five females at 5pm, and removed the next morning at 8am. Approximately 15% of female rats conceived using this approach.

It has been suggested that inter-litter variability may be largely reduced by breeding for a short period rather than overnight. Fujinaga et al (1990) compared the mean number of implantations and live embryos, the mean crown-rump length, the somite number and
protein content of the overnight and the morning short period breeding regimens {Fujinaga M, et al. 1990}. Their results showed no differences in mean number of implantations, live foetuses and resorptions between the two breeding regimens. The mean crown-rump length, somite number and protein content were significantly lower for embryos in the short breeding period group than those in the overnight breeding group. The differences were, however, small and of little practical importance for most experimental studies.

Pregnant rats were sacrificed at 15.5, 16.5, and 17.5 days respectively using overdosage of halothane anaesthesia. Uteri were excised and individual implantation sites were harvested and fixed in 4% paraformaldehyde in isotonic phosphate buffered saline (PBS) of pH 7.4. The embryonic sacs were egg-shaped structures with the pointed ends containing the embryos within the extra embryonic coelom and the blunt ends were mostly decidua tissues.

The foetuses were completely dissected out of the amniotic cavity using dissecting microscopy prior to fixation. The foetuses were then dissected in two planes, horizontally just above the mandibular plane and coronally just behind the ears. Foetal tissues were fixed overnight at 4°C, washed in cold PBS and dehydrated in graded alcohols. The foetuses were then orientated and embedded in paraffin contained by preformed embedding moulds. The planned orientation allowed the production of serial sections posterio-anteriorly.
Sectioning

Blocks were placed at 4°C for 15 minutes prior to mounting onto a microtome chuck. Serial paraffin sections were cut at 5μm and spread over the hot water-bath as a ribbon. Each serial paraffin segment which contained two to four sections was separated and mounted on a glass-slide with pre-determined orientation and numbering system.

The standard technique for examining microscopic slides is to stain them with the H & E staining system. The procedure for this is as follows:

- Treatment of slides: Every tenth slide was:
  
  (i) Oven heated to 60°C for 30 minutes
  (ii) Deparaffinised in xylol for 15 minutes

- The slides with pre-determined orientation and numbering system were then rehydrated through decreasing concentrations of alcohol solutions, i.e Absolute (2x), 95% (1x), 70% (1x) and washed well until clear in water.

- The slides were then stained with Mayer’s Haematoxylin and Eosin and observed under the light microscope.

The microscopic observation revealed important findings about the timing of palatal-shelf fusion. It was found that the pre-fusion stage occurred at around 15.5 days post-
conception in Sprague-Dawley rats. Post-fusion stage was seen to occur around 17.5 days, whereas, palatal-shelf fusion happened at 16.5 days. It must be pointed out that the timing of palatal fusion is an approximation due to the arbitrary gestational time with variation of up to 0.5 day.

Immunohistochemical Staining Technique

Background

Frozen hydrated sections should preserve antigens in a state close to physiological condition. Unfixed foetal tissues are highly hydrated and experience extensive ice crystal damage and distortion on freezing. Further, it is difficult to achieve precise orientation of foetal tissues for frozen sectioning. The best compromise was to accept the advantages of routine preparation in paraffin and to address subsequently, the issue of antigen preservation. Formalin preserves tissue morphology by creating protein cross-links, which can also affect protein conformation and accessibility or availability for antibody binding. Protein associations such as latency, can also be altered by the fixative, solvents or heat used during paraffin processing. Most antibodies exhibit some degree of decreased immunoreactivity in formalin sections, perhaps by virtue of a conformational change in latency that reduces access to the antigenic region {Brown, Wakefield, et al. 1990}.  

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Antibodies (Ab) can bind to antigens (Ag) either through specific or non-specific binding sites. The resulting complex of antibody-antigen can then be marked or labelled. TGF-β is found in many normal cells and tissues including liver, heart, brain, kidney, muscle, gut, uterus, and platelets {Roberts AB, et al. 1983} {Barnard, Beauchamp, et al. 1989}. Such tissues can be used as controls for the experimental technique. A positive control is essential to establish the validity of the technique. It validates the immunohistochemical reaction and demonstrates the specificity of the Ab-Ag complex. The control tissue utilised in these experiments was the rat small intestine.

It is necessary to block antibody binding to non-specific adhesive sites. Horse serum as a source of complex proteins was used to mask the non-specific multi-antigenic binding sites. This, in turn, exposes the binding sites on the required antigen for high affinity specific binding by the primary antibody.

Relevant slides of interest were selected by using those stained with H&E as a guide, to investigate and illustrate the presence of:

- Morphogenic proteins TGF-β1,2,3
- TGF-β RI,II
- Bax and Bcl-2

Normal rabbit serum was used as a control in place of affinity-purified rabbit antibody. This acted as a second control to validate the result.
Hydrogen Peroxide (H₂O₂) was initially utilized to block the endogenous peroxidase. When used at a concentration of 3%, H₂O₂ was shown to be quite destructive to the embryos. A lower concentration of 0.3% for 15 minutes was somewhat more acceptable for structural preservation. Comparative tests were carried out in sections with and without H₂O₂, and there was little difference in the stain intensity. Consequently, it was decided that H₂O₂ was to be excluded from the protocol in this research because of inadequate tissue structural preservation and the inconsistent results obtained.

1. **Proteinase Digestion.**

In the earlier stage of the research, trypsin was used in the digestive process with the intention of exposing more antigenic binding sites. Trials were carried out using varying concentrations of the enzyme. It was found that the digestive process proved to be extremely harsh on the delicate embryonic tissues. The optimal digestion concentration of trypsin without causing tissue damage was 0.1% at 37°C for 15 minutes. However, when the trypsin and non-trypsin treated sections were compared there was little difference in the stain intensity between the two.
2. Microwave Antigen Retrieval Technique (MART).

This is based upon microwave treatment combined with mordanting (post fixation) in heavy metal solutions. This technique has produced marked improvements in the immunohistological staining for many antigens in formalin-fixed paraffin-embedded tissues (Shi SR, et al. 1991). It is hypothesized that antigens “lost” during formalin fixation are masked by extensive cross-linking of proteins at the tertiary or quarternary structural level. Heating of tissue sections in excess of 100°C by high energy microwaves may break the cross linkages, thereby unmasking these epitopes.

The MART involves the following steps:

1. The slides are placed in a coplin jar containing 0.01M sodium citrate buffer (pH 6), covered with cling film (vented), and heated in an 800W microwave oven for two five minute cycles at full power. If the buffer evaporates after 5 minutes, it is necessary to top-up the fluid with hot, distilled water.

2. The sections are then left to stand for 20 minutes in the microwave.

Despite some early results which indicated that sections microwaved prior to immunohistochemical staining, stained more intensely than those which were not treated, the results were not consistent. It was observed that due to a combination of heat and pressure, together with the physical bubbling/boiling effect, the tissue was often ruptured.
Early studies utilized monoclonal antibodies and turkey polyclonal antibodies with broad specificity against transforming growth factor-beta isoforms. These produced weak reactions only and did not allow the discrimination of isoforms.

Recently, Santa Cruz Ltd released a range of polyclonal antisera raised against defined peptide sequences. This polyclonal antibody system was found to be highly sensitive and specific. The immunohistochemical results obtained were consistent and reproducible. Further, the stain recovered from the counter-staining technique was positive, strong and clear. The reagents and antibody system utilized in this research are listed in the Appendix.

Another antibody system which was on trial in the early stage of the research was monoclonal antibody to Keratin-8. This system was used in an attempt to demonstrate whether keratin expressed by the epithelial cells could be used as a phenotypic marker. However, the use of this antibody system was postponed due to inconsistent results and poor specificity.
3. General Protocol for Immunohistochemical Staining Technique

1. Sections deparaffinised in xylol for 15 minutes

2. Slides were rehydrated through decreasing concentrations of alcohol solutions, i.e.
   Absolute (2x), 95% (1x), 70% (1x) and washed well until clear in water

3. Sections were blocked with horse serum at room temperature (25°C) for 1 hour

4. Washed slides in PBS (three, 5 minutes cycles)

5. Appropriate dilutions of normal rabbit serum added to the marked control sections
   and incubated in the humidifier at room temperature for 1 hour

6. Primary antibody added to the marked test sections and incubated in the humidifier at
   room temperature for 1 hour

7. Washed slides in PBS (three, 5 minutes cycles)

8. Secondary antibody (peroxidase-labelled swine anti-rabbit) added to all sections and
   incubated in the humidifier at room temperature for 1 hour
9. Washed slides in PBS (three, 5 minutes cycles)

10. Metal enhanced diaminobenzidine reagent (Pierce Ltd) and H₂O₂ added to all sections and incubated at room temperature, in the dark, for 5 minutes

11. Slides washed thoroughly in water

12. Observed slides under light microscopy prior to counter-staining with Mayer’s haematoxylin for 15 seconds and blueing solution for 20 seconds

13. Glass-cover slip placed

14. Photography:

   Colour photographs were taken using an Olympus Vanox light microscope (C-35AD) and Konica Impressa colour print film (ASA 50).

   Magnification for slides: \(4x = 10x\)

   \(10x = 25x\)

   \(20x = 50x\)

   \(40x = 100x\)
Absorption with Specific Blocking Peptide

The specificity of the primary antibodies was confirmed by pre-incubation with peptides derived from the amino acid sequences of the proteins in question. This was carried out by adding the diluted peptide to the antagonist and the mixture was incubated at room temperature for 1 hour prior to blocking the control tissue. This test was performed at the same time and under similar experimental condition to that of the anti-peptide test. Such differences in the stain-intensity between peptide-protein and protein alone are important in interpreting the results both quantitatively and qualitatively.

The non-absorbed and absorbed antibodies were then used to stain sections of rat small intestine and the reduction of staining intensity following absorption noted.
Diagram 1: Illustration of Immunohistochemical reaction.
Explanation of Diagram 1 (using TGF-β as an example):

The amorphous mass in the center of the diagram represents the tissue of interest. This tissue contains multi-antigenic binding sites including TGF-β antigens. The first step of the immunohistochemical reaction involves a non-specific blocking procedure. This is done by adding horse serum (which contains non-specific polyclonal antibodies) to the tissue. This optimizes the exposure/expression of TGF-β antigens to a more highly specific interaction with the primary antibodies. High-affinity purified rabbit polyclonal primary antibody and TGF-β antigen complexes are formed immunohistochemically. Swine anti-rabbit immunoglobulins (secondary antibodies) are then added to complex with the rabbit primary antibodies. The swine anti-rabbit secondary antibodies are conjugated with horseradish peroxidase, which acts to amplify the expression of antibody-antigen complex. By adding Diaminobenzidine tetrahydrochloride/metal-hydrogen peroxide (DAB-H₂O₂) to the system, a brown insoluble deposit is labelled at the target sites of TGF-β antigens via the counter-staining technique. This brown staining will be illustrated in the subsequent immunohistochemical slides photographed throughout this thesis.

The immunohistochemical reaction is a highly sensitive system and is affected by a number of variables/factors:

1. The type and dilution of reagents
2. The number of antigen binding sites
3. The use of specific and non-specific blocking agents
4. The temperature and duration of incubation

5. Labeling (with DAB) and counter-staining technique

Refer to Diagram 1.
Diagram 2: Showing the sites of interest where Immunohistochemical stain-intensity score was taken:

1. Nasal epithelium of palatal-shelf
2. Oral epithelium of palatal-shelf
3. Epithelial seam
4. Palatal-shelf mesenchyme
Stain Intensity Score

1: Trace (refer to photograph 4)

2: Light (refer to photographs 6, 8)

3: Moderate (refer to photographs 9, 11)

4: Intense (refer to photographs 13, 15)

The subjective, semi-quantitative scoring system for the stain-intensity ranges from 1 to 4 with a score of 1 being the least intense and a score of 4 being the most intense. The net scores shown in the subsequent graphical illustrations are the means from six different embryo sections at the same stage of palatal development for TGF-β1,2,3 and TGF-βRI&II and Bax/Bcl-2. Sections were coded and the average intensity score was recorded blind.

These measurements were taken from the sections of the:

(1) Oral and nasal epithelium of palatal shelf

(2) Palatal shelf mesenchyme

(3) Epithelial seam

(4) Stellate reticulum

(5) Periderm

(6) Eye

Refer to Diagram 2.
Statistical Analysis

This was based on a systematic approach, whereby data obtained by blind and coded semi-quantitative assessment was analysed. The numerical scoring system for the stain-intensity ranged from 1 to 4, as described. Sections of six different foetuses (at the same stage of palatal development) were coded and the average intensity of staining scored blind. The results were graphed according to the staging of the palatal fusion sequence. The standard errors for the means were calculated and the data tested for any significant differences between the comparative samples for TGF-βs, TGF-βRs, and Bax/Bcl-2. Data were analysed by a two-tailed Student t-test. These values are indicated appropriately in the “Results” section whenever the differences are significant.

Refer to Results (Chapter 3) and Graphs 1 to 3.
CHAPTER 3

RESULTS AND DISCUSSION

A. RESULTS

1. Control Tissue, Rat Intestine

Positive staining for TGF-β1,2&3, TGF-βRI&II, and Bcl-2/Bax was observed in the control tissue, rat intestine. Further absorption with specific peptides reduced the stain intensity.

Reference: Photographs 4 showing positive stain for TGF-β1

5, 6 showing positive stain for TGF-β2 and absorbant activity

7, 8 showing positive stain for TGF-β3 and absorbant activity

9, 10 showing positive stain for TGF-βRI and absorbant activity

11, 12 showing positive stain for TGF-βRII and absorbant activity

13, 14 showing positive stain for Bcl-2 and absorbant activity

15, 16 showing positive stain for Bax and absorbant activity
2. TGF-\(\beta\) in Relation to Palatogenesis

(a) Nasal aspect of palatal epithelium

TGF-\(\beta1,2,3\) are expressed almost evenly throughout stages of the developmental sequence. On average, TGF-\(\beta2,3\) are slightly more dominant than TGF-\(\beta1\) and the difference between TGF-\(\beta2\) and TGF-\(\beta3\) is very slight, with TGF-\(\beta2\) slightly more dominant.

Reference: Graph 1 for graphical illustration of TGF-\(\beta1,2,3\)

Photographs 18, 19, 20 for IHC illustration of TGF-\(\beta1\)

21, 22, 23 for IHC illustration of TGF-\(\beta2\)

25, 26 for IHC illustration of TGF-\(\beta3\)

(IHC: Immunohistochemical)

(b) Oral aspect of palatal epithelium

TGF-\(\beta1,2,3\) are expressed throughout with an increasing intensity score toward the fusion and post-fusion stages of the developmental sequence. There is no difference between TGF-\(\beta2,3\) and TGF-\(\beta2,3\) are more dominant than TGF-\(\beta1\).

The two-tailed P value is 0.0087, considered highly significant.
(c) Epithelial seam

TGF-β1,2&3 are expressed only in the fusion and early post-fusion stages of the developmental sequence. There is no difference between TGF-β2&3 and TGF-β2&3 are more dominant than TGF-β1.

The two-tailed P value is 0.0313, considered significant.

Reference: Graph1 for graphical illustration of TGF-β1,2&3

Photographs 18, 19, 20 for IHC illustration of TGF-β1

21, 22, 23, 24 for IHC illustration of TGF-β2

25, 26 for IHC illustration of TGF-β3
(d) Palatal mesenchyme

TGF-β1,2&3 are expressed unevenly, with TGF-β2&3 absent in the pre-fusion stage and TGF-β1 absent in the late post-fusion stage of the developmental sequence. There are no significant differences between TGF-β2&3.

Reference: Graph1 for graphical illustration of TGF-β1,2&3

Photographs 18, 19, 20 for IHC illustration of TGF-β1

21, 22, 23, 24 for IHC illustration of TGF-β2

25, 26 for IHC illustration of TGF-β3

3. TGF-βRI&II in Relation to Palatogenesis

(a) Nasal aspect of palatal epithelium

TGF-βRI&II are expressed throughout the stages of the developmental sequence. TGF-βRII is more dominant in the pre-fusion and fusion stages, whereas, TGF-βRI is slightly more dominant in the post-fusion stage.

Reference: Graph2 for graphical illustration of TGF-βRI&II

Photographs 33, 34 for IHC illustration of TGF-βRI
(b) *Oral aspect of palatal epithelium*

TGF-βRI&II are expressed throughout the stages of the developmental sequence. On average, there is only a slight difference between the expression of TGF-βRI&II.

**Reference:** *Graph2* for graphical illustration of TGF-βRI&II

*Photographs* 33, 34 for IHC illustration of TGF-βRI

35, 36 for IHC illustration of TGF-βRII

(c) *Epithelial seam*

TGF-βRI&II are expressed only in the fusion and early post-fusion stages of the developmental sequence. There is no difference between the expression of TGF-βRI&II.

**Reference:** *Graph2* for graphical illustration of TGF-βRI&II

*Photographs* 33, 34 for IHC illustration of TGF-βRI

35, 36 for IHC illustration of TGF-βRII
(d) **Palatal mesenchyme**

TGF-βRI&II are expressed unevenly, with TGF-βRI&II absent in the late pre-fusion and early fusion stages of the developmental sequence. In addition, TGF-βRII is absent in the late fusion and late post-fusion stages. There is only a slight difference in expression between TGF-βRI&II.

**Reference:** *Graph2* for graphical illustration of TGF-βRI&II

*Photographs* 33, 34 for IHC illustration of TGF-βRI

35, 36 for IHC illustration of TGF-βRII

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4. **BCL-2 and BAX in Relation to Palatogenesis**

(a) **Nasal aspect of palatal epithelium**

Bcl-2 and Bax are expressed throughout the stages of the developmental sequence. Bax is slightly more dominant than Bcl-2, although the difference is not significant.

**Reference:** *Graph3* for graphical illustration of Bcl-2 & Bax

*Photographs* 31 for IHC illustration of Bcl-2
(b) *Oral aspect of palatal epithelium*

Bcl-2 and Bax are expressed throughout the stages of the developmental sequence. However, the expression of Bcl-2 and Bax is much less in the pre-fusion stage. There is no difference between the expression of Bcl-2 and Bax.

**Reference:**  *Graph3* for graphical illustration of Bcl-2 & Bax

*Photographs* 31, 32 for IHC illustration of Bcl-2

27, 29, 30, 28 for IHC illustration of Bax

(c) *Epithelial seam*

Bcl-2 and Bax are expressed only in the fusion and early post-fusion stages of the developmental sequence. There is no difference between the expression of Bcl-2 and Bax.

**Reference:**  *Graph3* for graphical illustration of Bcl-2 & Bax

*Photographs* 31, 32 for IHC illustration of Bcl-2

27, 29, 30, 28 for IHC illustration of Bax
(d) **Palatal mesenchyme**

Bcl-2 and Bax are expressed unevenly and are absent in the late fusion stages of developmental sequence. In addition, Bcl-2 is absent in the pre-fusion stages and Bax is slightly more dominant in the expression.

**Reference:** *Graphs* 3 for graphical illustration of **Bcl-2 & Bax**

*Photographs* 31, 32 for IHC illustration of **Bcl-2**

27, 29, 30, 28 for IHC illustration of **Bax**

Refer to Graphs 1 to 3.
**Photograph 1: H&E**

Showing apoptotic activity
in the cells of the epithelial seam
and at the palato-nasal junction of
an intact seam (10x).

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**Photograph 2: H&E**

Showing apoptotic activity
in the cells of the epithelial seam
and at the palato-nasal junction of
a disintegrated seam (10x).
Photograph 3:

Immunohistochemistry (IHC)

Showing a negative stain following application of normal rabbit serum in the control tissue, rat intestine (20x).

Photograph 4: IHC

Showing a positive stain for TGF-β1 in the control tissue, rat intestine (20x).
Photograph 5: IHC

Showing a positive stain for TGF-β2 in the control tissue, rat intestine (10x).

Photograph 6: IHC

Showing reduced staining for TGF-β2 in the control tissue, rat intestine following absorption of the antibody with the specific antigen peptide (10x).
Photograph 7: IHC
Showing positive a stain for TGF-β3 in the control tissue, rat intestine (10x).

Photograph 8: IHC
Showing reduced staining for TGF-β3 in the control tissue, rat intestine following absorption of the antibody with the specific antigen peptide (10x).
**Photograph 9: IHC**

Showing a positive stain for

\textbf{TGF-βRI} in the control tissue,

rat intestine (20x).

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**Photograph 10: IHC**

Showing reduced staining for

\textbf{TGF-βRI} in the control tissue,

rat intestine following absorption

of the antibody with the specific

antigen peptide (20x).
Photograph 11: IHC

Showing a positive stain for TGF-βRII in the control tissue, rat intestine (20x).

Photograph 12: IHC

Showing reduced staining for TGF-βRII in the control tissue, rat intestine following absorption of the antibody with the specific antigen peptide (20x).
Photograph 13: IHC

Showing a positive stain for **Bcl-2** in the control tissue, rat intestine (20x).

Photograph 14: IHC

Showing reduced staining for **Bcl-2** in the control tissue, rat intestine following absorption of the antibody with the specific antigen peptide (20x).
Photograph 15: IHC

Showing a positive stain for

**Bax** in the control tissue,
rat intestine (20x).

Photograph 16: IHC

Showing reduced staining for

**Bax** in the control tissue,
rat intestine following absorption
of the antibody with the specific
antigen peptide (20x).
**Photograph 17: IHC**

Showing a positive stain for TGF-β1 at the pre-fusion stage. Horizontal palatal shelves. Note the apoptotic activity in the shed periderm (10x).

**Photograph 18: IHC**

Showing a positive stain for TGF-β1 at the fusion stage, especially where there is intense apoptotic activity (10x).
Photograph 19: IHC

Showing a positive stain for TGF-β1 at the fusion stage.
Note the intense stain along the disintegrated epithelial seam (10x).

Photograph 20: IHC

Showing positive stain for TGF-β1 at the post-fusion stage. Note the intense stain in the island of the residual seam (10x).
Photograph 21: IHC

Showing a positive stain for TGF-β2 at the fusion stage.

Note the intense stain along the intact epithelial seam (10x).

Photograph 22: IHC

Showing a positive stain for TGF-β2 at the fusion stage.

Note the intense stain along the disintegrated epithelial seam (10x).
Photograph 23: IHC
Showing a positive stain for TGF-β2 at the fusion stage.
Note the intense stain along the intact epithelial seam (10x).

Photograph 24: IHC
Showing a positive stain for TGF-β2 at the post-fusion stage. Note the intense stain in the island of the residual seam (20x).
Photograph 25: IHC

Showing a positive stain for TGF-β3 at the fusion stage.

Note the intense stain along the intact epithelial seam (10x).

Photograph 26: IHC

Showing a positive stain for TGF-β3 at the post-fusion stage. Note the intense stain in the epithelial island of the residual seam (10x).
Photograph 27: IHC

Showing a positive stain for Bax at the fusion stage.

Note the intense stain along the intact epithelial seam (10x).

Photograph 28: IHC showing a positive intense stain for Bax in the residual islands of the epithelial seam (20x).
Photograph 29: IHC

Showing a positive stain for Bax at the fusion stage.
Note the intense stain observed in the disintegrated seam (10x).

Photograph 30: IHC showing a positive intense stain for Bax

in the residual islands of the epithelial seam (20x).
Photograph 31: IHC

Showing a positive stain for Bcl-2 at the fusion stage.

Note the disintegrated epithelial seam (10x).

Photograph 32: IHC showing a positive intense stain for Bcl-2 in the residual islands of the epithelial seam (20x).
Photograph 33: IHC

Showing a positive stain for

TGF-βRI at the fusion stage

(4x).

Photograph 34: IHC

Showing a positive stain for

TGF-βRI at the fusion stage.

Note the disintegrated seam (10x).
Photograph 35: IHC

Showing a positive stain for TGF-βRII at the fusion stage (10x).

Photograph 36: IHC

Showing a positive stain for TGF-βRII at the fusion stage.

Note the disintegrated seam (20x).
Photograph 37: IHC

Showing a positive stain for
TGF-β1 in the developing tooth
bud and surface epithelium (10x).

Photograph 38: IHC

Showing a positive stain for
TGF-β2 in the developing
tooth bud and surface
epithelium (10x).
Photograph 39: IHC

Showing a positive stain for

TGF-βRI in the developing tooth bud and surface epithelium (10x).

Photograph 40: IHC

Showing a positive stain for

TGF-βRII in the developing tooth bud and surface epithelium (10x).
Photograph 41: IHC

Showing a positive stain for Bax in the developing tooth bud and surface epithelium (10x).

Photograph 42: IHC

Showing a positive stain for Bcl-2 in the developing tooth bud and surface epithelium (10x).
Photograph 43: IHC showing a positive stain for TGF-β1 at the external epithelial lining in the proximity of the eye, (20x).
Graph 1: Showing stain-intensity scores of TGF-β1,2&3 at different stages of

Palatal developmental sequence: Pre-fusion A&B, Fusion C&D, and Post-fusion E&F.
Detection of TGFβR-(1, 2) in Relation to Palatogenesis

Graph 2: Showing stain-intensity scores of TGF-βR I&II at different stages of palatal developmental sequence.
Graph 3: Showing stain-intensity scores of Bax and Bcl-2 at different stages of developmental sequence of the palate.
B. DISCUSSION

Most of the documented studies on palatal development have been carried out on the mouse and to date not many experiments have been performed on the rat. Due to the inter-species differences, it is anticipated that the results obtained from the present study are not an absolute representation of the underlying developmental process in man. Although the causal mechanisms underlying growth and differentiation that occur during palatal development are not clearly understood, it is apparent that any disruption in the coordination of developmental events, at any level, could result in the malformation of the palate.

At the time of commencement of this research, there was rather scant information reported on morphogens, receptors and death proteins and the role they play in the process of palatal fusion. However, there has since been a bank of new findings marking a clear trend of interest regarding the behaviour of these important proteins in the field of development. As a result of the intensifying research there has been rapid progress recently in the evaluation of the relation of these proteins to the fusion sequence.

The serial coronal sectioning of the rat's palate performed in this study started in the posterio-anterior direction, and a comprehensive histological pattern of palatal formation was obtained. As a result, the staging of the palatal fusion sequence was fully captured and as consistent with the literature, the hard palate was found to be fusing in antero-posterior direction.
The epithelial lining of the palatal shelves is regionally heterogeneous and undergoes different cell fates. Regional specification of palatal epithelium is controlled by the underlying mesenchyme through the induction process. Further, Ferguson found that 50% of MEE cells were involved in an epithelial-mesenchymal transformation (EMT). These cells failed to express cytokeratins, but instead expressed the intermediate filament vimentin and thus became indistinguishable from the underlying mesenchymal cells {Ferguson MWL 1988}.

During shelf elevation and prior to contact, sloughing peridermal cells showed TUNEL-positive staining {Mori C, et al. 1994}. The peridermal layer appeared in this study as surface coating for the apposing palatal shelves. During the initial contact of the palatal shelves, the MEE remained negative for TUNEL staining. However, once the midline seam began to attenuate and break up into a discontinuous seam, TUNEL-positive staining was found in nuclei of cells within epithelial islands of the disintegrated palatal seam. It was interesting that some TUNEL-positive, keratin-negative cells just outside the epithelial islands were detected. DNA fragmentation was used in some cells along the palatal midline when the epithelial seam became discontinuous. It seemed that apoptosis was closely associated with the disruption of MEE but may not be required for initial contact or epithelial fusion of apposing palatal shelves {Mori C, et al. 1994}.

The three-dimensional reconstruction work of the palatal shelves carried out by Suzanne Brent in parallel with the present study showed that the basal lamina surrounding these
cells had disintegrated, and the epithelial cells lost epithelial characteristics and assumed fibroblast-like features.

There is a remarkable conservation of TGF-β sequences between species and they are known to be multifunctional growth factors that regulate many aspects of cellular activities, including cell growth and differentiation, and specific morphogenetic and histogenetic events in embryonic development. The effects of TGF-β on cells have been shown to be not a function of the peptide itself, but rather of the total set of growth factors and their receptors that is operant in the cell at a given time.

There is a complicated regulatory network whereby each of the TGF-β isoforms either upregulate or downregulate the expression of other isoforms which are represented by distinct genes on separate chromosomes. In the mouse, the three main TGF-β isoforms (TGF-β1,2,3) are expressed in a temporally and spatially regulated manner throughout palatogenesis. Epithelial expression of TGF-β1,2&3mRNA is associated with regions of active morphogenesis involving epithelial-mesenchymal interactions. In general, the expression patterns are non-overlapping suggesting that each isoform may have a specific function. Each TGF-β has diverse biological activities in vitro and each can influence cell growth and/or differentiation in a positive or negative manner. The specific action of the growth factor is dependent on the cell type and culture conditions {Roberts AB, et al. 1990}. Whether epithelially-derived TGF-βs are mainly involved in autocrine regulation of growth and differentiation and/or in paracrine interactions between the epithelium and the underlying mesenchyme, remains to be determined.
TGF-β1 has been demonstrated to be synthesised by epithelium and is predominantly localised in the adjacent mesenchyme. TGF-β1mRNA is abundant in the epithelial component of structures that are actively involved in the process of morphogenesis such as tooth bud and secondary palate {Akhurst, FitzPatrick, et al. 1990}. In general, it has been shown that the embryological localisations of mRNA transcripts and protein product for TGF-β1 show a strong correlation indicative of both autocrine and paracrine mechanisms. Furthermore, where epithelial TGF-β1mRNA expression is seen, this mRNA is co-expressed with either TGF-β2 and/or TGF-β3 mRNA. Therefore, TGF-β2 and β3 might similarly be involved in the general control of morphogenesis {Lehnert SA, et al. 1988}.

TGF-β3 has a specific role in the events that control transdifferentiation of the medial edge epithelial cells including degradation of the underlying basement membrane. Data indicate that TGF-β3 affects palatal shelf fusion by an intrinsic, primary mechanism rather than by effects secondary to craniofacial morphometrics {Kaartinen, Cui, et al. 1997} {Proetzel, Pawlowski, et al. 1995}. It has previously been demonstrated that the application of specific antisense oligodeoxynucleotides (ODN), to prevent mRNA expression, and neutralizing antibodies to block the activity of the active peptide of TGF-β3, but not TGF-β1 or TGF-β2, prevented the epithelial-mesenchymal transformation of the medial seam and inhibited fusion {Brunet, Sharpe, et al. 1995}. This inhibition could be rescued by exogenous TGF-β3. Organ culture studies indicate that TGF-β1 and TGF-β2 accelerate palatal shelf fusion and that antisense oligodeoxynucleotides or neutralizing
antibodies to TGF-β3, but not to TGF-β1 or TGF-β2, block the fusion process {Brunet, Sharpe, et al. 1995} {Potts JD, et al. 1991}.

Unlike tgf-β1&3, epithelial expression of the tgf-β2 gene is not only limited to morphogenetically active tissue, but is also seen in epithelial cells of established structures that are in the process of differentiation. These include alveolar epithelium and hyperplastic nodules of palatal epithelium {FitzPatrick, Denhez, et al. 1990}. TGF-β2 levels have been shown to elevate in vitro and in vivo in keratinocytes growth-inhibited or induced to differentiate by retinoic acid or by calcium ions {Glick AB, et al. 1989}. It has been suggested that the endogenous in vivo function of TGF-β2 in some epithelia is in autocrine modulation of growth and/or differentiation {Pelton RW, et al. 1989}.

Complete confidence in the specificity of the antibody reactions in the present study was essential to allow valid interpretation of the data. The series of controls performed in this study provided the necessary confidence that specific identification of TGF-β isoforms had been achieved.

In the present study, the TGF-β family (1,2&3) and the death proteins were detected in relation to discrete stages and defined localization in palatogenesis. There was a consistent strong expression of TGF-β’s at late pre-fusion and throughout the fusion stages. Overall, the family of TGF-β1,2&3 were observed to be expressed at the same time in a coordinated manner, and there was no clear evidence to suggest that TGF-β3 was the dominant isoform. This differs from the literature which primarily reports data
for the mouse suggesting that TGF-β3 is more dominant in palatogenesis, especially, in the fusion process {Kaartinen, Cui, et al. 1997} {Brunet, Sharpe, et al. 1995}. It is interesting to note a similar pattern of expression between TGF-β1,2&3 and TGF-βRI&II in the present study, especially in the midline epithelial seam. This emphasizes strongly the potential autocrine and/or paracrine actions of TGF-β’s.

If TGF-β3 dominates in regulating the fusion process in the rat as in the mouse, there is a requirement to explain the basis for this when the data indicate near synchronous expression of the three isoforms during palatogenesis in the rat (see Results). Related to the structural identity of each isoform, a possible mechanism would be preferential activation of TGF-β3 by specific proteinases or glycosidases. Alternatively, TGF-β3 may not play a decisive role in palatogenesis in the rat and perhaps also, in man.

Most cell lines display all three receptor types, although the relative proportions vary. The expression of receptors is consistent across most species and tissues of origin, with no apparent correlation with the derivation of the cell lines. Current literature supports the hypothesis that one receptor mediates multiple TGF-β responses, rather than multiple receptors mediating plural responses {Massague J 1992}. The type II receptor complex as well as the type I-II receptor complex can co-exist in cells that express all three TGF-β receptors, whereas the type III receptor binds and presents TGF-βs to type II receptors. Different TGF-β responses may require different levels of signalling and thus be inhibited to varying degrees by a dominant-negative receptor {Massague J, et al. 1996}. The results obtained from the experiment showed an intense and strong expression of
receptors critically located in a temporal and spatial context in the period of palatal fusion. This raised a high possibility of the potential autocrine and/or paracrine actions of TGF-β’s.

Bcl-2 is widely expressed early in mouse foetal development in tissues derived from all three germ layers and this expression becomes restricted with maturation. During organ differentiation, Bcl-2 expression becomes increasingly restricted to certain cell types within each tissue (Lebrun DP, et al. 1993). The wide distribution of Bcl-2 in the developing mouse suggests that many immature cells require a death repressor molecule or that Bcl-2 may have roles beyond regulating developmental cell death. This may reflect a common need for immature cells to express survival factors in order to overcome a stage-specific vulnerability to cell death. There is supportive evidence for the importance of inducible cell survival as a regulatory process in normal homeostasis and morphogenesis in many foetal tissues and structures, but the exact biological function of the protein is unknown. This implication for Bcl-2 in regulating the developmental process, such as active cell death during the fusion process of palatal shelves is considered important in biological research. The restricted localization of Bcl-2 to the tissues involved in the fusion process as reported in this study, appears to be the first detailed information available.

Bcl-2 must bind Bax to exert its effect. Furthermore, the ability of Bcl-2 to bind Bax alone can be insufficient for an anti-cell death function. The existence of Bcl-2/Bax heterodimers implies that Bcl-2 functions as a homodimer or oligodimer. Over-
expression of Bax accelerates apoptosis induced by cytokine deprivation in an IL-3 dependent cell line and counters the repressor effect of Bcl-2 on apoptosis. Thus the ratio of Bcl-2 to Bax determines the amount of Bcl-2/Bax heterodimers versus Bax/Bax homodimers and is important in determining susceptibility to apoptosis {Oltvai ZN, et al. 1993} {Yin XM, et al. 1994} {Krajewski S, et al. 1994} {Gratiot DJ, et al. 1993}.

The results from the present study show that there is a strong expression of the TGF-β family and of Bcl-2/Bax in the epithelial seam, especially in the presence of dead epithelial seam cells. It is interesting to note that heavy staining in the residual islands of mid-line epithelial cells preceded the lateral spread in the oral and nasal aspects of the palatal epithelia. In addition, Bcl-2/Bax also exhibited similar expression in the midline epithelial seam at the same stage of palatal development. This further consolidates the proposed theory of the role of apoptosis in contributing to the breakdown of the seam which leads to confluency of the mesenchymal compartment. Further, there is a close similarity in the expression of Bcl-2 and Bax. This suggests that they play a fine tuning role in regulating cellular activities as opposed to an “antagonistic” role which has previously been suggested in the literature. Further, the expression of Bcl-2 and Bax appears to correlate with the sequence rather than with the death of a specific cell or a group of cells. Importantly, there was much less expression of Bcl-2 and Bax in the pre-fusion stage. This further supports the highly specific action and timing of these death proteins and their antagonists in palatogenesis.
The expression of TGF-β's and receptors, as well as that of Bcl-2 and Bax, was much less intense and more scattered in the palatal mesenchyme. Morphogens and death proteins were, however, moderately stained particularly during the fusion and early post-fusion stages of palatal development. This, perhaps, illustrates the precise timing of the inductive potential of the underlying mesenchyme during the fusion process of the overlying epithelium.

In parallel TGF-β's and the receptors and Bcl-2/Bax were also examined in the adjacent structures including developing tooth buds and the eyes. It was interesting to observe that a similar pattern of expression to that of palatogenesis was recorded. This finding suggests a wave of induction through the oro-facial structures and strongly suggests the role of morphogens and death proteins in the morphogenesis of many foetal tissues.

In summary, the detailed analysis in the present study using a blind, coded and semi-quantitative measurement did not support the highly restricted temporal and spatial expression of growth factors in palatogenesis. In contrast, the expression of the morphogens was demonstrated (refer to Results) to be in synchrony. The receptors were found to be expressed in harmony with the transforming growth factors, particularly at the critical stages of the palatal fusion. Further, there was a fine tuning in the expression of the death proteins in regulating cellular activities in palatogenesis. The present research data did not support the reported “antagonistic” role played by these highly specific death proteins.
Survey of the current literature reveals a significant lack of information about the
decisive and specific roles played by morphogens and death proteins in palatogenesis in
the rat and possibly also, in man. This points to the need for further future studies on the
comparative biology of palatogenesis. There are still many unanswered questions relating
to this increasingly appreciated aspect of embryological development that require further
investigation.
APPENDIX

1. Reagents.

There was an extensive list of reagents used in our protocol. These were as follow:

- **Horse serum:** 20%.

- **Foetal calf serum:** 10%.

- **Phosphate Buffered Saline:** 0.02M, pH 7.0
  
  This solution was prepared by dissolving the following salts in 1 liter of distilled water:

  (i) Dibasic sodium phosphate anhydrous (Na₂HPO₄) 1.92g

  (ii) Monobasic sodium phosphate monohydrate (NaH₂PO₄.H₂O) 0.92g

  (iii) Sodium chloride (NaCl) 5.90g

- **Tris Buffer:** 0.05M, pH 7.6

  This solution was prepared by dissolving 6.1g Tris (hydroxymethyl amino methane) in 50ml of distilled water and added 37ml of 1N hydrochloric acid.

- **Hydrogen peroxide:** 3%

- **Sodium Citrate Buffer:** 0.01M, pH 6.0
This solution was prepared by adding 2.94g of tri-sodium citrate (BDH product code 10242) to 1 litre of distilled water

- **Normal Rabbit serum: x.092 (Dako)**
  20mg/ml stock concentration. This product was used in the double control studies.

2. **Antibody System.**

(a) **Primary antibodies:**

1. **TGF-β₁ (V): cat # sc-146 (Santa Cruz)**
   - Rabbit polyclonal IgG, 100 µg/ml in 0.05M Sodium-Phosphate buffer containing 0.1% Sodium-Azide and 0.2% gelatin, stored at 4°C
   - Epitope corresponding to amino acids 328-353 mapping at the carboxy terminus of the precursor form of human TGF-β₁ (identical to corresponding mouse sequence)
   - Specific for TGF-β₁; non cross-reactive with TGF-β₂ or TGF-β₃
   - Mouse, rat and human reactive
   - To be used for Western blotting, Immunoprecipitation, and Immunohistochemistry (paraffin-embedded tissues)

2. **TGF-β₁ (V): control peptide sc-146 P (Santa Cruz)**
• 100 ug/0.5 ml

3. TGF-β2 (V): cat # sc-90 (Santa Cruz)
   • Rabbit polyclonal IgG, 100 ug/ml
   • Epitope corresponding to amino acids 352-377 mapping at the carboxy terminus of the precursor form of human TGF-β2
   • Specific for TGF-β2; non cross-reactive with TGF-β1 or TGF-β3
   • Mouse, rat and human reactive
   • To be used for Western blotting, Immunoprecipitation, and Immunohistochemistry (paraffin-embedded tissues)

4. TGF-β2 (V): control peptide sc-90 P (Santa Cruz)
   • 100 ug/0.5 ml

5. TGF-β3 (V): cat # sc-82 (Santa Cruz)
   • Rabbit polyclonal IgG, 100 ug/ml
   • Epitope corresponding to amino acids 350-375 mapping at the carboxy terminus of the precursor form of human TGF-β3 (identical to corresponding mouse and chicken sequences)
   • Specific for TGF-β3; non cross-reactive with TGF-β1 or TGF-β2
   • Mouse, avian and human reactive
• To be used for Western blotting, Immunoprecipitation, and Immunohistochemistry (paraffin-embedded tissues)

6. TGF-β3 (V): control peptide sc-82 P (Santa Cruz)
   • 100 µg/0.5 ml

7. TGF-β RI (V-22): cat # sc-398 (Santa Cruz)
   • Rabbit polyclonal IgG, 100 µg/ml
   • Epitope corresponding to amino acids 158-179 of the precursor form of TGF-β RI (also designated ALK-5) of human origin (identical to corresponding rat sequence)
   • Specific for the ALK-5 form of TGF-β RI p55; non cross-reactive with TGF-β RII p70
   • Mouse, rat and human reactive
   • To be used for Western blotting, Immunoprecipitation, and Immunohistochemistry (paraffin-embedded tissues)

8. TGF-β RI: control peptide sc-398 P (Santa Cruz)
   • 100 µg/0.5 ml

9. TGF-β RII (L-21): cat # sc-400 (Santa Cruz)
   • Rabbit polyclonal IgG, 100 µg/ml
• Epitope corresponding to amino acids 246-266 of the precursor form of TGF-β RII of human origin (identical to corresponding rat sequence)

• Specific for TGF-β RII p70; non cross-reactive with TGF-β RI p55

• Mouse, rat and human reactive

• To be used for Western blotting, Immunoprecipitation, and Immunohistochemistry

10. TGF-β RII: control peptide sc-400 P (Santa Cruz)

• 100 ug/0.5 ml

11. Bcl-2 (N-19): cat # sc-492 (Santa Cruz)

• Rabbit polyclonal IgG, 100 ug/ml

• Epitope corresponding to amino acids 4-21 mapping at the amino terminus of Bcl-2 of human origin (identical to the corresponding domain of mouse Bcl-2)

• Specific for Bcl-2; non cross-reactive with Bcl-x or Bax

• Mouse, rat and human reactive

• To be used for Western blotting, and Immunohistochemistry

12. Bcl-2 (N-19): control peptide sc-492 P (Santa Cruz)

• 100 ug/0.5 ml
13. **Bax (N-20):** cat # sc-493  
   (Santa Cruz)
   - Rabbit polyclonal IgG, 100 μg/ml
   - Epitope corresponding to amino acids 11-30 mapping at the amino terminus of Bax of human origin (identical to the corresponding domain of mouse sequence)
   - Specific for Bax; non cross-reactive with Bcl-x or Bcl-2
   - Mouse, rat and human reactive
   - To be used for Western blotting, and Immunohistochemistry

14. **Bax (N-20): control peptide** sc-493 P  
   (Santa Cruz)
   - 100 μg/0.5 ml

15. **Keratin 18:** c-8541  
   (Sigma Immuno Chemicals)
   - Monoclonal Anti-cytokeratin peptide 18
   - Mouse Ascite fluid 0.2ml

\(b\)  **Secondary antibodies:**

1. **Swine anti-rabbit, Peroxidase-conjugate Immunoglobulins/HRP:**
   (Santa Cruz)
   - Affinity-isolated
   - Solvent 0.05M Tris/Hcl, 15mM NaN₃, pH 7.2
• 0.24g/L concentration of specific antibodies, mainly IgG reacts with rabbit Igs

2. Goat Anti-Mouse IgG (whole molecule), Peroxidase-conjugate:

(Sigma Immuno Chemicals)

• Affinity Isolated Antigen Specific Antibody (Antibody Developed in Goat)
• 0.01M phosphate buffered saline, pH 7.4, containing 1% BSA with 0.01% thimerosal as a preservative

• Diaminobenzidine (DAB)/metal concentrate with stable Peroxide Buffer

DAB Chromogen solution 1mg/ml:

(i) 1 tablet 10mg diaminobenzidine tetrahydrochloride
(ii) 10ml of diluent buffer (Tris-HCL or PBS)

(a) Dilutions of reagents:

• Antibody concentration:

  Primary and secondary antibodies- 1:20 dilution

  Diluent: 10% Fetal calf serum (FCS) + 90% PBS

• Normal rabbit concentration: 1:1000 dilution
- **Peptide concentration**: 1:10 dilution

- **DAB: H₂O₂ concentration**: 1:10
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