

A Single Major-Gene Defect Underlying Cardiac Conotruncal Malformations Interferes with Myocardial Growth during Embryonic Development: Studies in the CTD Line of Keeshond Dogs

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Summary

The common forms of isolated congenital heart disease are usually not inherited in a Mendelian pattern, and most are considered multifactorial threshold traits. A large subset consisting of a group of malformations of the ventricular outflow region, termed “conotruncal defects” (CTDs), include subarterial ventricular septal defects, tetralogy of Fallot, and persistent truncus arteriosus. Similar aggregations of CTDs have been reported in human families and in the keeshond breed of dog. The results of our early breeding experiments utilizing noninbred keeshonds were not consistent with any hypothesis of a fully penetrant monogenic inheritance. Here we report a recent series of genetic and embryologic studies conducted after more than 10 generations of selective inbred matings between affected-CTD-line dogs. The results are now consistent with a defect at a single autosomal locus, the Mendelian pattern of transmission having been obscured prior to selective inbreeding by genetic background. On the basis of morphometric embryologic studies, the mutant CTD allele causes conotruncal malformations in homozygous animals by interfering with myocardial growth in the conotruncus during the critical window when the conotruncal cushions fuse to form the conotruncal septum.

Introduction

Congenital malformations of the heart constitute one of the largest classes of human birth defects, with the prevalence of confirmed cardiac defects of all types reported to be around 7/1,000 live births in studies conducted in the United States and Europe (reviewed in Ferencz et al. 1985, 1991; Pexieder et al. 1989a). Early epidemiologic and family studies pointed out that the common types of isolated cardiac malformations tend to run in families and that affected relatives frequently have the same or anatomically related defects

(McKeown et al. 1953; Lamy et al. 1957; Campbell 1965; Ehlers and Engle 1966; Davison 1967; Fuhrman 1968; Nora 1968; Sanchez-Cascos 1978). These findings were consistent with the conclusion that many forms of congenital heart disease have a lesion-specific genetic component in their etiology, but the patterns of inheritance were usually not consistent with any hypothesis of fully penetrant monogenic inheritance. Interpreted in the light of concepts of polygenic inheritance that were developed by Wright (1934) and Gruneberg (1952) and that were applied to human disease by Falconer (1960, 1965), Edwards (1960, 1969), and Carter (1961, 1969), this evidence suggested the hypothesis that most cardiac malformations are “multifactorial” (Nora 1968; Nora and Nora 1978a, 1978b)—i.e., are caused by the combined effects of alleles at a number of gene loci interacting with stochastic and/or environmental factors. In early discussions, the genetic component was assumed to be polygenic (i.e., due to many genes with small additive effects). However, more

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recently, the term "multifactorial" has been used to refer to situations in which a defect is caused by a combination of genetic, stochastic, and environmental "factors," without reference to the number or action of the genes involved (Fraser 1976; Nora and Nora 1978a, 1980; Kurnit et al. 1985, 1987). It has been estimated that about 8% of congenital heart defects are due to chromosomal anomalies or single mutant genes, that 2% are due to teratogenic agents in the environment, and that the remaining 90% are multifactorial (Nora 1978a).

Epidemiologic and family studies of congenital heart disease in the dog that were conducted in the late 1950s and early 1960s indicated that the common cardiac malformations are similar to those in humans and supported the hypothesis that lesion-specific genetic factors are important in the etiology of a number of forms of congenital heart disease that occur with high frequency in certain breeds (Patterson 1965, 1968; Mulvihill and Priester 1973). These forms included a defect in keeshond dogs that closely resembles tetralogy of Fallot in man. Family studies (Patterson 1968) showed that keeshond dogs with tetralogy of Fallot frequently had close relatives with type I (subarterial) ventricular septal defects (Soto et al. 1980), which are considered to be related to failure of fusion of the conal cushions (Clark 1987).

A small group of related keeshonds with tetralogy of Fallot and isolated ventricular septal defects was obtained from breeders and used to found a breeding colony to investigate the genetic and developmental basis of these malformations. Subsequent studies confirmed that a series of malformations, all attributable to varying degrees of maldevelopment of the conotruncal septum, were inherited in the keeshond line and that defects of other organ systems were not present (Patterson et al. 1974). The spectrum of conotruncal defects (CTDs) included subclinical defects of the crista supraventricularis, small and large ventricular septal defects, ventricular septal defects with pulmonic valve stenosis, and ventricular septal defects with pulmonic valve atresia. After further studies showed that affected dogs occasionally had persistent truncus arteriosus, this rare malformation, not previously reported in the dog, was added to the spectrum of conotruncal malformations produced in the CTD line (Patterson 1978; Van Mierop et al. 1978). The cardiac defects observed in the CTD line include most of those considered to be conotruncal malformations in humans (Clark 1987), and it has been noted that some human families have aggregations of CTDs, including types seen in the keeshond

dog (Fraser and Hunter 1975; Miller and Smith 1979; Takao et al. 1980; Corone et al. 1983).

Results of Mendelian experiments in the initial colony of noninbred keeshonds were not consistent with any fully penetrant single-gene hypothesis and were fitted to a threshold model in which multiple genes act additively to determine the liability to CTD (Patterson et al. 1974). Descendants of the original colony of keeshonds were maintained in a closed breeding colony and used for a series of embryologic studies. These studies confirmed that the malformations observed are associated with hypoplasia of the conotruncal cushions, resulting in abnormal septation of the conal and truncal outflow portions of the heart; the degree of abnormal development varied in a way that explained the spectrum of lesions found at term (Van Mierop et al. 1977, 1978; Van Mierop 1978). The anatomic lesions and underlying embryonic pathology form a continuum, with subclinical defects of the crista supraventricularis at one end and persistent truncus arteriosus at the other (table 1 and fig. 1). On the basis of genetic studies prior to selective inbreeding, it was assumed that the spectrum of anomalies and embryonic pathology observed in the CTD line represented thresholds along a continuous scale of liability to abnormal development produced by multiple genes (Patterson et al. 1974).

A portion of the CTD line has been propagated by matings between affected dogs, utilizing animals with the most severe defects (e.g., isolated ventricular septal defects and acyanotic tetralogy of Fallot) compatible with survival and reproduction. In a series of matings extending over approximately 10 generations, inbreeding coefficients of offspring in the colony reached levels exceeding .50 in some animals. Having produced, by selective inbreeding, an increased degree of genetic homogeneity in the CTD line, we recently conducted a series of breeding experiments designed to retest the inheritance hypotheses. These studies, described in the present paper, show that abnormal conotruncal development in the CTD line can now be explained as the effect of a mutant allele at a single genetic locus. Additional embryologic studies, also reported here in summary, confirm that the mutant allele interferes with normal myocardial growth in the conotruncal region during the critical developmental window of conotruncal septation.

Material and Methods

Genetic Studies

The CTD line of keeshond dogs has been maintained in a closed colony at the University of Pennsylvania

Table 1**Classification and Embryopathogenesis of Conotruncal Lesions in CTD Line**

Anatomic Lesion ^a	Embryologic Correlation ^b
Grade 1—subclinical anomalies of the crista supraventricularis	Mild hypoplasia of conus cushions; delayed fusion of conotruncal septum at its proximal (upstream) border; and failure of papillary muscle of conus to form
Grade 2—grade 1 defects plus ventricular septal defect (small to large)	Moderate to severe hypoplasia of conus cushions, with failure of fusion at proximal border
Grade 3: Grade 2 defects	Severe hypoplasia of conus cushions, with incomplete conus septum
Grade 2 defects plus pulmonic valve stenosis or atresia	Severe dysplasia of truncus cushions, with right ventricular outflow obstruction
Grade 4—persistent truncus arteriosus, type I	Severe hypoplasia of conus cushions with absence of conus septum; severe hypoplasia and failure of fusion of truncus cushions; and normal aorticopulmonary septum

^a Based on pathologic studies by Patterson et al. (1974) and Patterson (1978).

^b Based on embryologic studies by Van Mierop et al. (1977, 1978) and Van Mierop (1978).

School of Veterinary Medicine since 1968. Normal beagles with no family history of congenital heart disease were purchased from White Eagle Laboratories (Pottstown, PA), for breeding experiments and embryologic studies. All dogs were maintained in a temperature-controlled animal colony, and all care, treatment, and euthanasia were carried out in accordance with the humane principles and procedures recommended by the Guide for the Care and Use of Laboratory Animals (1985). Clinical diagnosis of cardiac malformations was made by a combination of cardiac auscultation, electrocardiography, radiography, ultrasonography, cardiac catheterization, and angiography, using standard methods for the dog (Ettinger and Suter 1970; Detweiler et al. 1979). In initial outcrosses to produce an F1 generation, affected CTD-line parents were crossed to normal beagles. The CTD-line dogs were as follows: males, pedigree numbers 987 (tetralogy of Fallot) and 1010 (ventricular septal defect); and females, pedigree numbers 975 (tetralogy of Fallot) and 988 (ventricular septal de-

fect). In the F1 backcross generation, three F1 males were backcrossed to a number of different affected (grade 2 or grade 3) CTD-line females, and 10 F1 females were backcrossed to either CTD-line male 987 or CTD-line male 1010, to produce an F1 backcross generation of 183 offspring. The F1 offspring of CTD female 988 were utilized in F2 crosses but not in backcrosses. F1 males and females were mated inter se to produce an F2 generation of 62 offspring. In addition, five matings were made between affected (grade 2) members of the F1 backcross generation, producing 28 offspring.

Of the 304 offspring produced in these matings, 88 pups (29%) were stillborn or died in the first week after birth. After clinical examination, all but 15 of the surviving pups were euthanatized. Five clinically normal F1 offspring and 10 clinically affected F1 backcross dogs were retained for further breeding. Cardiovascular diagnoses in the 289 animals that died or were euthanatized were made by detailed postmortem examination of the heart, using a dissecting microscope for smaller hearts.

Embryologic Studies

CTD-line embryos from matings between two affected keeshonds (grade 2 CTD) and from control embryos from matings between normal beagles were removed by uterotomy under general anesthesia. After fixation in Bouin's solution, the embryos were staged as in previous studies (Van Mierop et al. 1977), using the criteria for developmental stages originally established for human embryos by Streeter (1948). After standard processing (dehydration, clearing, paraffin infiltration, and embedding), serial sections were prepared and stained with Meyer's hematoxylin and eosin. Five control and five CTD-line embryos at stage 17, totaling 261 and 239 serial sections, respectively, were compared. With the image-analysis system, Quantimet 920 (Cambridge Instruments), the contours of each of the following cardiac-tissue compartments were traced with the help of a digitizing pad: lumen, endocardial cushion tissue, and myocardium. At various levels of the conotruncus, the relative volume percent of each compartment was computed using a program written in the metalanguage Quips (Pexieder et al. 1989b). Point-by-point comparisons between CTD-line and control embryos were evaluated by a version of the small-sample Mann-Whitney nonparametric test (Siegel 1956) adapted from the Mann-Whitney test in the Hewlett-Packard statistical library (HP09845-15251).

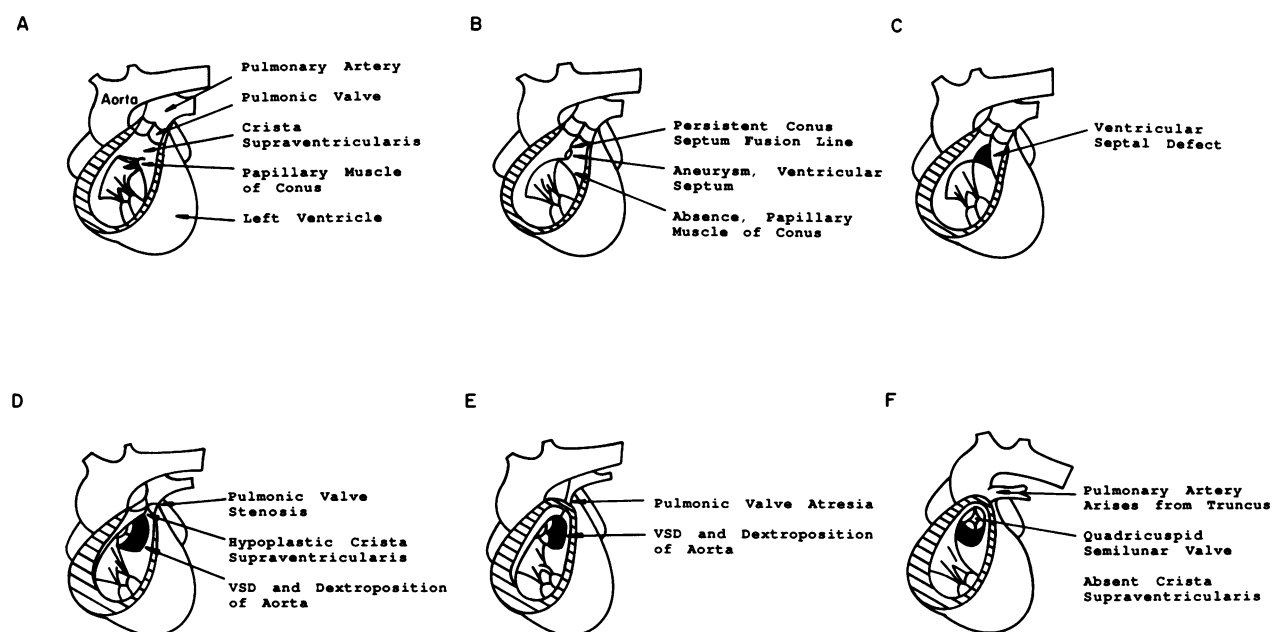


Figure 1 Anatomy of conotruncal anomalies in the CTD line. The heart is viewed in the frontal plane, with the right ventricular wall removed. A, Normal. B, Grade 1 CTD—subclinical anomalies of crista supraventricularis. C, Grade 2 CTD—ventricular septal defect. D, Grade 3 CTD—tetralogy of Fallot. E, Grade 3+ CTD—pseudotruncus arteriosus. F, Grade 4 CTD—persistent truncus arteriosus, type 1.

Results

Genetic Studies

The anatomic types of CTDs found in the offspring were classified as grade 1–4 CTDs, according to our previous observations in the CTD line (Patterson et al. 1974; Patterson 1978). The grading system used in classification of anatomic lesions is summarized in table 1 and illustrated in figure 1.

The results of breeding experiments are presented in table 2, which shows the number of offspring in each category of anatomic diagnosis in F1, F1 backcross (F1BC), and F2 generations, as well as those in five matings between affected offspring of the F1 backcross generation.

The litter size did not differ significantly between mating types when compared with the average of 5.8 pups/litter for all matings ($\chi^2 = 2.4$, $df = 3$, $.7 > P > .5$). There was no difference between the frequency of defects in male offspring (75/155) and that in female (74/149) offspring ($\chi^2 = 0.5$, $.7 > P > .5$). The sexes were combined for further analysis in table 2.

F1 generation.—All 31 F1 generation offspring were normal on clinical examination. Postmortem studies were performed on 26 of the F1 offspring, and 5 were retained for further breeding. Two (7.7%) of the 26 dogs studied postmortem had grade 1 subclinical de-

fects (fig. 1B), and the remainder had hearts that were anatomically normal.

F1 backcross (F1BC) generation.—Affected animals with grade 1–3 CTD occurred in the F1 backcross generation originating from each of three affected CTD-line keeshonds (table 2). A pedigree depicting the matings originating from CTD-line male 1010 is shown in figure 2. One grade 4 defect (persistent truncus arteriosus type I) occurred in the group originating from male 987. When the grade 4 defect was combined with the grade 3 defect, the proportions of normal and affected hearts in the various anatomic grades were not significantly different between the three F1 parent groups ($\chi^2 = 8.8$, $df = 6$, $.20 > P > .10$).

In the combined F1 backcross generation, 105/183 dogs (57.4%) were affected, slightly exceeding the 50% expected in autosomal recessive inheritance ($\chi^2 = 3.98$, $P < .05$). When the F1 backcross matings are subdivided and examined according to the individual CTD-line parents that produced the F1 offspring used in the backcrosses, it can be seen that the percentages of affected pups in parental groups 1010 and 975 are consistent with the 50% expected under the autosomal recessive hypothesis. In the 987 group, the proportion of affected offspring (69.4%) exceeds that expected ($\chi^2 = 9.3$, $P < .01$).

Table 2**Breeding Experiments**

MATING	NO. OF MATINGS	TOTAL NO. OF OFFSPRING	NO. OF NORMAL OFFSPRING	NO. OF CTDs					% WITH CTD
				Grade 1	Grade 2	Grade 3	Grade 4	Total	
F1	5	31	29	2	0	0	0	2	7.7 ^a
F1 backcross:									
1010 Male	18	96	46	20	22	8	0	50	52.1
987 Male	12	62	19	18	21	3	1	43	69.4
975 Female	3	25	13	7	5	0	0	12	48.0
All F1 backcross	33	183	78	45	48	11	1	105	57.4
F2	9	62	48	8	5	1	0	14	22.5
Affected to affected	5	28	0	10	7	10	1	28	100.0
All matings	52	304	155	65	60	22	2	149	49.0

^a Of the 31 offspring in the F1 generation, all were clinically normal; 26 were examined postmortem, and 5 were retained for further breeding. Of those examined postmortem, 2/26 (7.7%) had grade 1 defects. The other 24 had hearts that were anatomically normal.

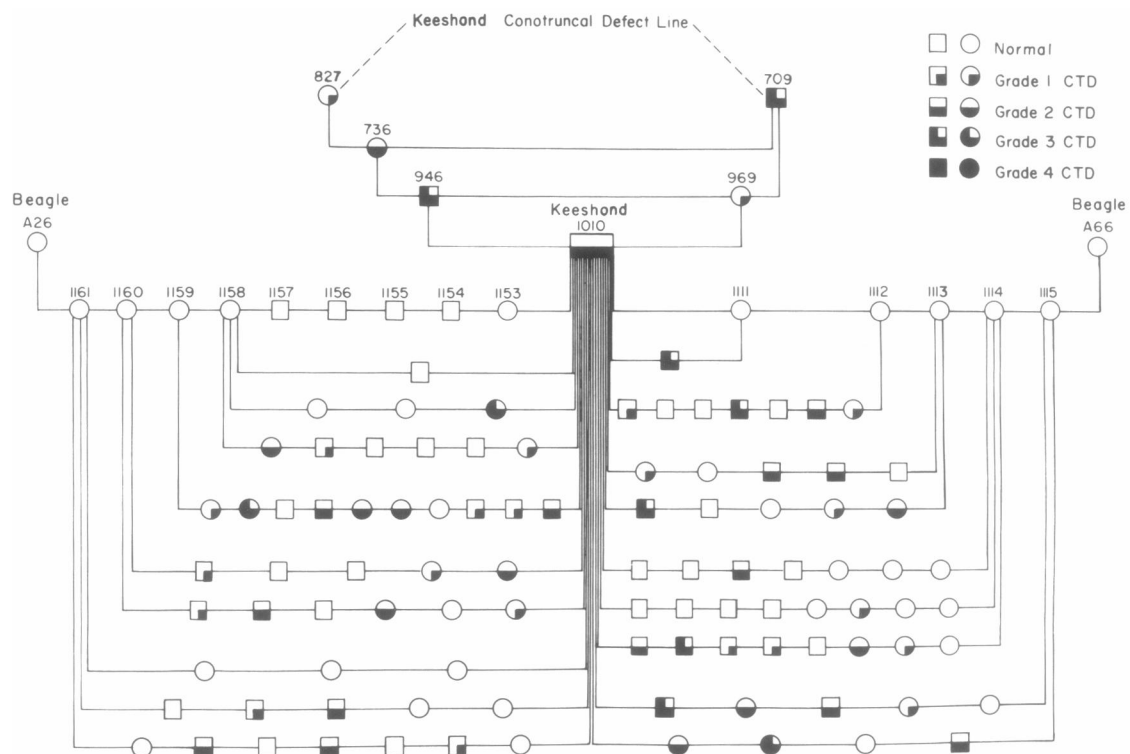


Figure 2 F1 outcross and backcross matings to CTD keeshond 1010. Male 1010, which has a grade 2 CTD, was the offspring of a brother-sister mating between two affected CTD dogs that were also inbred. If breeding contributions from common ancestors in previous generations (not shown) are included, the inbreeding coefficient of male 1010 is .45. Male 1010 was mated to two normal female beagles, and the F1 females were backcrossed to 1010.

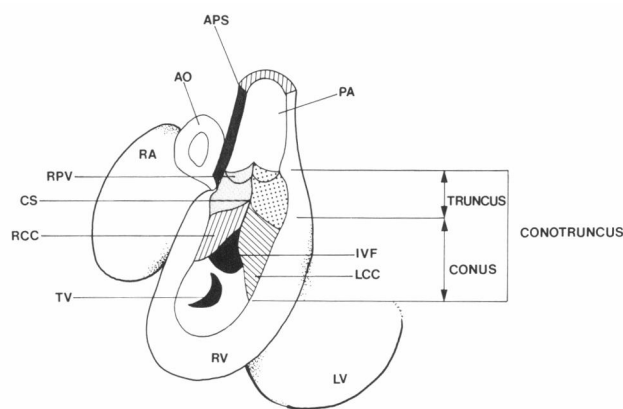


Figure 3 Landmarks in the embryonic heart, stage 17—schematic representation of the opened right ventricle of a normal canine embryo at stage 17. The aortic sac has already been partitioned into the aorta and pulmonary artery distal to the semilunar valves, and the conotruncal cushions have formed and partially fused. In the distal (downstream) portion of the conotruncus, part of the right and left truncus cushions form the right and left cusps of the pulmonic valve. The right and left conus cushions are fused in the distal and mid portion of the conus but, as yet, are not fused in their proximal portions, leaving an interventricular opening, i.e., interventricular foramen III, at the future site of the lower border of the crista supraventricularis (for normal anatomy of the heart at term, see fig. 1A). AO = aorta; APS = aorticopulmonary septum; CS = conotruncal septum (entirely fused truncus and partially fused conus); IVF = interventricular foramen III; LCC = left conus cushion (unfused, proximal portion); LV = left ventricle; PA = pulmonary artery; RA = right atrium; RCC = right conus cushion (unfused, proximal portion); RPV = right pulmonary valve cusp; RV = right ventricle; and TV = tricuspid valve orifice.

F2 generation.—Nine F2 matings were made between normal F1 males and females derived from outcrosses of the following affected CTD-line dogs to normal beagles: CTD-line male 987 (two matings), CTD-line male 1010 (three matings), and CTD-line female 988 (four matings). In the combined F2 generation, the proportion of affected pups was 14/62 (22.5%), not significantly different from the 25% expected under the autosomal recessive hypothesis ($\chi^2 = 0.2$, $.70 > P > .50$). If one assumes, as an alternative, the hypothesis that recessive alleles at two separate unlinked loci are involved, the expected proportion of affected in F2 would be 1/16. The observed proportion of affected is significantly greater than expected under this hypothesis ($\chi^2 = 28.2$, $P < .001$).

Affected-to-affected matings.—To further examine the hypothesis of autosomal recessive inheritance, five matings were performed between surviving offspring with clinically verified ventricular septal defects (grade 2 CTD). Of the 28 offspring produced, all had CTDs,

ranging from grade 1 to grade 4. Among affected offspring, the proportion of severe defects (grades 3 and 4) was greater in affected-to-affected matings (11/28 = 39.3%) than in the F1BC (12/105 = 11.4%) and F2 (1/14 = 7.1%) generations ($\chi^2 = 13.6$, $df = 3$, $P < .01$).

Embryologic Studies

The embryonic conotruncal septum arises as two opposing endocardial outgrowths from the myocardium, the conotruncal cushions or swellings. These grow and eventually fuse along a path that divides the primitive single-outflow region of the ventricles (conal region) into right and left ventricular outflow tracts. The cushions divide the more distal truncal region into the roots and semilunar valves of the aorta and pulmonary artery. Embryologic studies in the dog, as in man (Van Mierop et al. 1977), have shown that fusion of the conotruncal cushions normally occurs first in the truncal region, forming the pulmonary and aortic semilunar valves, and proceeds proximally to form the conus septum. The conus septum gives rise to the muscular medial wall of the right ventricular outflow tract, the crista supraventricularis. At embryonic stage 17, the aortic and pulmonary valves have already formed, and the conus cushions are fused in the more distal (downstream) part of the conal region, leaving a large interventricular foramen bordered by the unfused conal cushions in the proximal region of the conus (fig. 3). The aorta and pulmonary artery distal to the level of the semilunar valves are separated by the aorticopulmonary septum, a structure derived from cells migrating from the neural crest (Kirby et al. 1983).

In previous studies, we noted by microdissection and scanning electron microscopy that the first morphologic sign of deviation from normal cardiac development in the CTD-line embryos was apparent at stage 15. At this stage, hypoplasia of the right ventricle, with accompanying elongation of the conotruncus, was present (Pexieder and Patterson 1984). At this stage, the conotruncal cushions are just beginning to form, and the right and left ventricular outflow tracts are as yet undivided (Van Mierop et al. 1977). In subsequent preliminary studies reported in abstract form, we analyzed a limited number of CTD-line and control embryos by morphometric methods (Alaili et al. 1985; Pexieder et al. 1989b). These studies suggested that the right ventricular hypoplasia seen at stage 15 and also observed at subsequent stages in association with failure of normal development and fusion of the conotruncal cushions was due primarily to a deficiency of myocardium. To confirm these preliminary observations, in the studies

Table 3**Comparison of Tissues Composing the Entire Conotruncus at Stage 17**

TISSUE	MEAN $\pm$ SD RELATIVE VOLUME IN (%)		SIGNIFICANCE (α)
	Controls	CTD Line	
Myocardium	27.9 $\pm$ 10.6	23.8 $\pm$ 17.8	.001 ^a
Cushion tissue	43.1 $\pm$ 18.4	40.4 $\pm$ 22.0	.070
Lumen	28.9 $\pm$ 24.0	35.7 $\pm$ 29.8	.003 ^a

^a Significantly different.

reported here we examined in detail the tissues constituting the embryonic conotruncus, by computer-assisted morphometric analysis at stage 17, the stage immediately prior to that when conotruncal septation is normally completed (Van Mierop et al. 1977). The normal heart at this stage is depicted schematically in figure 3. Embryos from affected-to-affected CTD-line matings were compared to normal beagle embryos at the same developmental stage, first with respect to the proportions of the entire conotruncus comprised by myocardium, cushion tissue, and lumen (table 3). When the volumes of each tissue type integrated over the entire conotruncus are compared, it is apparent that the CTD-line conotruncus contains significantly less myocardium, slightly less cushion tissue, and a larger lumen than do the normal beagle embryos of the same stage.

Because these tissues vary over the length of the conotruncus, local variations in tissue composition in the CTD-line and control embryos were then compared at three levels: proximal, mid, and distal conotruncus (fig. 3 and table 4). The hypoplasia of both myocardium and cushion tissue in the CTD line is most pronounced in the proximal conotruncus, where the right and left conus cushions are not fused. In the mid conotruncus, where the conal cushions are fused in controls, the myocardium is deficient in CTD-line embryos, but cushion tissue in CTD-line embryos is not significantly different from that in control embryos. In the distal conotruncal region, proportions of myocardium are not significantly different between CTD-line embryos and control embryos. However, there is an increase in the cushion tissue and a narrowed lumen in the CTD-line embryos, consistent with the narrowed outflow tract and subpulmonic stenosis observed in some affected dogs at term. In these and previously reported embryologic studies in the CTD line, no morphologic

abnormalities were found in the aorticopulmonary septum.

Discussion

The multifactorial hypothesis for the etiology of congenital heart disease has been attractive because it explains the lack of clear Mendelian ratios in human families and holds the promise that cardiac malformations might be prevented by avoiding specific environmental triggers. However, it has been difficult to test the multifactorial hypothesis, because of problems inherent in investigating the genetics of human cardiac malformations. They represent a diverse group of anatomic defects, and accurate diagnostic data spanning generations of a family are usually not available. Nonsymptomatic individuals are frequently not available for study, and minor subclinical lesions such as those observed in the CTD-line dogs by postmortem examination go undetected. Although modern imaging techniques would undoubtedly detect some of the grade 1 CTDs seen in these studies of the dog, the more subtle anomalies would probably not be detectable, in vivo, by any present technique. Additional difficulties in studying the genetics of congenital heart disease in

Table 4**Local Variations in the Stage 17 Conotruncus Composition**

LEVEL AND TISSUE	MEAN $\pm$ SD RELATIVE VOLUME IN (%)		SIGNIFICANCE (α)
	Controls	CTD Line	
Proximal conotruncus, right and left conus cushions (unfused):			
Myocardium	25.7 $\pm$ 7.1	16.3 $\pm$ 6.4	.008 ^a
Cushion tissue	39.7 $\pm$ 12.5	8.2 $\pm$ 13.4	.001 ^a
Lumen	34.5 $\pm$ 16.8	55.4 $\pm$ 19.4	.001 ^a
Mid conotruncus, conotruncal septum (fused):			
Myocardium	31.7 $\pm$ 3.9	27.5 $\pm$ 9.1	.005 ^a
Cushion tissue	58.6 $\pm$ 6.5	61.1 $\pm$ 11.7	.127
Lumen	9.7 $\pm$ 4.1	11.4 $\pm$ 4.8	.059
Distal conotruncus, conotruncal septum (fused):			
Myocardium	35.1 $\pm$ 5.9	36.6 $\pm$ 13.5	.267
Cushion tissue	45.7 $\pm$ 16.9	53.2 $\pm$ 13.9	.024
Lumen	19.2 $\pm$ 12.8	10.1 $\pm$ 8.1	.001 ^a

^a Significantly different.

human families include lack of assurance, when families are pooled, that they have homologous developmental defects. Since, over time, there is great environmental variation both within and between families, it is also difficult to assess the possible effects of nongenetic factors. Thus, while patterns of fully penetrant Mendelian inheritance are usually not apparent, it has been difficult to determine in human families whether the genetic component in a given type of congenital heart defect consists of many genes, a few, or, in some cases, a single major gene. This is an important distinction, for, if there are single loci of major importance in the etiology of specific malformations, and if they can be identified, it might then be possible for the morphologic abnormalities in cardiac development to be traced to the underlying molecular derangements.

The results of our studies indicate that a defect in a single autosomal gene underlies the developmental abnormality leading to conotruncal malformations in the CTD line of keeshond dogs. The model that best explains the observations is one in which the mutant CTD allele is partially penetrant in heterozygotes, as indicated by the lack of clinically significant defects but the occurrence of a few subclinical anomalies of the crista supraventricularis in the F1 generation (2/26 = 7.7% of animals examined postmortem). The slight excess of affected offspring in the F1 backcross matings is also consistent with partial penetrance in heterozygotes. There is complete penetrance of the CTD gene in the homozygous state, in that the observed proportions of offspring with some degree of anatomical evidence of abnormal conotruncal development fit those expected for fully penetrant autosomal recessive inheritance in the F1 backcross, F2, and affected-to-affected matings. The degree of abnormal development differs among homozygotes, as is most evident in the offspring of matings between two affected individuals, where all four grades of CTD are represented (table 2).

Both the slight penetrance of CTD in heterozygotes and the complete penetrance but variable expression in homozygotes can be viewed as evidence of the same phenomenon: a single-major-gene effect on conotruncal development, with superimposed additional minor variation. The latter can, in theory, be due to either the effects of genes at other loci, the environment, or chance. The finding of an excess of affected offspring in F1 backcross matings involving one CTD-line male (1987) suggests the possibility that genes at other loci may affect the severity of the developmental abnormality. Kurnit et al. (1985, 1987) have proposed a stochastic single-gene model for defects in cardiac septation, as

well as for other congenital malformations in which variation in penetrance/expressivity is due largely to random (chance) effects. According to their model, developmental processes in the early embryo, such as growth and fusion of endocardial cushions, can be made sensitive to early perturbations that become magnified as development progresses. When the genotype at a single locus shifts an embryologic process to a point near a critical developmental threshold, two individuals with identical genotypes can have different phenotypes, simply because of chance effects.

The findings in the present study differ from those obtained in the original breeding experiments using noninbred keeshonds in crosses to dogs of a number of different breeds (Patterson et al. 1974). In the earlier studies, the results were not consistent with any fully penetrant monogenic hypothesis. By selectively inbreeding affected animals to affected animals for over 10 generations and utilizing purebred beagles in outcrosses, it appears that we have reduced the effects of minor genes (genetic background) that previously obscured Mendelian transmission. This is the first strong evidence that a common type of cardiac malformation that behaves in a non-Mendelian fashion in an outbred population has a single-gene defect as the major determinant.

Our earlier embryologic studies at the organ level indicated that the CTD allele produces a spectrum of conotruncal anomalies by interfering with growth and fusion of the conotruncal cushions (Van Mierop et al. 1977, 1978). Subsequent preliminary morphometric studies at the tissue level suggested that conotruncal cushion hypoplasia in CTD dogs is associated with a generalized deficiency of myocardial growth in the conotruncal region during the normal period of conotruncal septation (Pexieder and Patterson 1984; Alaili et al. 1985; Pexieder et al. 1989b). In the present, more extensive morphometric study, we have confirmed the presence of hypoplasia of myocardium in the proximal and mid portions of the embryonal conotruncus, just prior to the stage when conotruncal septation is normally completed (tables 3 and 4). Conotruncal cushion tissue is also hypoplastic in the proximal, unfused cushions, but not in the mid and distal portions of the conotruncus. In fact, there is an increase in the cushion tissue in the distal conotruncus. Whether this is a primary phenomenon or perhaps is related to hemodynamic effects and functional adaptations (Pexieder 1981) due to the more proximal developmental abnormalities is not clear. However, the increased cushion tissue and narrowed lumen in the distal conotruncus are consis-

tent with the narrowed right ventricular outflow tract and pulmonic stenosis observed in CTD-line dogs with grade 3 defects (tetralogy of Fallot and pseudotruncus arteriosus) at term.

Thus it appears that myocardial, rather than cushion-tissue hypoplasia, is the primary developmental abnormality in the CTD-line embryos. Recent evidence indicates that the embryonic myocardium has a primary inductive role in controlling the morphogenesis of endocardial cushion tissue (Markwald et al. 1990; Mjaatvedt et al. 1991; Sinning et al. 1992). Hypoplasia of conus cushions in the unfused regions of the conus septum may be secondary to the myocardial defect leading to myocardial hypoplasia.

Our findings support the hypothesis that the normal allele at the CTD locus is important in growth of the conotruncal myocardium during the critical phase of conotruncal septation, but its mode of action at the molecular level is as yet unknown. Isolation of the CTD gene and analysis of its structure could lead to an understanding of both its function and the molecular pathogenesis of hereditary CTDs. Relevance to congenital heart defects in humans is suggested by the reported aggregation, in human families, of cardiac malformations similar to those in the CTD line (Pitt 1962; Fuhrman 1968; Real 1968; Emanuel 1970; Fraser and Hunter 1975; Miller and Smith 1979; Takao et al. 1980; Corone et al. 1983). A few human families with conotruncal malformations have been reported to have aggregations of affected offspring consistent with single-gene inheritance (Pitt 1962; Miller and Smith 1979).

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References

- Alaili R, Pexieder T, Patterson DF (1985) Image analysis of tissue mechanisms in the conotruncal hypoplasia in the keeshond dog. *Eur Heart J* 6, Suppl 1:38
- Campbell M (1965) Causes of malformations of the heart. *Br Med J* 2:895-904
- Carter CO (1961) The inheritance of congenital pyloric stenosis. *Br Med Bull* 17:251-254
- (1969) Genetics of common disorders. *Br Med Bull* 25:52-57
- Clark EB (1987) Mechanisms in the pathogenesis of congenital cardiac malformations. In: Pierpont ME, Moller JH (eds) *Genetics of cardiovascular disease*. Martinus Nijhoff, Boston, pp 3-11
- Corone P, Bonaiti C, Feingold J, Fromont S, Berthet-Bondet D (1983) Familial congenital heart disease: how are the various types related? *Am J Cardiol* 51:942-945
- Davison BCC (1967) Concordance and discordance of congenital heart disease in 20 families. *J Med Genet* 4:245-250
- Derweiler DK, Patterson DF, Luginbuhl H, Rhodes WH, Buchanan JW, Knight DH, Hill JD (1979) The cardiovascular system. In: *Canine medicine*, 4th ed. American Veterinary, Santa Barbara, pp 813-949
- Edwards JH (1960) The simulation of Mendelism. *Acta Gene Stat Med* 10:63-70
- (1969) Familial predisposition in man. *Br Med Bull* 25:58-64
- Ehlers KH, Engle MA (1966) Familial congenital heart disease. I. Genetic and environmental factors. *Circulation* 34:503-516
- Emanuel R (1970) Genetics and congenital heart disease. *Br Med J* 32:281-291
- Ettinger SJ, Suter PF (eds) (1970) *Canine cardiology*. WB Saunders, Philadelphia
- Falconer DS (1960) *Introduction to quantitative genetics*. Oliver & Boyd, Edinburgh
- (1965) Inheritance of liability to certain diseases as estimated from the incidence among relatives. *Ann Hum Genet* 29:51-71
- Ferencz C, Correa-Villaseñor A, Rubin JD, Boughman JA, Loffredo C (1991) The epidemiology of cardiovascular malformations (CVM): ten years later. *Teratology* 43:450
- Ferencz C, Rubin JD, McCarter RJ, Brenner JL, Neill CA, Perry LW, Hepner SI, et al (1985) Congenital heart disease: prevalence at live-birth. *Am J Epidemiol* 121:31-36
- Fraser FC (1976) The multifactorial/threshold concept—uses and misuses. *Teratology* 14:267-280
- Fraser FC, Hunter ADW (1975) Etiologic relations among categories of congenital heart malformations. *Am J Cardiol* 36:793-796
- Fuhrman W (1968) Congenital heart disease in sibships ascertained by two affected siblings. *Humangenetik* 6:1-12
- Gruneberg H (1952) Genetical studies on the skeleton of the mouse. IV. Quasicontinuous variations. *J Genet* 51:95-114
- Guide to the care and use of laboratory animals (1985) PHA NIH publication 85-23, revised. US Government Printing Office, Washington, DC
- Kirby ML, Gale TF, Stewart DE (1983) Neural crest cells contribute to normal aorticopulmonary septation. *Science* 220:1059-1061
- Kurnit DM, Aldridge JF, Matsuoka R, Matthysse S (1985) Increased adhesiveness of trisomy 21 cells and atrioventricular canal malformations in Down syndrome: a stochastic model. *Am J Med Genet* 30:385-399

- Kurnit DM, Layton WM, Matthysse S (1987) Genetics, chance, and morphogenesis. *Am J Hum Genet* 41:979-995
- Lamy M, DeGrouchy J, Schweisguth O (1957) Genetic and non-genetic factors in the etiology of congenital heart disease. *Am J Hum Genet* 9:17-41
- McKeown T, MacMahon B, Parsons CG (1953) The familial incidence of congenital malformations of the heart. *Br Heart J* 15:273-277
- Markwald RR, Mjaatvedt CH, Krug EL, Sinning AR (1990) Inductive interactions in heart development: role of cardiac adherens in cushion tissue formation. *Ann N Y Acad Sci* 599:13-25
- Miller ME, Smith DW (1979) Conotruncal malformation complex: examples of possible monogenic inheritance. *Pediatrics* 63:890-893
- Mjaatvedt CH, Krug EL, Markwald RR (1991) An antiserum (ES1) against a particulate form of extracellular matrix blocks the transition of cardiac endothelium into mesenchyme in culture. *Dev Biol* 145:219-230
- Mulvihill JJ, Priester WA (1973) Congenital heart disease in dogs: epidemiologic similarities to man. *Teratology* 7:73-78
- Nora JJ (1968) Multifactorial inheritance hypothesis for the etiology of congenital heart diseases: the genetic environmental interaction. *Circulation* 38:604-617
- Nora JJ, Nora AH (1978a) The evolution of specific genetic and environmental counseling in congenital heart diseases. *Circulation* 57:205-213
- (1980) The genetic contribution to congenital heart disease. In: Nora JJ, Takao A (eds) *Congenital heart diseases: causes and processes*. Futura, New York, pp 3-13
- (1978b) Genetics and counseling in cardiovascular diseases. Charles C Thomas, Springfield, IL
- Patterson DF (1965) Congenital heart disease in the dog. *Ann N Y Acad Sci* 127:541-569
- (1968) Epidemiologic and genetic studies of congenital heart disease in the dog. *Circ Res* 23:171-202
- (1978) Lesion-specific genetic factors in canine congenital heart disease: patent ductus arteriosus in poodles, defects of the conotruncal septum in the keeshond. *Birth Defects* 14:315-347
- Patterson DF, Pyle RL, Van Mierop L, Melbin J, Olson M (1974) Hereditary defects of the conotruncal septum in keeshond dogs: pathologic and genetic studies. *Am J Cardiol* 34:187-205
- Pexieder T (1981) Cellular abnormalities leading to congenital heart disease. In: Godman M (ed) *Paediatric cardiology*, vol 4. Churchill Livingstone, Edinburgh, pp 24-32
- Pexieder T, Bloch D, Beuret A (1989a) Eurocat collaborative study on congenital heart disease—1986 data. *Eur J Epidemiol* 5:254
- Pexieder T, Patterson DF (1984) Early pathogenesis of conotruncal malformations in the keeshond dog. In: Nora JJ, Takao A (eds) *Congenital heart disease: causes and processes*. Futura, New York, pp 439-457
- Pexieder T, Vuillemin M, Alaili R, Veuthey S, Patterson DF, Scott WJ Jr (1989b) Experimental studies in the pathogenesis of conotruncal defects. In: Aranega A, Pexieder T (eds) *Correlations between experimental cardiac embryology and teratology and congenital cardiac defects*. University Granada Press, Granada, pp 37-92
- Pitt DB (1962) A family study of Fallot's tetrad. *Australas Ann Med* 11:179-183
- Real F (1968) Familial incidence of cyanotic congenital heart disease. *Proc Assoc Eur Pediatr Cardiol* 4:46
- Sanchez-Cascos A (1978) The recurrence risk in congenital heart disease. *Eur J Cardiol* 7:197-210
- Siegel S (1956) *Nonparametric statistics for the behavioral sciences*. McGraw-Hill, New York
- Sinning AR, Krug EL, Markwald RR (1992) Multiple glycoproteins localize to a particulate form of extracellular matrix in regions of the embryonic heart where endothelial cells transform into mesenchyme. *Anat Rec* 232:285-292
- Soto B, Becker AE, Moulart AJ, Lie JT, Anderson RH (1980) Classification of ventricular septal defects. *Br Heart J* 43:332-343
- Streeter GL (1948) Developmental horizons in human embryos: description of age group XV, XVI, XVII, XVIII, being the third issue of a survey of the Carnegie Collection. *Contrib Embryol* 32:113-203
- Takao A, Ando M, Cho K, Kinouchi A, Murakami Y (1980) Etiologic categorization of common congenital heart disease. In: Van Praagh R, Takao A (eds) *Etiology and morphogenesis of congenital heart disease*. Futura, New York, pp 253-268
- Van Mierop LHS (1978) The pathogenesis of spontaneously occurring anomalies of the ventricular outflow tract in keeshond dogs: embryologic studies. *Birth Defects* 14:361-375
- Van Mierop LHS, Patterson DF, Schnarr WR (1977) Hereditary conotruncal septal defects in keeshond dogs: embryologic studies. *Am J Cardiol* 40:936-950
- (1978) Pathogenesis of persistent truncus arteriosus in light of observations made in a dog embryo with the anomaly. *Am J Cardiol* 41:755-762
- Wright S (1934) The results of crosses between inbred strains of guinea pigs differing in numbers of digits. *Genetics* 19:537-551