Experimental Studies of Vascular Development in Normal and Cleft Palate Mouse Embryos

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Introduction

Between the third and tenth weeks of human embryonic life, a complex and critical transformation must occur in the blood vessels that supply structures of the first branchial arch. First the primitive aortic arch dwindles and is replaced by the stapedial artery, a branch of the embryonic internal carotid (*16*). Then the stapedial artery loses its attachment to the internal carotid and its function is partially taken over by the ingrowing external carotid artery. McKenzie (*15*) has suggested that a deficiency or failure in timing within this vascular relay system is the underlying etiologic mechanism in major first arch anomalies inclusive of cleft palate. The concept that primary embryonic vascular deficiency can result in congenital anomaly has, in fact, received ample experimental support. Brent and Franklin (*1*) clamped the uterine vessels of rats on the ninth day of gestation and produced a wide range of anomalies. Vogel and McClenahan (*23*) as well as Stark, *et al.* (*21*) have interfered with embryonic chick carotid arterial supplies and have noted bizarre craniofacial anomalies inclusive of anencephaly, anophthalmia, microphthalmia, malocclueded mandibles, and other beak deformities. In addition, many investigators have found it difficult to separate the teratologic effects of hypoxia from those of defective vascularity. Byerly (*2*) produced suffocation effects in chick embryos by varnishing the shells. This resulted in the production of malformations that were associated with large venous extravasation sinuses. Since that time a number of other experiments have dealt with the relationship of hypoxia and congenital anomalies. Degenhardt (*4*) induced craniofacial dysplasias by subjecting rabbit embryos to hypoxia, and Ingalls and Curley (*9*) produced high incidences of cleft

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palate in mice. Grabowski (*6*) has also shown that embryonic hypoxia and vascular toxicity in the chick embryo induces edema and lactic acid accumulation. He proposed that an osmoregularity imbalance between intra and extraembryonic capillaries ultimately results in the formation of edema blisters which, when situated adjacent to rapidly developing organs, could produce death or malformation by mechanical distension. He hypothesized that hypoxia may force cells into anaerobic cycles which may then initiate maldevelopment.

While investigating normal palatal closure in the A/jax mouse, J. Little (*13*) discovered a prominent vascular plexus situated on the medial aspects of shelves that were in the process of active closure. He speculated that an engorgement of these vessels could produce the elevating action of the palatal shelves. It could also follow that a deficiency in this system would act etiologically in cleft palate. Previous studies of vascular anatomy in normal and cleft palate humans have yielded conflicting evidence. Slaughter, *et al.* (*20*) reported vascular immaturity adjacent to the cleft margins, but Maher and Swindle (*14*) have found no vascular deficiency.

The present study was designed to determine the nature of vascular development in the palatal region and to study particularly a possible relationship between blood vessel maldevelopment and the occurrence of cleft palate in experimental animals.

Materials and Methods

A preliminary series of eight A/jax mice, four of which were term and four adult, were prepared by intracardiac perfusion with India ink for study of the gross anatomy of blood vessels that enter the palatal region. The perfused specimens were fixed, dehydrated, cleared and microdissected. The findings from this series were then used to prepare illustrations of basic vascular morphology of the A/jax cranial-palatal regions (Figures 1, 2) and to serve as a basis for comparison with the vascular morphology of the experimental embryo series.

The main experimental series consisted of thirty A/jax mice embryos that were divided equally into normal and cleft palate groups. Clefting was induced by injecting the gravid females with 1.25 milligrams of cortisone acetate on the eleventh through the fourteenth days of gestation. Both groups were further divided into groups of five embryos, each according to the calculated gestation age at time of vascular perfusion (Table 1). In this manner both normal and cleft palate embryos were to be studied at three stages of development: a period prior to palatal closure, thirteen and three quarter days, a second time that corresponded to the approximate time of palatal shelf elevation, fourteen and three quarter days, and the final gestation time of sixteen days corresponding to a post-palatal closure time period.

At these specified stages of embryonic development the normal and cortisone treated female mice were prepared for arterial perfusion of the embryos. General anesthesia was induced with ether inhalation and

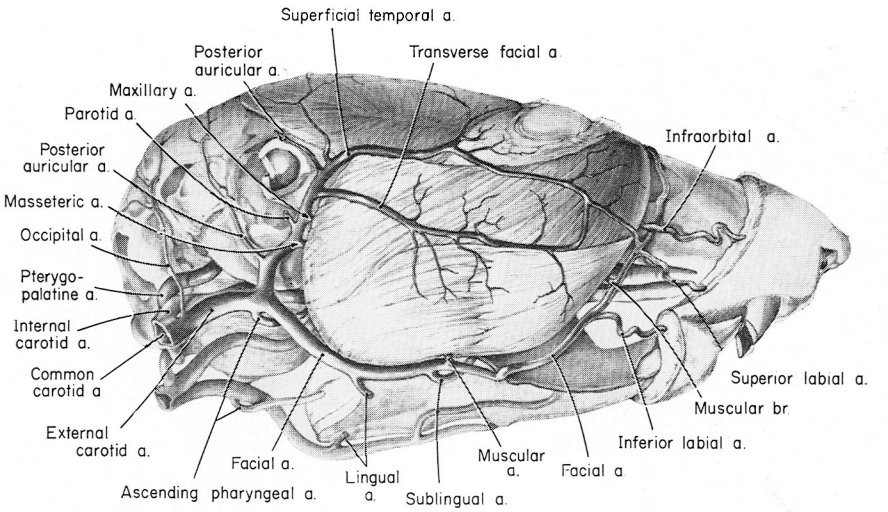

FIGURE 1. Illustration of superficial arteries based on perfused term and adult A/jax mice. Note the deep origin of the pterygopalatine artery from the common carotid trunk.

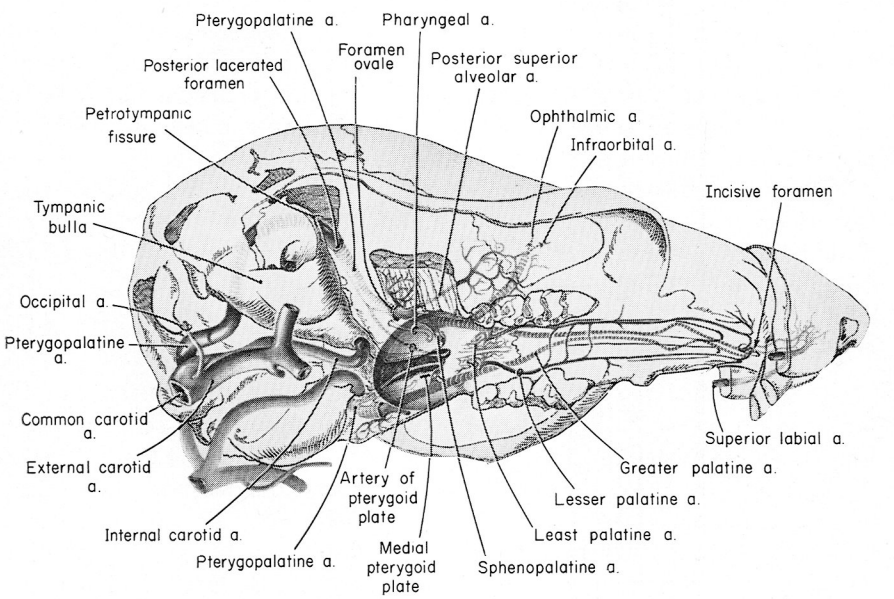

FIGURE 2. Illustration of oblique basilar skull to demonstrate the course and distribution of the pterygopalatine artery into palatal and nasal regions.

spinal anesthesia of the caudal third of the body was achieved by spinal cord separation. The unconscious animal was secured to a dissecting board and a laparotomy was performed to expose the contents of the uterus. The uterus was then elevated through this incision, encased in sterile gauze

TABLE 1. Gestation age at time of vascular perfusion

Normal Control Group: (15 embryos)	13 Days, 18 Hours (5 embryos)	14 Days, 18 Hours (5 embryos)	16 Days (5 embryos)
Cortisone Treated Group: (15 embryos)	13 Days, 18 Hours (5 embryos)	14 Days, 18 Hours (5 embryos)	16 Days (5 embryos)

and kept flooded with warm mammalian Ringers solution. In this manner the living individual embryos were made readily accessible. Each embryo with attached placenta was dissected free and placed under a dissecting microscope in a wax-lined well of Ringers solution. The embryo with attached placenta was pinned securely to the wax and the umbilical vessels were exposed for perfusion.

The perfusion apparatus consisted of fine heparinized needles drawn from capillary pipettes which were attached to twelve inches of fine rubber tubing. The tubing was used in conjunction with mouth pressure to control the injection of the India ink dye. The umbilical cord was looped over crossed pins and embryonic vitality was determined by noting pulsations of blood within the umbilical vessels. The umbilical vein was entered by the capillary needle tip at the point at which the vessel looped over the crossed pins. In the initial phases of perfusion small amounts of dye were introduced into the blood of the umbilical vein on its way to the atrium and the embryonic heart was allowed to aid in the perfusion. In the later stages of perfusion drops of digitoxin solution were added in order to potentiate myocardial contraction.

Following perfusion, the upper face and cranium were divided from the rest of the body and these cranial-palatal tissues were then fixed, dehydrated, cleared and embedded in clear plastic for purposes of dissecting microscope analysis.

Results

Gross Dissections. Two illustrations of the A/jax craniofacial vascular anatomy have been prepared on the basis of microdissections performed on four perfused term and four adult specimens (Figures 1, 2). Within the superficial tissues the facial artery complex was uniformly large with multiple masseteric muscular branches (Figure 1). The maxillary artery system was usually more variable. In some cases it was very small and was accompanied by adjacent parotid, masseteric and temporalis vessels. More frequently, however, a common maxillary arterial trunk gave rise to all of the vessels in the area.

Deeper dissection revealed the pterygopalatine artery to be a vessel of diameter equal to the internal carotid and diverging from the cervical common carotid to enter the skull at the jugular foramen (Figure 2). The pterygopalatine artery (Stapedial) coursed laterally and then anteriorly

beneath the tympanic bulla to finally leave the skull at the petrotympanic fissure before dividing into its major branches. Two small and highly variable branches, which previously have been grouped together as the "pterygoid" division in the rat (Greene) (7), were seen to arise at this point. First, the artery of the pterygoid canal passed through the medial pterygoid plate to ultimately supply the nasal pharynx and large portions of the nasal fossa. Second, a pharyngeal artery was observed in some cases to course anteriorly and inferiorly to supply the lateral pharynx, palatine tonsil and portions of the soft palate. The second major division of the pterygopalatine, the "palatine" division, was seen to be the direct continuation of the main trunk and quickly gave off posterior superior, pterygoid, perforating masseteric and buccinator branches. In addition, ophthalmic and orbital branches were seen as large terminals. The final large terminal trunk gave rise to the sphenopalatine artery and a variable number of palatine vessels. The sphenopalatine artery itself arose in the floor of the orbit, penetrated into the nasal fossa and supplied nasal turbinates and the nasal septum in association with the artery of the pterygoid canal. The total number of palatine arteries was variable. Typically, a greater palatine artery was seen diverging inferiorly in the infraorbital groove and coursing anteriorly within the substance of the lateral third of the palate before reaching its primary area of distribution in the premaxilla. An anteriorly directed lesser palatine artery was seen to break up into an anastomosing plexus in the posterior hard palate as well as establishing anastomoses across the palatal suture. A typical system of terminal vessels was also well demonstrated in the sixteen days' gestation embryo (Figure 3).

Perfused Embryos. Examination of normal embryos of the thirteen and three-fourths to fourteen day age group revealed a pterygopalatine arterial complex complete with all major branches (Figures 4, 5). Of particular note was the predominance of terminals in the premaxilla. The sphenopalatine, the artery of the pterygoid canal and especially the greater palatine artery were seen to branch and anastomose extensively in the incisive canal region. In contrast, vascularity in the posterior palatal shelf regions appeared to be sparse at this time. A delicate plexus was observable within the medial shelf tissues and appeared to be derived anteriorly from the greater palatine trunk and posteriorly from the lesser palatine branches (Figure 5).

In the India ink perfused embryos of fourteen and three-fourths days' gestation, which corresponds to the approximate time of shelf elevation, no major changes were observed in the large artery patterns. However, there was found to be an expansion of the plexus within the medial portions of the palatal shelves. At the extreme medial aspects of the elevating shelves the confluent arcades were dilated into large, bead-like sinusoids (Figure 6).

Compared to the normal pre-closure perfused embryos the palates of

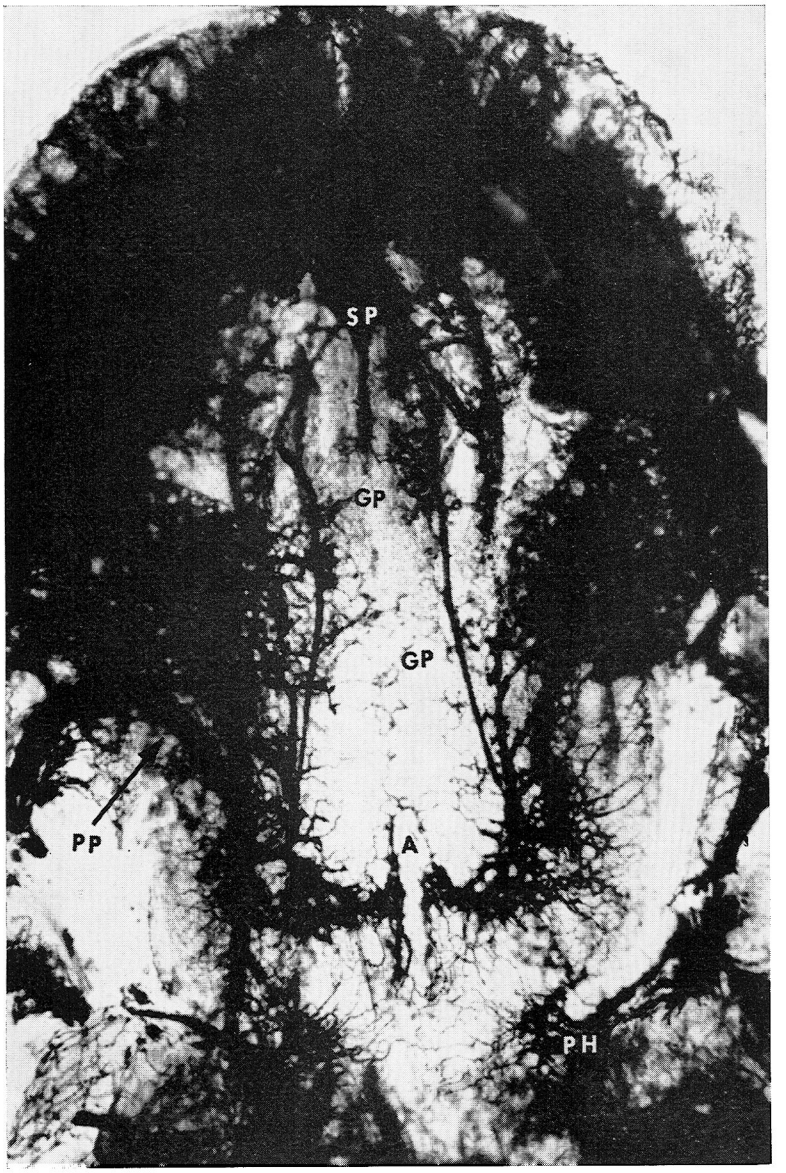

FIGURE 3. Perfused palate of a normal embryo of sixteen days' gestation. The tongue and mandible have been removed to expose the ventral surface of the anterior cranium. Note the gross configuration of the greater palatine artery (GP) and its length compared to that seen in Figure 4. The lesser palatine (LP) arcade systems are richly developed with the exception of an oval avascular area (A). Pterygopalatine (PP), pharyngeal (PH), sphenopalatine (SP). 10×

the post-closure sixteen day specimens gave the overall impression of a great lengthening in the greater palatine arteries (Figures 3, 4). Gross measurements confirmed that there was little change in the transverse distance from point of greater palatine artery entrance, but that the

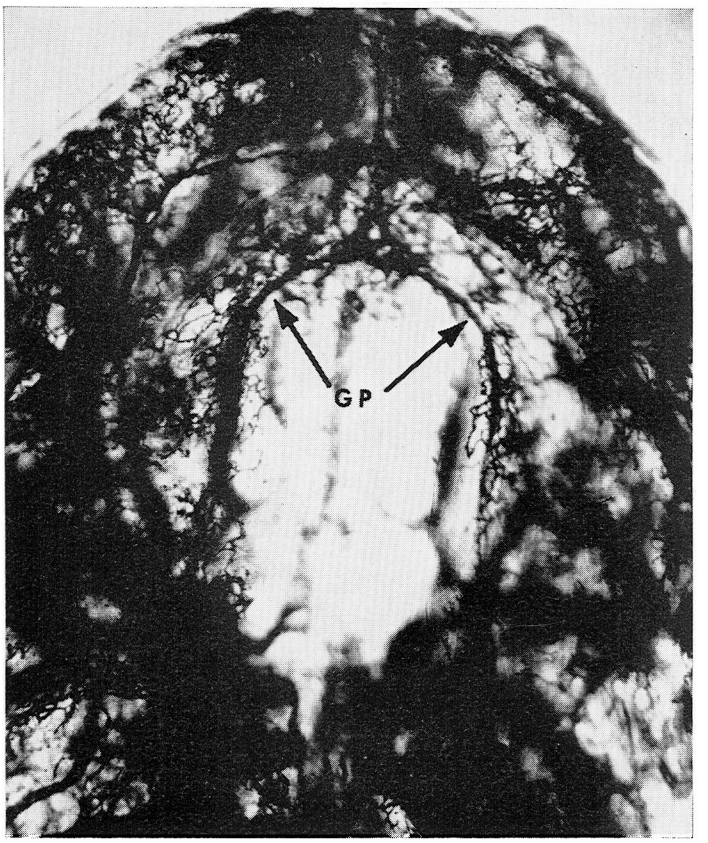

FIGURE 4. Perfused palate of a normal embryo of thirteen and three-fourths days' gestation. In this pre-closure specimen the greater palatine arteries (GP) are prominent with extensive anastomoses in the premaxilla. A delicate plexus of vessels is seen within the shelves although the most medial portions of the shelves are void of perfused vessels. 10×

length of the "post-closure" greater palatine artery was more than twice that of the "pre-closure" (Figure 4). The gross configuration of the greater palatine arteries was of interest also. When entering the premaxillary region, the arterial trunk was seen to curve sharply laterally and then recurve again to its midline terminus. When viewed bilaterally, this gave the pattern of an arrow head outline (Figure 3). In further contrast to the vascularity seen prior to palatal closure, the vessels in the posterior palate were numerous and complex. The lesser palatine vessels were conspicuous in that an oval area of avascularity was frequently observed in the approximate region of the future hard and soft palate junction.

Cortisone treated embryos which were perfused at the thirteen and three-fourths to fourteen day interval demonstrated no variance in the course of major branching of pterygopalatine vessels. In many of the pre-cleft specimens, however, a difference in degree of gross perfusion was

FIGURE 5. Higher power view of the palatal shelf of a normal embryo of thirteen and three-fourths days' gestation. Note the delicate plexus within the shelf and the terminal buds (arrows) which suggest active vascular sprouting. 30×

noted between the contralateral palatal shelves (Figure 7). Anastomosing networks of vessels with vascular budding were visible within the shelves of one side whereas the contralateral shelf appeared almost void of perfused vessels. Another rather constant finding was that of an angular or square outline appearance of the greater palatine arteries (Figure 7) in contrast to the more graceful inverted "U" shape of the normal arteries of comparable age (Figure 4).

Cortisone treated embryos of fourteen and three-fourths days' gestation demonstrated prominent vascular plexuses with greatly engorged vascular structures within the palatal shelves (Figure 8). Although the vascular plexuses appeared similar in origin and structure to those observed in the normal palates during the comparable stage of closure, the plexuses in the cortisone group were not bilaterally equal.

Cleft palate specimens from the cortisone group of embryos demonstrated a gross alteration in the emphasis of palatal and nasal blood supply. There was considerable enlargement of sphenopalatine artery and

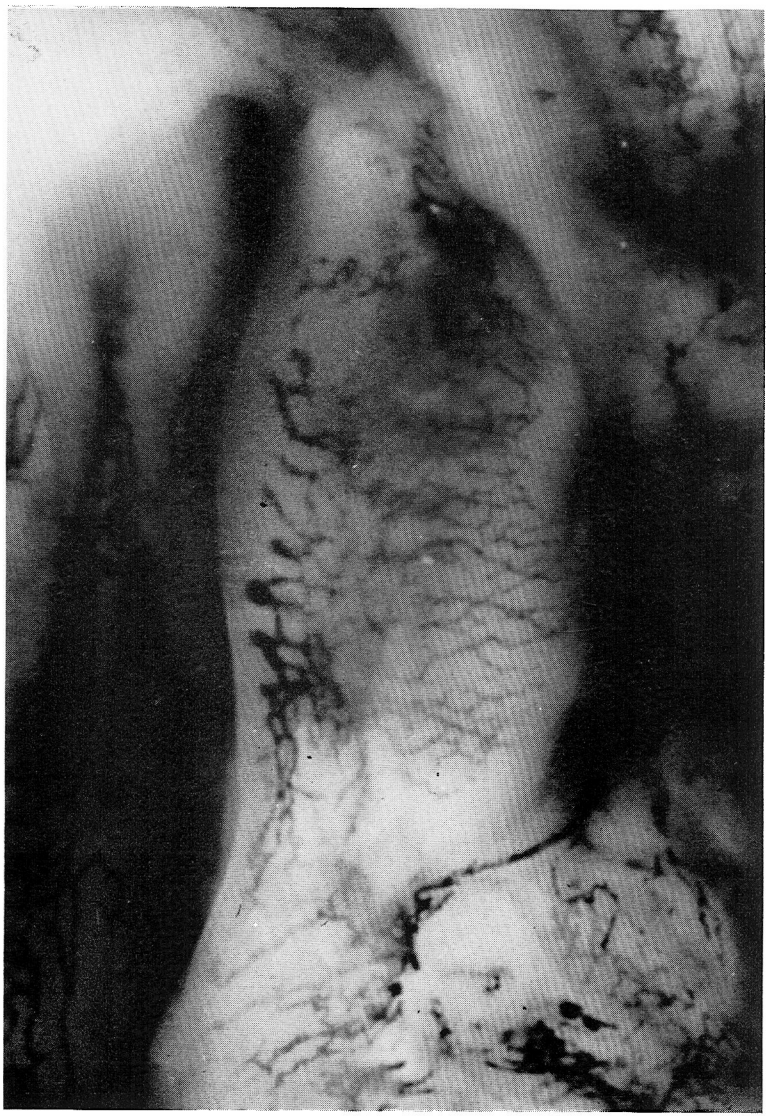

FIGURE 6. Normal embryo of approximately fourteen and three-fourths days' gestation. Note the prominent sinusoids and vascular plexus within the palatal shelf. 20×

the artery of the pterygoid canal with a concomitant diminution in the trunk size of the greater palatine artery (Figures 9, 10). A delicate anastomotic plexus was seen at the medial aspects of the atrophic cleft palatal shelves.

Discussion

Vascular perfusion of mouse embryos before, during and after the period of palatal closure and cleft palate induction has permitted a wide

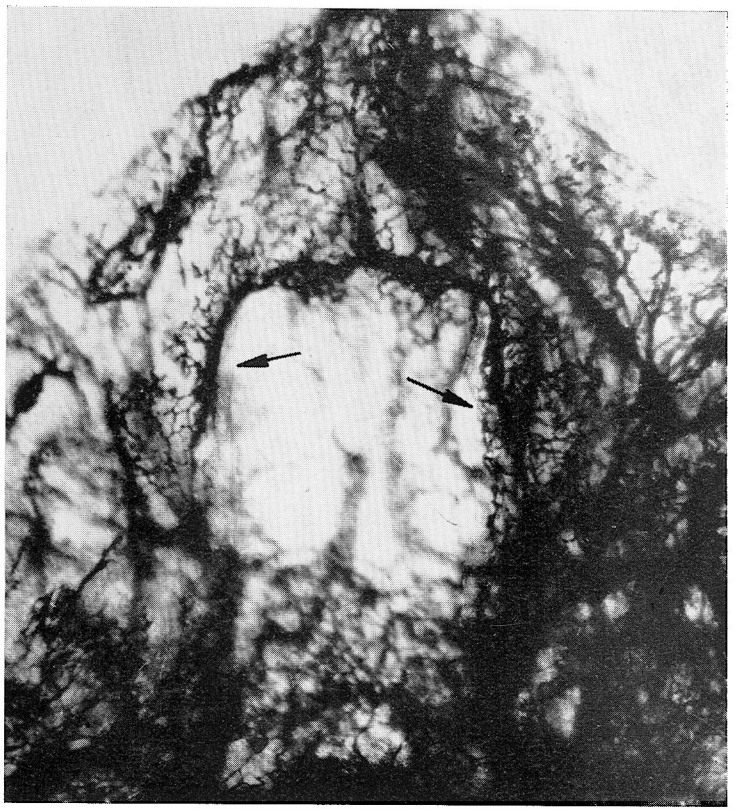

FIGURE 7. Cortisone treated embryonic palate of thirteen and three-fourths days' gestation. Note the difference between the degree of perfusion in the palatal shelf on the right as compared with the poorly perfused medial shelf at the left. 10×

variety of observations that may have significance in the understanding of palatal, nasal and facial development. For example, in the premaxillary regions of pre-closure embryos the greater palatine arteries were seen to be highly developed with extensive anastomoses with labial and nasal vessels. (Figure 11) This is in contrast to the apparent lack of complexity in the greater and lesser palatine arteries within the shelves at the same time. On this basis, it might be concluded that the internal carotid-based greater palatine artery of the mouse embryo is probably a significant early blood supply to the premaxillary mesenchyme and may provide needed impetus for the fusion of prelabial processes.

In normal embryos following palatal closure an oval shaped avascular area was observed in the mid-posterior palate. Although palatal fusion is thought to begin in the anterior secondary palate region in both mice (Little) (*13*) and human (Robinson) (*13*), (Reeve) (*17*) has observed that the last region of actual fusion in rats is represented by an oval area in the posterior hard palate. Therefore, the oval area of avascularity

FIGURE 8. Perfused palate of a cortisone treated embryo of fourteen and three-fourths days' gestation. Large dilated vascular sinusoids are seen in the medial aspect of the shelf at the right and a highly branching network is visible laterally. 15×

observed in the midpalate of mice immediately following closure may represent the final area of fusion which is still in the process of mesenchymal penetration.

The gross configuration of the anterior portions of the greater palatine arteries prior to palatal closure was that of a graceful inverted "U" shape (Figure 4). However, following normal closure the gross pattern resembled an inverted "arrow head" in the pre-maxillary region (Figure 3). Although a similar pattern may be observed in the adult mouse and rat palate, it does not appear in the primate. It is possible that this peculiar vascular pattern exists in order to accommodate the presence of the vomeronasal, or Jacobson's organ, which occupies the pre-maxillary and septal regions of the rodent. Unlike the mouse or rat, in which the vomeronasal organ persists into adulthood, the comparable structure in the human appears sometime during the fifth week, spans the period of palatal closure and retrogresses at thirteen to fifteen weeks (Kraus) (*11*). Confirmation is lacking for a vascular pattern in humans similar to that described

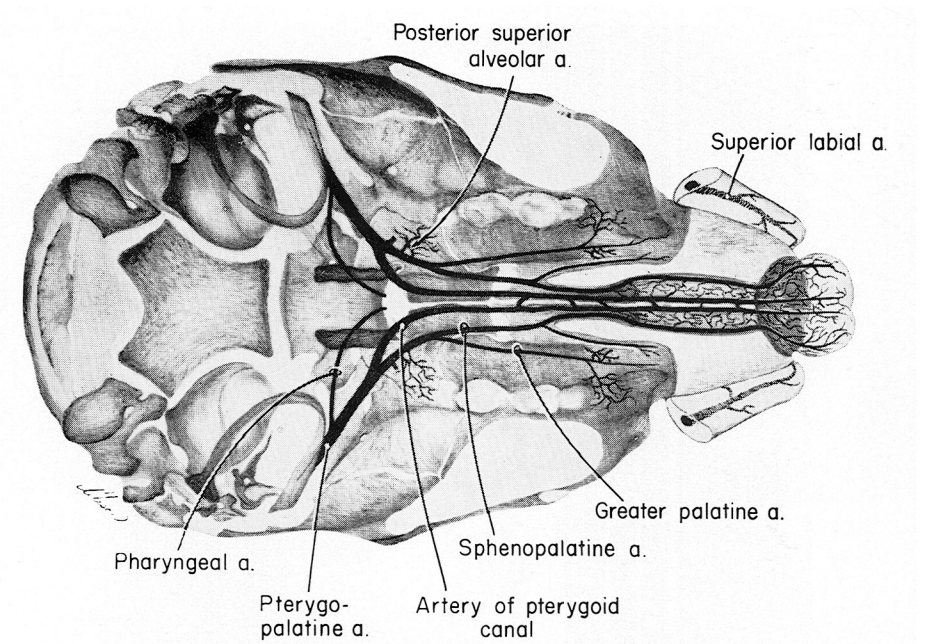

FIGURE 9. Illustration of palatal-nasal patterns observed in the sixteen day cortisone treated bilateral cleft lip and palate specimen.

for the embryonic mice during the period of palatal closure and in possible association with the transient vomeronasal organ.

Cleft palate embryos from the cortisone group displayed an apparent diversion of vascularity from the laterally placed palatine shelves into the septal and nasoglobular tissues. At the same time there appeared to be a retention of the lacy, delicate vascular reticulum on the atrophic palatal shelves which resembled the network seen prior to the time of usual palatal closure. Both of these findings are consistent with findings in human necropsy specimens. Sanvenero-Rosselli (*19*) observed an apparent enlargement of vessels in the nasal septum and diminution in the size of vessels in the cleft palatal tissues. Slaughter (*20*) has furthermore observed that vessels in those areas immediately adjacent to human clefts are very immature and possess both arterial and venous characteristics.

A most significant observation of the current study was that of a distinctive plexus of vessels which appeared along the medial border of the growing palatal shelves approximately twenty-four hours prior to closure. The plexus consisted of small, tortuous vessels which frequently terminated medially in blind expanded nobs or sacs. This network appeared greatly expanded at the approximate time of palatal shelf elevation. At that time the terminal nobs were greatly dilated and dominant structures. It is probable that these terminal vascular structures observed in the embryonic palates were dilated vascular sprouts of the variety described

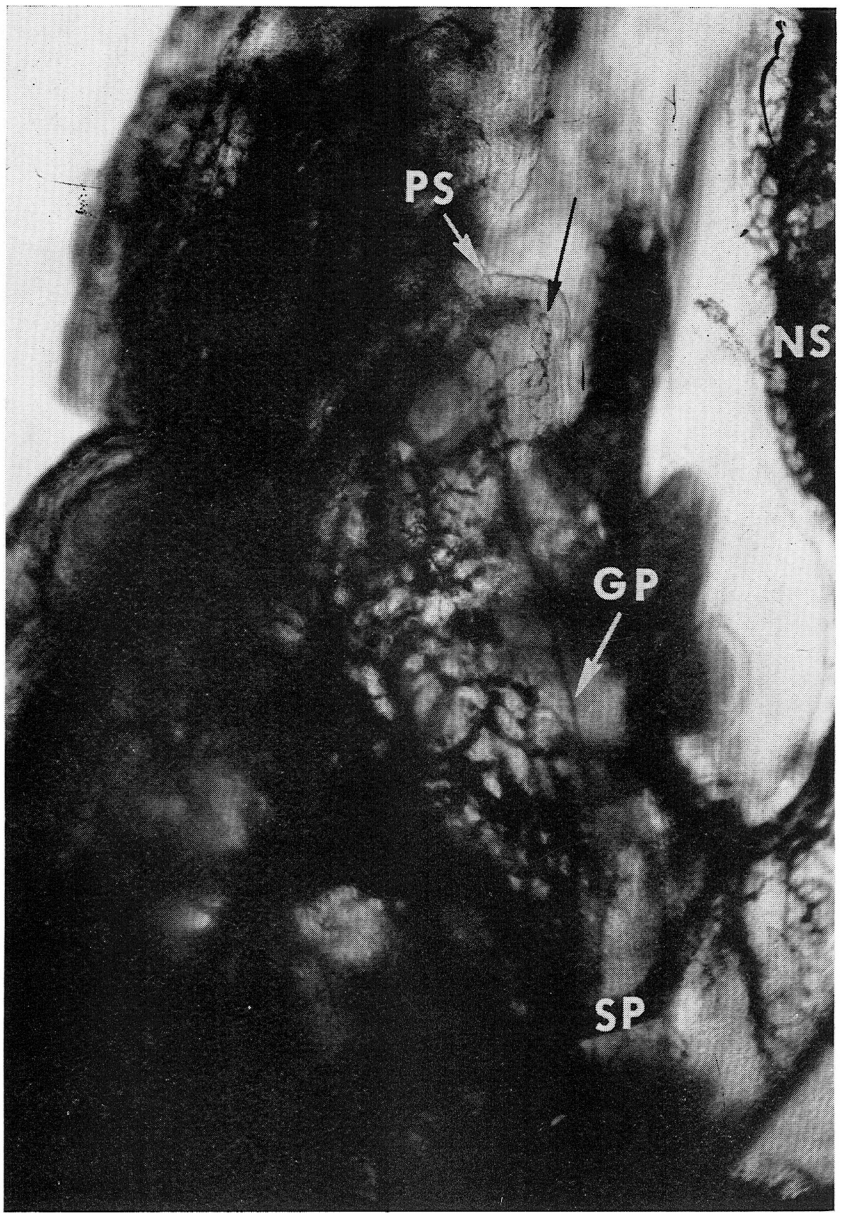

FIGURE 10. High power view of the palatal cleft in a 16 day cortisone treated embryo. Note the small palatal shelf (PS) and the persistant primitive appearing vascular network within the shelf (arrow). The greater palatine artery (GP) is small in comparison to the sphenopalatine artery (SP) which is seen entering the nasal septum (NS). 20×

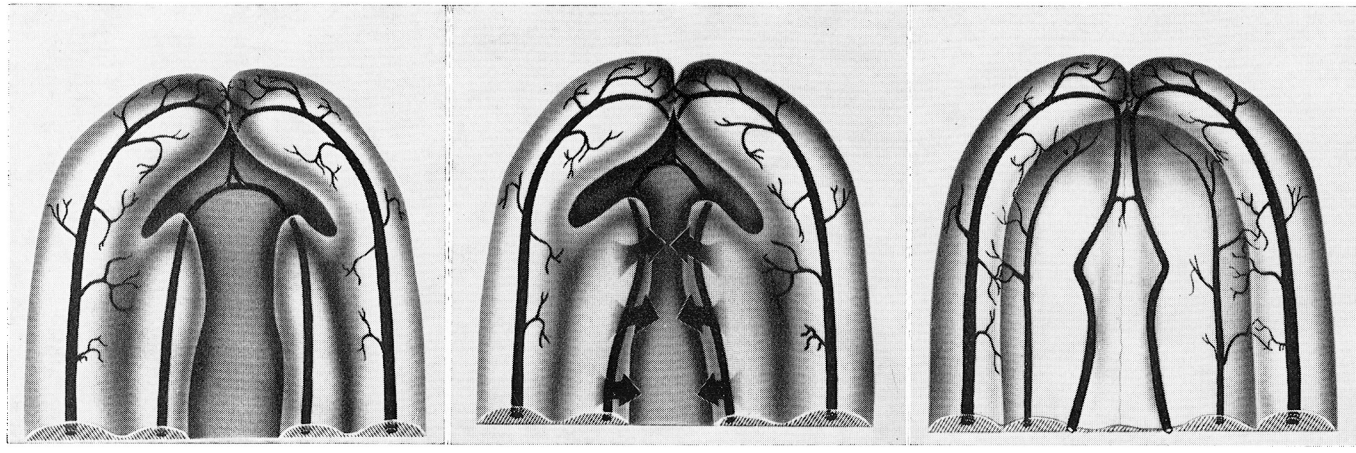

FIGURE 11. Illustration of the proposed outline changes that take place in the greater palatine arteries during critical stages of development. In the left diagram the greater palatine arteries are seen entering the premaxilla in a medially directed arc. As the shelves elevate (middle diagram) the trunks are also carried medially. However, the point of entrance of the greater palatine arteries into the premaxilla maintains its lateral position. This accounts for the gross configuration seen in the diagram at the right and observed in normal post-closure embryos.

by Clark and Clark (*3*). They observed temporary vascular regions at the point of normal sprout anastomosis which had the curious capacity for alternately distending and collapsing in response to changes in blood flow. It is possible that the dimensional changes of terminal dilatations such as those observed in the palatal shelves are passive phenomena since the necessary smooth muscle and sympathetic innervation of mammalian vessels would not have been established at the time of their observation.

The prominence of the developing palatal plexus is suggestive of a high degree of function. Little (*13*), who first described the structures on the medial aspects of mouse palatal shelves, suggested that they may play an important role in the poorly understood process of shelf elevation. For example, the vascular networks might participate in shelf elevation and later growth by simply providing a framework on which fibroblastic activity could occur. Harrison's experiments (*8*) had emphasized the need for a proper substratum on which mesenchymal cells may migrate into a given area. Another possible means by which vascular structures could participate in palatal closure is through a form of controlled edema in which extracellular fluids would exert tension on the palatal shelves and facilitate their elevation. Jacobs (*10*) has discussed the effects of altering the fluid balances within embryonic mouse palates. He demonstrated that the palatal shelves of embryos subjected to cortisone acetate treatments became excessively swollen, edematous and less cohesive. He attributed these changes to alterations in the tissue water binding capacity caused by an overproduction of hydrophilic open-coiled hyaluronate macromolecules.

Another mechanism by which the vascular plexus could participate in shelf elevation is through a simple mechanical surging of blood into the passive vascular buds resulting in an erective force. A number of investigators have looked for an "intrinsic shelf force" as proposed by Lazzaro (*12*) which would build up within the vertical palatal shelves until strong enough to overcome the resistance of the tongue and then quickly produce a flow of the shelves superiorly into the closed position. In the current experiment the palatal shelves before and during closure contained vascular plexuses with terminal buds which appeared capable of extensive passive dilatation. It might be theorized that with the release of pressure on palatal shelves, such as might occur when the tongue is pulled from between the shelves by reflex movement, a compensatory dilatation would occur in the relative vacuum provided. In this manner an overall wavelike erective action could occur. Although the hemodynamics of such a proposed system have not been adequately investigated, Walker and Fraser (*24*) showed that a form of controlled hypotension in the gravid mouse did not inhibit palatal shelf elevation.

In one of the major observations of the current study it was seen that the palatal shelves of cortisone treated embryos usually contained very well developed vascular plexuses and terminal dilatations. However, in

these same specimens the contra-lateral shelves frequently demonstrated extremely poor vascular perfusion. It is possible that this apparent disparity in palatal vascularity within the same embryo was related to variation in rates of development between opposing sides of the embryo. However, this observation may, in fact, reflect a point of teratologic influence by cortisone on the developing palatal vascular tissues. Frederiks (*5*) has advanced a concept of biphasic blood vessel development in which autonomous capillary networks develop *de novo* within the newly expanding tissue masses and these peripheral networks are then secondarily joined by branches of the central vascular tree. Based on this concept, Vogel (*23*) has argued that a faulty establishment of internal carotid connections with intrinsic vasculature of the cephalic anlage is the teratologic mechanism behind anencephaly. Stark, *et al.* (*21*) has suggested that such a faulty integration of central and peripheral vascular components may be precipitated by hypoxia. In the present experiment a dramatically active process of vascular "sprouting" was observed within the developing palatal shelves. In view of the known anti-angioblastic properties of cortisone in the developing organism (*22*), it might be proposed that cortisone interferes with the timely integration of the sprouting central vascular system with the peripheral networks of the palatal shelves.

Therefore, observations in the present experimental series lead to the conclusion that cortisone treatment does not interfere grossly with the ultimate development of vascular structures which may be vital to the process of shelf elevation. However, cortisone may actually delay the effective sprouting and integration of systemic and palatal systems to the point that shelf elevation is rendered out of phase insofar as the overall dynamics of palatal closure and fusion are concerned.

Summary

Developing cranio-palatal vascular patterns were studied in both normal and cortisone treated A/jax mice using a technique of embryonic perfusion. A complex vascular plexus appeared twenty-four hours prior to normal palatal closure and then expanded greatly by the time of palatal closure to exhibit prominent terminal dilatations within the shelves. These vascular plexuses may be significant as a force for palatal shelf elevation. Although similar plexuses were observed within the shelves of cortisone treated embryos, the plexuses were not bilaterally equal, and contralateral shelves were frequently deficient in perfused vessels. This suggests a possible point of teratogenic influence on the rate of angiogenesis and on the integration of local palatal networks with systemic circulation. Cleft embryos also displayed a persistence of the more primitive vascular structures adjacent to the cleft margins as well as a diversion vascularity from the cleft palatine shelves into the septal and premaxillary tissues.

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