MORPHOGENESIS OF THE BRAIN AND CRANIOFACIAL COMPLEX IN TRISOMY 16 MICE

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INTRODUCTION

Autosomal trisomy in mammals is a frequent cause of fetal death and significant debilitating malformations in newborns. In humans, the most frequent trisomy surviving birth is trisomy of the smallest autosome, chromosome 21 (HSA 21). Trisomy 21 (Ts21) results in Down Syndrome (DS). Down Syndrome is the most common known genetic cause of mental retardation and congenital heart disease (de la Cruz and Oster-Granite, 1990). Although no feature of DS is pathognomonic, there frequently are defects in neural, craniofacial, ocular and audiovestibular development which have characteristic features (Balkany et al, 1979a, b; Brown et al, 1989; Caputo et al, 1989; Coyle et al, 1986; Glass et al, 1987; Harada and Sando, 1981; Igarashi et al, 1972; Miller et al, 1986; Scherbenske et al, 1990; Shibahara and Sando, 1989; Tangerud et al, 1990; Warkany et al, 1966). The mechanisms by which these defects arise are unknown, and require elucidation by sequential analysis of embryonic and fetal development of the organ systems in question.

Abnormalities associated with structures of the head and neck region in DS individuals have been described by many investigators (see above), but few authors report systematic, comparative, correlative analyses of the constellation of defects observed frequently in a single individual or among individuals in the cohort being studied. Further, little effort has been made to quantify differences which do
exist. Despite these inadequacies, it is clear that the nature and extent of deficits in particular systems are highly variable in DS individuals, that particular defects occur frequently, and that many more subtle pathologic changes go unrecognized.

To examine the pathogenesis of defects occurring in the craniofacial apparatus and nervous system of DS individuals effectively, analysis of human conceptuses from the 4th to 20th week of development would be necessary. Such longitudinal studies cannot ethically or realistically be undertaken. However, since DS is a genetic condition, resulting from triplication of a cluster of genes present on the distal part of the long arm of HSA 21, examination of other mammals with triplication of the same cluster or similar clusters of genes may afford the opportunity for systematic, prospective, potentially quantitative, developmental studies (Cooper and Hall, 1988; Epstein, 1986; Gearhart et al, 1986). A portion of this cluster has been preserved in the same order in mice and human beings, and in mice is located on chromosome 16 (MMU16) (Reeves et al, 1987, 1988, 1989). Studies of trisomy 16 (Ts16) mouse have revealed many similarities to DS individuals, as reviewed recently (Epstein, 1986; Coyle et al, 1988, 1991).

The purpose of this study was to analyze the morphology of the brain and craniofacial complex in fetal Ts16 mice and their normal littermates. Initially, we focussed on qualitative differences that exist grossly, histologically, and ultrastructurally with particular emphasis on aspects of cell proliferation and neurogenesis, cell migration patterns, and initiation of differentiate events. We then began to quantify, and thus to describe more fully, some of the differences that exist in external and skeletal anatomy, particularly of the craniofacial complex of normal and trisomic fetal mice through the use of discriminant function analysis (Klecka, 1980; Grausz et al, in preparation).

METHODS AND MATERIALS

Procedures associated with the various techniques involved in these studies including the generation of trisomic conceptuses (Ts16 and Ts19, trisomy of the smallest autosome in the mouse), cell birthdating, cell cycle analyses, histology, electron microscopy, northern analyses, and in situ hybridization analyses for mRNA species associated with genes encoded on MMU 16 have been presented elsewhere (Bendotti et al, 1987, 1990; Gearhart et al, 1986; O'Hara et al, 1989; Oster-Granite, 1983, 1986; Oster-Granite and

Discriminant function analyses (Klecka, 1980) involved study of various internal skeletal structures, some of which were associated with craniofacial development. Aneuploid and normal littermate control mice from E14-E18 were stained with alcian blue and alizarin red S (Inouye, 1976; Macleod, 1980; Grausz et al., in preparation). Ninety-three specimens (23 trisomic; 70 littermate control) collected between E14 and E17 were chosen for study. These samples were analyzed without consideration of sex, kinship, or litter size.

Three types of data were collected for the discriminant function analysis. At necropsy, crown rump length and weight were measured. In addition, 8 external features including presence or absence of edema, relative digital development, whisker formation, and eyelid closure were scored. After whole embryo processing, the relative degree of development of 17 internal or skeletal variables were measured. Ten of these were associated with craniofacial or cervical structures (atlas, axis, nasal capsule, mandible, sphenoid, basiocciput, exoccipitalis, parietals, tympanicum, and maxilla). The other 7 represented postcranial features (femur, humerus, etc.). Scoring was done using an interval level rating scale of 0 to 3 in 0.1 unit increments, with 0 representing a state where mesenchyme, but no cartilage, was present and 3 a state of full ossification of a given skeletal structure. Since some variables appear or disappear as the animals age, not all 17 features could be evaluated at each gestational age.

RESULTS

Many of the trisomies in mice have characteristic, as well as shared, features (Gearhart et al., 1986; Gropp et al., 1975). In Ts16 conceptuses at midgestation, shared features include growth retardation, anasarca, and intrauterine death. Characteristic features include open eyelids, dysplastic ears, a high incidence of endocardial cushion defects in the developing heart, and poor hematopoietic stem cell colonization of the liver. When examined at the cellular level, hypoplasia, dysmorphogenesis, and/or overt malformation occurs in virtually every organ examined in the Ts16 mouse.

To ascertain the basis of the cellular hypoplasia, we first analyzed whether the reduction in cell number was due to altered cell cycle duration (Oster-Granite et al., 1987, unpublished results). No significant differences were observed either during gastrulation (E8.5-10) or in various portions of the neural tube on E10-12, although fewer cells
were already present in the neural tube (and other tissues). Similarly, when neuronal cell birthdates were determined, as we did in an analysis of the basal forebrain (Sweeney et al., 1988), no differences were noted in the initiation of neurogenesis, but changes in the duration of neuron generation and in the mitotic index accounted for a reduction of 30-40% among the putative cholinergic, but not other neuron, populations. This reduction corresponded to that observed in cholinergic markers in previous neurochemical studies (Ozand et al., 1984; Saltarelli et al., 1987; Singer et al., 1984). Analyses of the hippocampal formation and cerebellum have proved more difficult, since much of their neurogenesis occurs during postnatal periods, when Ts16 mice are not viable.

Morphogenesis of the Retina and Brain

Delay in the development of the retina and its choroidal layer was accompanied by delayed differentiation of the lens. By E14, lens fibers in the normal littermate control mice were well organized in parallel arrays, the nuclei were located in a basal (retinal) position, and the lens fibers were becoming attenuated. In contrast, the lens fibers in the E14 Ts16 mice were not yet organized into parallel arrays, the nuclei were still dispersed, and the lens fibers were thickened. The developing optic nerve contained fewer processes than did that of normal littermates.

Morphogenesis of various brain regions has been examined in Ts16 mice from E10-E18 (Kornuth et al., 1986; Oster-Granite, 1986; Oster-Granite et al., 1983; 1987; Plioplys and Bedford, 1989). Common findings are best illustrated by ultrastructural examination of the developing hippocampal anlagen between E13 and E15. While striking differences were not observed in these neuroblasts prior to the formation of neurofilaments and accumulation of neurotubules at E15, some striking differences were observed with respect to the preservation of cellular membranes and the extent of extracellular space in Ts16 mice compared to littermate control mice. Preservation of membranes in Ts16 specimens at all ages was more difficult and frequently disrupted membranes were observed; such membrane disruption was not seen frequently in normal littermate specimens processed at the same time. Similarly at all gestational ages examined, extracellular space was greater in Ts16 mice than in their normal littermates.

At E13, Ts16 neuroblasts were characterized by their loosely reticulated nucleoplasm and abundant polyribosomes. Cell processes contained few microtubules and there was a sparse accumulation of Golgi membranes in the cytoplasm. In
normal littermates, many of the nuclei additionally were polymorphous and cytoplasmic density was increased.

At E14, numerous mitotic figures could be distinguished at the pial surface. Cellular processes increasingly contained microtubules, but polyribosomes and sparse Golgi membranes were also present. Nucleolar condensation was more apparent, and the neuroblasts appeared like more mature migratory cells at this stage. Although cell apposition was commonplace, no desmosome-like processes (which precede synaptogenesis) were readily evident. Extracellular space was much reduced in the normal littermates, and desmosome-like contacts occurred infrequently. Occasional dense cells, reminiscent of microglia, were found readily in the normal specimens. While cellular junctions between ependymal cells were complete at the ventricular surface of normal mice, both the ependymal barrier and the external glial limits were still discontinuous in many Ts16 specimens.

At E15, migratory cells and their processes were readily visible in the hippocampal anlagen, but few of the processes contained well developed microtubules. The amount of Golgi membranes in the cells increased markedly and the number of polyribosomes became reduced, giving the appearance of a more lucid cytoplasm in the neuroblasts.

**Morphogenesis of the Craniofacial Complex**

Although the faces of Ts16 mice look nothing like DS individuals, the Ts16 mice exhibited platybasia and hypoplasia of the midface and nares, mandible, and basi-cranium. Much of the hypoplasia of the face resulted from delay in first and second pharyngeal arch development on E10-12 relative to that of the tongue (Oster-Granite et al, 1983). By E14 (See Figure 3 in Gearhart et al 1986), the mandible was small and the maxilla hypoplastic when compared to littermate control animals. This produced a shortened, somewhat flattened snout and shortened dysplastic external ears. These features were exaggerated greatly in Ts16 fetuses that survived to E18.

In the Ts16 mouse the most common ocular and orbital abnormalities were microophthalmia with retinal and choroidal dysplasia and persistent open eyelid. Hypocellularity of the periorbital tissues, coupled with transient edema, may have contributed to the failure of the eyelids to close during midgestation. The extraocular muscles remained hypocellular.

The hypoplasia in the maxillary region was also evident in the reduced sizes of the maxilla and nasal septum, the decreased complexity of the turbinate bones, and the narrowing of the lateral palatal shelves in histologic sec-
tions. Cell numbers, but not cell sizes, were reduced. Similarly, the mesenchymal tissue inferior to the orbit was hypocellular, but glandular cells in the orbital and nasal regions and hair follicles in the vibrissal region were present and developing. No changes were noted in the pattern or number of whisker vibrissae.

The masticatory, facial, pharyngeal muscles and their associated connective tissues were also reduced by decreased cell number and density. Reduction of the nasopharyngeal space and delay in the closure of the secondary palate contribute to a relatively common choanal stenosis in Ts16 mice. In part these muscular reductions limited the expansion of the tongue at its base and at its forward extension at E12-14. Thus, the tongue occupied much of the shared nasal and oral space, separating the lateral palatine shelves for an extended period of time. By E17, the osseous mesenchyme of the mandible elongated and the tongue dropped forward in the mouth. Thus, the palatal shelves usually closed by E17, so that cleft palate was rare in Ts16 mice, in contrast with Ts19 mice (Gearhart et al., 1986).

Chondrification and/or ossification of the bones associated with the skull were also altered in Ts16 mice (Oster-Granite et al., 1983; Patestas et al., 1990). Mesenchymal condensations that precede chondrification were hypocellular in each bony primordium examined. The initiation of chondrification was delayed slightly, but never more than 24 hours. The pattern, size, and shape of endochondral bones of the basicranium, however, differed between Ts16 and normal littermates at both E14 and E18. By E14, the length of the basicranium was reduced, but the volume of skeletal structures present were not significantly smaller. By E18, the significant hypoplasia of the basiocciput, and to a lesser extent the basisphenoid accompanied an alteration in the angle formed between the basisphenoid and the basiocciput, resulting in relative platybasia. The timing of the appearance of ossification in either endochondral or intramembranous bones was slightly delayed, but the sequence of ossification was not. Ossification of the cranial bones began with the basiocciput and progressed anteriorly and sequentially through the basisphenoid, prepaenoid, and the mesoethmoid. In contrast, the quality and character of the deposition of intramembranous bone was much more variable and diffuse in Ts16, particularly in the parietal bones.

Although exhaustive reconstruction of the atlanto-occipital and atlanto-axial joints was not done, the cervical vertebrae of Ts16 mice were smaller, the ligaments more mesenchymal, and the neck shortened relative to littermate controls. This shortening distorted the relationship between the basiocciput, the atlas, and the axis. The cartilaginous
primordia of the developing atlas and axis were distorted in shape and reduced in size. The infrahyoid muscles, the colli muscles, and their associated connective tissues were also hypocellular. The connective tissues of the thymus, parathyroids, and thyroid were reduced and hypocellular. Excessive fluid in the neck region expanded connective tissue spaces in the neck and distorted muscular and connective tissue relationships.

Morphogenesis of the Audiovestibular Complex

Morphogenesis of the three parts of the audiovestibular complex in the Ts16 mouse began with the establishment of the primordia during the third gestational trimester. Unfortunately, much of the development of the audiovestibular complex was not completed, because most Ts16 mice die in utero. The development that does occur, however, is consistent with that observed in DS patients.

Significant hygroma of the neck and anasarca of the body occurred in most Ts16 mice by E13-E15, distorting the position of the external ear relative to the eye and mandible. By E14, the epidermis of the head and neck was already separated from the adjacent dermal and connective tissues and muscles of the body. Both cellular hypoplasia and edema contributed to the "tucked in" appearance of the head, and the size of the fetus compared with its littermate control was distorted. In those Ts16 mice surviving to E18, the edema was much reduced, but the skin of the upper torso was wrinkled considerably with redundant folds of skin evident in the nuchal region. Multiple hemorrhagic petecchia were evident in the skin of the head and neck. We did not examine carefully whether abnormal development of lymphatic channels might have contributed to these features.

Both the external ear and meatus were dysplasia and hypoplastic. The hillocks of the pinna of Ts16 mice were hypocellular, but present and appeared between E11 and E12, consistent with the reduced tissues of the first and second arches. By E18, the pinna may have extended across the forming external auditory meatus, but did not cover more than 1/2 of the external ear canal, and its external shape was distorted and folded abnormally. In contrast, between E14 and E18, littermate normal mice developed a pinna that covered the external ear canal completely.

In the middle ear, the mesenchymal condensations of all the ossicles were hypocellular in Ts16 mice at E14, but became cartilaginous by E18 in surviving Ts16 mice. The alignment of the ossicles was altered by the lack of expansion of the first pharyngeal pouch and of the base of the skull. The forming ossicles were embedded in a loose,
sparsely cellular mesenchyme. The angle formed between the forming tubotympanic recess and the meatal plate of the external auditory apparatus was more acute in Ts16 mice and was exaggerated by the platybasia.

The inner ear complex was particularly altered in structure and overall relationships. At the earliest stage examined (E10), the cartilaginous spherical capsule, derived from the otocyst, was markedly hypocellular, and had few associated neural crest cells. The maculae of the saccule and utricle were slow to organize, were smaller, and were structurally less well organized than those of normal littermates. Some of the semicircular canals of many of the animals were dehiscent, but no consistent pattern of dysgenesis was observed. By E18, surviving Ts16 mice had malformations of the cochlear complex; the modiolar cartilage was distorted with fewer spiral ganglion cells. The elements of the Organ of Corti were also distorted, and abnormal non-parallel relationships occurred among adjacent cochlear cells. In all Ts16 mice examined, the cochlea contained fewer than 1.5 turns at E18, while the cochlea of normal littermate mice contained the expected 2.5 cochlear turns. In many Ts16 mice, the facial canal was dehiscent and the promontory was reduced in size.

Discriminant Function Analysis

At E14, 14 of the 17 variables were appropriate for comparison between the Ts16 mice and their littermate controls. The best discriminant variables at this age were the relative degree of development of the atlas and the femur, followed by the relative separation of the digits of the forelimb. At E14, all skeletal characters examined represent cartilaginous or mesenchymal structures, since osseous material was rare in any of the specimens. A combination of these variables by way of discriminant function analysis showed the separation between the groups to be highly significant (p<0.001).

At E15, both Ts16 and normal mice began to ossify their skeletons. Many of the characters examined were associated with the presence of osseous material. Of the 10 variables that were suitable for analysis at this age, the best discriminators proved to be the relative degree of development of the sphenoid and of the humerus. Differences between the Ts16 and normal mice did not reach statistical significance, according to the variables used in the discriminant function analysis (p<0.70).

At E16, all major skeletal features had begun ossification, and significant bony tissue was present in the normal mice. Of the 16 variables analyzed, the development of
the maxilla and mandible showed the greatest discriminant quality and a high degree of statistically significant difference between groups (p<0.001).

At E17, 11 variables could be examined of which the degree of ossification of parietal bones was the best discriminator between the groups, followed by femur and nasal capsule (p<0.001). For the first time, both weight and length proved to be strong discriminators.

The statistical results were then used to classify the specimens into groups, as a test of the success of the discriminant analysis. All specimens were assigned appropriately on E14, 16, and 17, but two Ts16 specimens were classified as within normal range in the E15 group.

Thus, the variables that prove most discriminant differ from day to day between the Ts16 and normal littermate animals. To attempt to determine whether a pattern could be distinguished developmentally, 4 internal variables that were applicable to all four gestational ages were compared with length and weight (mandible, sphenoid, humerus, and femur). These variables were selected by examining the 7 most important discriminants in each of the previous analyses. At E14, for example, length and degree of development of the femur were most discriminant (p<0.005). At E15, the sphenoid and humerus were the most important discriminators, but the differences were not statistically significant (p<0.28). At E16, the relative degree of development of the humerus proved most able to distinguish between normal and Ts16 groups (p<0.001), but other discriminant variables were closely clustered. At E17, mandible and length were the most discriminant (p<0.001). Again, the results of each day save E15 were statistically significant, but no distinct pattern appeared among the discriminators.

**DISCUSSION**

We made an extensive qualitative evaluation of brain and craniofacial development in the Ts16 mouse and extended our observations by beginning to quantify differences that exist by use of discriminant function analysis. Many of these features appear to be similar qualitatively between individuals with DS and mice with Ts16, and to represent most likely, the results of gene dosage effects rather than the nonspecific perturbation of developmental events by increased genetic material per se.

**Retinal and Brain Abnormalities**

Many of the features of the brains of individuals with DS have been reviewed previously (Coyle et al, 1986) and at
this meeting. They include reduced brain weight, changes in gyral and sulcal patterns, alterations in neurotransmitters, and reductions in specific neuronal populations with the appearance of the pathologic features of Alzheimer’s disease. Ocular abnormalities observed frequently in DS individuals include oblique, narrow palpebral fissures, epicanthus, hypoplastic iris, focal condensation of the iris (brushfield spots) cataracts, retinal dysplasia, strabismus, and myopia (Caputo et al, 1989; Scherbenske et al, 1990). Thus, tissues derived from ectoderm, neuroectoderm, and neural crest were all affected adversely. While these are common observations, the genesis of many such defects has not been examined in any detail in DS conceptuses.

In the Ts16 mouse, common alterations observed in the development of the cerebellum, hippocampus, and cortical plate and in ocular structures include changes in the amount of extracellular space, in the number of cells and the friability of their membranes, and in cell-cell interactions (Kornguth et al, 1986; Oster–Granite et al, 1983, 1987; Plioplys and Bedford, 1989). While edema may contribute to the increased extracellular space observed in nervous tissues of Ts16 mice, such changes may also reflect altered cell-cell interactions or changes in the composition of membranes (see Plioplys and Bedford (1989)).

Problems in membrane preservation occurred in Ts16 mice (Kornguth et al, 1986; Plioplys and Bedford, 1989). In addition, Plioplys and Bedford (1989) observed changes in neuron cross sectional area and in microtubules and neurofilaments in the cortical plate at stages later than those we examined. At least in part some of these changes may be due to dosage effects of genes encoding specific nuclear proteins (such as HMGI4) (Pash et al, 1990) and cell surface molecules such as the α and β interferon cell surface receptor (Kornguth et al, 1986; Epstein, 1986) or the amyloid precursor protein (O’Hara et al, 1989; Fisher et al, 1991).

Membrane composition changes have been observed previously in neurochemical and electrophysiological studies of Ts16 mice as well. For example, electrical membrane properties of cultured dorsal root ganglion neurons were compared among DS individuals, Ts16, and Ts19 mice (Caviedes et al, 1990), using both intracellular electrode recordings and whole cell patch-recording pipette techniques. Similar changes in action potential parameters and passive membrane properties were observed in both DS individuals and in Ts16 mice, but not in neurons cultured from Ts19 fetal mice (Ault et al, 1989; Orozco et al, 1987, 1988; Caviedes et al, 1990). These alterations may result from changes in Na+ channels or currents, which may alter calcium influx and thus to affect neurotransmitter release, protein phosphory-
lation, and/or synaptic plasticity. Thus, increases in depolarization and repolarization and decreases in spike duration appear to be specific to Ts16 mice and to DS individuals.

When we examined the eyes of Ts16 mice, we found the periorbital connective tissue was reduced and the eyelids remained open. Microphthalmia was common, with reduced choroidal pigmentation. Like individuals with DS, these features varied in occurrence and in severity. For example, we and Lipski and Bersu (1990) found that the eyes of Ts16 mice were positioned more superiorly in the head. We also observed that there was increased extracellular space with mild developmental delay between E10 to 15. In contrast, Lapasha et al (1990) found absence of the transient nerve fiber layer of the retina, as well as increased intercellular space and cell death in the inner neuroblast layer of the retina.

Lipski and Bersu (1990) further found that eyelid closure appeared to be prevented by a transient, large cell mass that continuous with the cornea and located near the inner canthus at E15. Lapasha et al (1990) observed much more severe ocular deficits including aphakia, pseudolens formation, and malaccumulation of ectomesenchyme forming the vitreous. Corneal abnormalities included dense aggregations of irregularly arranged mesenchyme cells centrally with cell reduction peripherally, much as we observed.

Such severe ocular alterations appear to be specific to Ts16, since Ts19 mice exhibit only developmental delay of 2 days in neuronal and glial development of the retina (Lorke and Winking, 1986) and in myelin formation in the optic nerves (Lorke and Lauer, 1990). While the diameters of the optic nerve axons were not changed, the cross-sectional area was reduced because there were fewer and more densely packed myelinated axons (Lorke and Lauer, 1990). In Ts16 mice, axonal numbers were reduced, but packing density was not increased.

Craniofacial and Cervical Abnormalities

Common craniofacial abnormalities observed in DS individuals include brachycephaly, platybasia, hypoplasia of the midface and nares, mandible, and basicranium, and increased incidence of choanal atresia (Bersu, 1980; Glass et al., 1989; Walkany et al., 1966; Miller et al., 1986). In Ts16 mice, these same features develop, perhaps due in large part to relative hypoplasia of the first and second pharyngeal arches at E10-12, which may arise as a result of failure of neural crest cells to populate the archæs, and thus to induce the differentiation of muscles and to organize skeletal
structures associated with the arch derivatives, such as the maxilla and mandible of the first arch, and the hyoidal processes of the second arch.

Both we and Patestas et al (1990) observed delay in palatogenesis in Ts16 mice between E14-E18. While secondary palate closure is completed between E14-E15 in normal mice, it is delayed at least 24 hours in Ts16 mice. In part, an abnormally shaped tongue and hypoplastic and grossly misshaped intermaxillary segment and nasal septum contributed to this delay. Further growth of the mandible after E16 in the Ts16 mouse permitted the tongue to ‘drop’ into the oral cavity, and palate closure to proceed rapidly to completion.

When the skulls of DS individuals were examined, the most significant developmental alterations occurred in the basicranium and cervical region (Roche et al, 1972; Miller et al, 1986). For example, sphenoidal sinuses failed to aerate or achieved only partial aeration in a large number of DS individuals (Miller et al, 1986). Since many of the basicranial abnormalities occur in relation to the audiovestibular complex, they will be discussed there.

Cervical abnormalities observed frequently in DS individuals include shortening of the neck, which may arise in part as a result of skeletal abnormalities and of generalized tissue fluid accumulation due to edema during the first trimester. Atlantooccipital subluxation with significant ligament laxity occurs more frequently in males, but the overall incidence is increased in both males and females with DS relative to normal individuals (Miller et al, 1986; Tangerud et al, 1990). Further, fusion of the vertebral bodies and facets, flattening of cervical vertebrae, and degenerative changes were common in the first 5 cervical vertebrae, particularly at C2-3 (Miller et al, 1986; Tangerud et al, 1990). In normal individuals, such degenerative changes are usually associated with C5-6.

In Ts16 mice, Sterz et al (1989) observed abnormalities of the occipital bone with a high incidence of premature fusion of the ossa exoccipitales with the os basioccipitale. They also found distinct retardation of the fusion of the processus palatini with the os palatinum. In our sample, all skeletal characters had begun to ossify by E16, but the character of the cartilaginous preforms was distorted for many structures. Ossification of calvarial bones was likewise altered, with decreased mineralization and altered patterns of trabeculation (Grausz et al, in preparation). While only the sphenoid, mandible, and maxilla were sufficiently different between Ts16 and normal littermate mice to be distinguished by discriminant function analysis, all bony structures were affected. In view of the observed alterations in the sphenoid, coupled with the nasal cavity
deficits we observed, it is perhaps not surprising that incomplete or absent aeration of the sphenoidal sinuses occurs at later developmental stages in human beings.

Similar distortions of the cervical vertebrae in the Ts16 mice are also consistent with positional distortion and altered 3D relationships that would have persisted had the animals survived the perinatal period. More thorough reconstruction techniques are needed, though, to assess how much these positional changes might have resulted from the considerable edema of the neck region that is common to trisomies in mouse other than Ts16.

**Audiovestibular System.**

Peripheral and central audiovestibular abnormalities are found in > 80% of DS individuals examined audiologically (Balkany et al, 1979 a, b) and contribute to premature onset of presbyacusis (Buchanan, 1990). While abnormalities vary widely in character and extent, common ones include dysplastic and hypoplastic external ears, meatal hypoplasia and stenosis, congenital and acquired ossicular defects, mastoidal stenosis or hypoplasia, dehiscent facial canals, tubotympanic recess functional abnormalities, and mixed hearing loss (Aase et al 1973; Balkany et al, 1979a, b; Glass et al, 1989; Harada and Sando, 1981; Shibahara and Sando, 1989).

Chronic otitis media is a common problem among DS children and may be due to anatomic malalignment, smaller nasopharyngeal fossa and space, and hypotonia of the tensor veli palatini muscle (Brown et al, 1989; Schwartz and Schwartz, 1978). Although muscle development may be normal, cartilages in the Eustachian tube are smaller, the semicanal of the tensor tympani may be dehiscent, and the lumen of the tube may be reduced, occluded, or collapsed. Mesenchymal tissue in the middle ear space may occur without external ear canal abnormalities in DS (Shibahara and Sando, 1989), and may contribute to decreased movement of the round window (Harada and Sando, 1981; Igarashi et al, 1977).

Inner ear abnormalities observed include a variable amount of shortening of the cochlear coils and hypoplastic or dehiscent semicircular canals (Harada and Sando, 1981; Igarashi et al, 1977) but these features are by no means universal (Shibahara and Sando, 1989). Thus, as with the ocular complex and the craniofacial complex, developmental interactions of ectoderm, neuroectoderm, and neural crest cells appear to be affected adversely.

In Ts16 mice, common features that occur throughout the audiovestibular complex include failure of cartilaginous structures to form properly and hypocellularity of adjacent
mesenchymal tissues. This leads to the ‘lop’ or abnormally folded external ear, the altered alignment of the middle ear ossicles, and the persistence of loose mesenchyme in the tubotympanic recess. Delay in first and second arch development affects audiovestibular structures significantly, since there is a failure of expansion of the first pharyngeal pouch which subsequently alters the angle of the developing tympanic membrane (meatal plate) and distorts its relationship with both the malleus and incus. Hypocellularity of the structures associated with the developing Eustachian tube may lead to the alterations observed in individuals with DS, since persistent mesenchyme in the middle ear cavity is associated with increased incidence of effusion in the perinatal period in human beings.

Particularly notable were the considerable alterations that occurred in the developing inner ear. Peripheral sensory structures associated with the audiovestibular complex were reduced in number and complexity. During normal genesis of these cochlear and vestibular structures, both placodal (otocyst) and neural crest cells interact extensively to induce not only neurogenesis of the spiral and vestibular ganglia, but also to induce proliferative changes that reshape the spherical otocyst into the definitive cochlea and semicircular canals associated with the utricle and saccule. In Tg16 mice, hypocellularity of the inner ear complex most likely results from a failure of rhombencephalic neural crest cells to associate with the otocyst between E8.5 to E9.5. As a result at later stages, cochlear development lags and is associated with poor colonization of the spiral ganglion cells in the modiolus, and thus contributes to a failure of the cochlea to achieve an appropriate number of coils. Secondarily, this failure is associated with reduced bone formation in the petrous portion of the temporal bone.

To ascertain how much of the alterations observed in the audiovestibular complex is due to reduced colonization by the neural crest cells of the rhombencephalon, as well as those associated with the mesencephalon, which colonize the first and second arches, reconstruction analysis is needed between E8.5 and E10.

Other Skeletal Abnormalities

In view of the alterations in bony structures associated with the craniofacial complex observed in DS individuals, it is somewhat surprising that the only skeletal anomalies described include slight deformation of the skull, anomalies of the pelvis and metatarsophalangeal sesamoid bones, and missing ribs (as discussed by Sterz et al, 1989).
In Ts16 mice, Sterz et al (1989) found extensive asymmetry or abnormal fusion of the vertebral centers and alterations in the vertebral arches along with rib malformations consistent with rib-vertebra syndrome. Malformations of the spinal column included missing, twinned, asymmetric, or abnormally fused vertebral bodies and pronounced alterations in vertebral arches in the thoracic and lumbar regions. Malformations of the sternum were common. While we did not examine thoracic and associated vertebral structures with the care that Sterz et al (1989) did, the specimens we did study were consistent with their observations. As we also observed, the bone of the humerus, femur, and tibia appeared primordial and retarded in development with greatly reduced substantia compacta and decreased mineralization. These changes resulted in the humerus showing greatest discriminant quality on E15 and the femur on days E14 and E17. At later gestational stages, we too found a reduction of humeral length, concomitant with the overall decrease in size of the embryo.

Conclusions

Craniofacial and ocular malformations observed in Ts16 mice are much more like those observed in DS individuals than the defects observed in the head and neck region of other mouse trisomies, such as Ts1, Ts12, Ts14, and Ts19 (Oster-Granite, 1986). In Ts19, for example, the 'mildest' of the aneuploid conditions, the size of the craniofacial complex is smaller, but there are not the gross distortions of skeletal and mesenchymal structures observed in Ts16 mice. Consistently in Ts19 mice, development is delayed 24 to 48 hours in both the central nervous system and in the ocular complex. Once initiated, though, development proceeds quite normally.

Perhaps the most severe craniofacial defects occur in Ts1, where there are midline facial defects, cyclopia or aprosopia, and holoprosencephaly. These severe malformations may arise from failure of mesodermal cell migration into the cranial and cardiogenic regions of the embryo during gastrulation. Mesenchymal deficits are less severe in Ts12 and Ts14, where craniofacial development is quite normal, but exencephaly frequently results. Both these types of craniofacial abnormality are observed very rarely in Ts16 mice.

Thus, the constellation of alterations of the craniofacial complex of Ts16 mice appears to be as distinctive among the mouse trisomies as similar alterations are in DS individuals among human trisomies. In general, tissues which require prolonged periods of cell
proliferation, cell migration, and extensive interaction between mesenchyme and epithelium to organize successfully are particularly vulnerable in the Ts16 mouse, e.g., brain, retina, heart, and immune system. One significant cell population which may be vulnerable to dosage effects of genes located on MMU16, and thus, may contribute to the significant hypoplasia of the craniofacial apparatus is the neural crest of the cranial and cervical region. From the constellation of defects that are observed in the Ts16 mouse, all components of the cranial neural crest appear to be affected. Subsequent studies in our laboratory will focus on the period of generation of such neural crest cells (E8.5-E10) to determine their fates in Ts16 mice, and thus their roles in the generation of these developmental abnormalities.

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