mors. Whether any of these changes are causally related to the malignancy in which they occur remains a fundamental, unanswered question.

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27. We thank C. J. Tabin and R. Weinberg for providing us with the pEJ plasmid; J. D. Minna, A. F. Gazdar, and D. N. Carney for providing the NCI lung culture lines; H. C. Hoover for help in obtaining the tissues used in this study; D. Gomolka and R. Thompson for technical assistance; and S. Smith for preparing the manuscript. Supported by PHS grants 09071, 31053, 24592, 18404, awarded by the NCI, DHHS, and grant PDT-ACS 108 from the American Cancer Society.

29 March 1983; revised 2 May 1983

Hyaluronate in Vasculogenesis

Abstract. *Limb buds of chicken embryos contain within the peripheral mesoderm an avascular zone that is rich in hyaluronic acid. Epithelial tissues that synthesize large amounts of hyaluronic acid relative to other glycosaminoglycans caused avascularity when implanted into normally vascular wing mesoderm. Epithelia that synthesize little hyaluronic acid did not cause avascularity. Elvax implants containing hyaluronic acid caused the formation of avascular zones, whereas similar implants containing other glycosaminoglycans did not give rise to avascular zones. Hyaluronic acid may thus play a role in determining the location of blood vessels in the embryo.*

Vasculogenesis, the development of the vascular system, has been studied by embryologists (*1*) and by those interested in tumor vascularization (*2*). Factors that promote or inhibit blood vessel growth have been described in the tumor system (*2, 3*), but little is known about the way in which tissues interact to form the vascular system in embryos.

Injection of India ink into the vascular system of chicken embryos at successive stages (*4*) of development and clearing (making the embryo transparent with oil of wintergreen) revealed vascular and avascular regions (*1, 5, 6*). The limb bud, as well as the entire trunk region, has a definitive avascular zone within the peripheral mesoderm. This shell of avascular mesoderm lies subjacent to the ectoderm and surrounds a central core of highly vascular mesoderm (Fig. 1, A and B). The only histological difference between these regions is the presence or absence of blood vessels (Fig. 1C). The ectoderm is responsible for the formation and maintenance of this peripheral avascular zone (*7*). When embryonic back-skin ectoderm was implanted into the normally vascularized mesoderm of the wing bud, the tissue surrounding the implants became avascular. The width of this avascular region was similar to that underlying normal wing bud ectoderm.

To determine whether the formation of avascular zones in underlying mesoderm was a universal characteristic of embry-

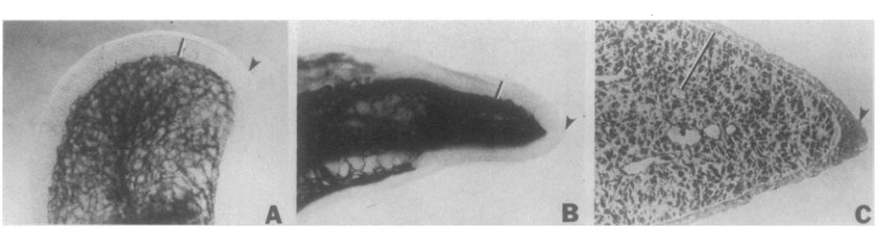

Fig. 1. (A) Dorsal view of the distal portion of a wing bud at stage 25 showing the peripheral mesodermal avascular zone (bar) subjacent to the ectoderm (arrowhead). (B) Side view of a wing bud showing the mesodermal avascular zones subjacent to the dorsal (bar) and ventral surfaces of the limb. These avascular domains merge beneath the apical ectodermal ridge at the distal tip of the limb (arrowhead). (C) A section (1 μm) of a similar subridge region shows the location of the vascularized mesodermal core. The mesenchyme appears similar within the vascular and avascular (bar) parts of the limb. The arrowhead points to the apical ectodermal ridge, which is continuous with the limb ectoderm.

onic epithelia, we implanted epithelial tissues from various regions of the embryo into slits cut in the vascularized region of wing bud mesoderm. After 24 hours, the embryos were injected with India ink, fixed, and cleared. A number of epithelial tissues (7-day corneal endothelium, 5-day amnion, 4-day back-skin ectoderm, 5-day chorion, and 4-day limb ectoderm) caused the formation of avascular zones, whereas others (5-day retinal pigment epithelium, 5-day lens epithelium, 7-day corneal epithelium, and 4-day ectoderm overlying the yolk sac) had no effect on vascularity (Fig. 2). Epithelia that caused the formation of avascular zones in the limb bud–implant assay were usually adjacent to avascular mesoderm. Epithelia normally associated with highly vascular mesoderm in the embryo never formed avascular zones.

Embryonic ectoderm synthesizes relatively large amounts of hyaluronic acid (HA), a glycosaminoglycan differentially distributed in the extracellular matrix surrounding embryonic mesenchymal cells (*8*). An HA-rich region between the ectoderm and the mesodermal vascular bed in the chicken limb bud has been described (*9*). This region corresponds to the peripheral avascular zone of mesoderm described above. All of the implanted tissues were tested for HA synthesis. A larger relative amount of labeled glucosamine was incorporated into hyaluronate by all of the tissues that formed avascular zones than by those that did not form avascular zones (Fig. 2).

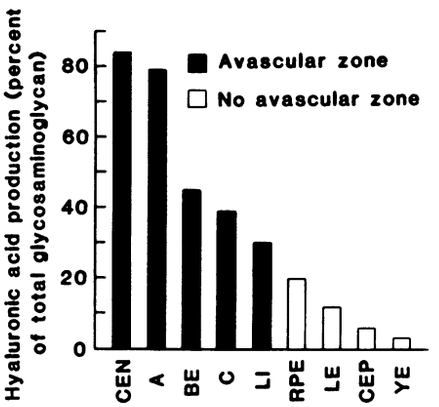

Fig. 2. Histogram showing the relative amounts of labeled glucosamine incorporated into hyaluronate. Tissues that formed avascular zones were *CEN*, 7-day corneal endothelium; *A*, 5-day amnion; *BE*, 4-day back-skin ectoderm; *C*, 5-day chorion; and *LI*, 4-day limb ectoderm. Tissues that did not form avascular zones were *RPE*, 5-day pigmented retinal epithelium; *LE*, 5-day lens epithelium; *CEP*, 7-day corneal epithelium; and *YE*, 4-day ectoderm overlying the yolk sac. Since amnion and chorion contain both ectodermal and mesodermal components, it is not clear whether HA is synthesized exclusively by one layer or by a combination of the two. The percentages are the means of quadruplicate determinations and are typical results of assays of replicate samples. Isolated tissues were placed into 0.4 ml of Ham's F-12 medium (Gibco) containing 100 μCi of D-[6-^{3}H(N)]glucosamine hydrochloride (New England Nuclear) and incubated at 37°C (*8*). Duplicate 20-μl portions of [^{3}H]glycosaminoglycan received 10 μl of buffer alone or buffer containing 1 TR unit of *Streptomyces* hyaluronidase (Calbiochem). The samples were incubated for 1½ hours at 37°C, boiled briefly, and mixed with 0.125 μl of unlabeled carrier HA (1 mg/ml) and chondroitin sulfate (1 mg/ml). The undegraded glycosaminoglycan was precipitated by adding 155 μl of 1 percent cetylpyridinium chloride (CPC). This mixture was poured into a sampling manifold (Millipore) and vacuum-filtered through a glass fiber filter (Whatman GF/C). The filters were washed in methanol and dried and the radioactive material retained on the filters was counted in a toluene-based scintillation fluid. The difference in CPC-precipitable counts between *Streptomyces* hyaluronidase-treated samples and controls was divided by the total CPC-precipitable counts and multiplied by 100 to give the percentage of HA incorporation.

These data suggested a relationship between the distribution of HA and the distribution of blood vessels in embryonic mesenchyme. The local concentration of HA within limb mesenchyme was increased by using Elvax-40 (*10–12*) as a slow-release vehicle. Two-phase mixtures of Elvax and HA were cast (*13*), cut into fine slivers, and implanted into the vascular mesoderm of wing buds from chicken embryos of stages 20 to 22 (*4*). After 24 hours, embryos were injected with India ink, fixed, and cleared. Control implants consisted of Elvax alone or Elvax impregnated with chondroitin sulfate or heparin, two glycosaminoglycans also found in the extracellular matrix. Avascular zones surrounded only those implants that had HA incorporated into the polymer (Fig. 3). The HA used in these assays was extracted from human umbilical cord (grade 111-P, Sigma). After being further purified by treatment with alkali and borohydride (*14*), HA produced avascular zones similar to the one shown in Fig. 3A. These data suggest that the distribution of blood vessels in the embryo may be determined in part by the relative amounts of HA contained within embryonic tissue.

An additional set of implants containing high molecular weight DNA (calf thymus, Sigma) was used to test whether another negatively charged polymer was able to prevent the formation of blood vessels in embryonic mesoderm. Implants containing DNA did not form avascular zones. This suggests that the avascularity associated with hyaluronate is not solely caused by the steric properties of the molecule.

Hyaluronic acid, a constituent of most connective tissues, is found in greatest abundance in tissues that are normally avascular. Both umbilical cord and rooster comb, from which HA is most often purified, have large vessels running through their cores but their HA-rich peripheral regions are completely avascular. Hyaline cartilage and the vitreous

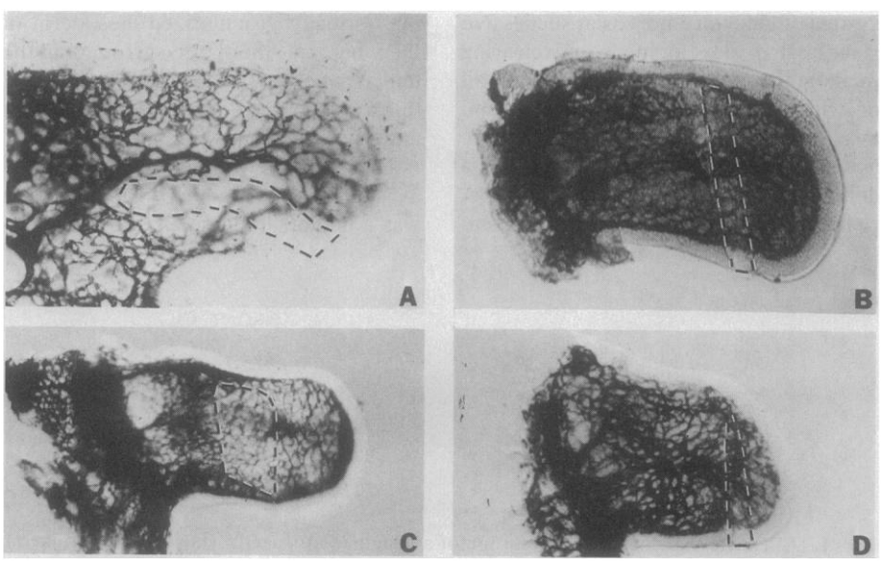

Fig. 3. (A) Position of a polymer implant containing HA is outlined on this wing bud (*16*). A large area of avascularity extends around the implant. Vessels beneath the implant, which appear out of focus, are on the opposite (ventral) side of the limb and have not been affected by the implant. (B–D) Control implants of Elvax alone (B) or Elvax containing chondroitin sulfate (C) or heparin (D) did not form an avascular zone. These specimens are shown 24 hours after implantation. The location of the implant is outlined (*16*).

body of mammalian eyes also contain high concentrations of HA and are avascular throughout most of life. The association of large concentrations of HA with these avascular tissues may indicate a continuing role for HA in establishing and maintaining avascularity.

Extracellular matrix molecules, particularly hyaluronate, are believed to participate in various developmental processes (*15*). We have correlated the presence of increased levels of hyaluronate with the absence of blood vessels. If HA acts in other regions of the embryo as it appears to act in the peripheral mesoderm of the chicken wing bud, it will influence the location of embryonic capillary networks. Since all of the major blood vessels are formed from such capillary networks, HA may have a critical role in determining the location of these vessels during normal development.

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13. Material to be implanted (25 mg) was dissolved in 10 ml of acetate buffer (0.15*M* NaCl and 0.02*M* sodium acetate, *p*H 5). The mixture was frozen in liquid nitrogen, pulverized, and lyophilized. The lyophilization chamber was vented with nitrogen, and dry methylene chloride was added. The suspension was concentrated to a volume of 0.5 ml by centrifugation. The polymer casting solution was made by dissolving 125 mg of Elvax-40 in the methylene chloride mixture at 37°C for 45 minutes. This viscous mixture was placed on a glass slide and immediately immersed in liquid nitrogen. The plastic was stored overnight at −20°C and then stored at reduced pressure until needed.
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16. The polymer implants become transparent when the embryo is cleared in wintergreen. The position of the implant can be determined with a substage mirror.
17. We thank J. Piatigorsky and A. H. Reddi for reading the manuscript. Supported by grant RO7033 from the Uniformed Services University of the Health Sciences.

12 October 1982; revised 21 March 1983

Somatic Mutations of Immunoglobulin Variable Genes Are Restricted to the Rearranged V Gene

Abstract. *An important question concerning the mechanism of somatic mutation of immunoglobulin variable (V) genes is whether it involves all of the numerous V genes in a differentiated B cell, independent of location, or if it is restricted to a particular chromosomal site. Comparison of the sequence of two alleles of a given V gene shows that the mutations are limited to the rearranged V gene, while the same V gene on the other chromosome has not undergone mutation. This indicates that a V gene sequence alone is not sufficient for somatic mutation to take place. The mutation is therefore restricted to the rearranged V gene and consequently does not occur before rearrangement.*

Immunoglobulin genes are striking in their sequence diversity and in the DNA rearrangements associated with their expression. The productive rearrangement required for light chain expression involves the positioning of one of the several hundred variable (V) genes next to the joining (J) region of the constant (C) gene. For heavy chain expression, two rearrangements are necessary since a third region, the diversity (D) region, is incorporated in the product. Usually, only one immunoglobulin molecule is produced per cell, a phenomenon called allelic exclusion. The exclusion of one of the two alleles results from either the absence of a V-J rearrangement or from an aberrant form of rearrangement.

The diversity of immunoglobulin genes results from (i) the existence of a large number of different V genes and a smaller set of D and J regions in germline DNA, (ii) variability introduced at a limited number of sites in the process of V-J or V-D joining (or both), and (iii) the occurrence of somatic mutations affecting V genes. Somatic mutation of immunoglobulin V genes was originally described for V lambda genes (*1*) and is now well established for V kappa (*2–4*) and V heavy genes (*5–8*). However, the nature of the mechanism responsible for these V gene–specific somatic mutations and the extent of its action are still not understood (*9*). Since there are several hundred different V genes in the mouse genome, an important question concerning the mutation of these genes is: are most or all V genes mutated because the mechanism responsible for mutation recognizes V gene sequences in a differentiated lymphocyte or are mutations limited to the V genes that have undergone V-J joining?

If somatic mutations result from a generalized mechanism in differentiated lymphocytes, based on the recognition of V gene sequences, then most or all V genes should be found mutated when compared with their germ-line homologs. However, if mutations are restricted to the site of V-J joining, then only the particular V gene involved in the rearrangement would be subject to mutations. An unambiguous distinction between these alternatives can be obtained by a DNA sequence analysis of the same gene, rearranged and mutated on one chromosome and not rearranged on the other chromosome. The V kappa light chain genes from mouse myeloma T (Fig. 1) are well suited for such a study because in this myeloma (i) V-J joining has taken place on both chromosomes (*10, 11*), thus excluding any possible difference in susceptibility between V genes on a rearranged and on a nonrearranged chromosome; (ii) on each chromosome the V gene involved in the V-J rearrangement has undergone somatic mutation (*2*), showing that the mutation mechanism has operated on both chro-

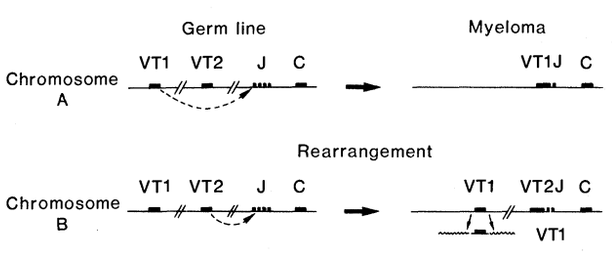

Fig. 1. Schematic description of myeloma T. Rearrangement has taken place on both chromosomes (*8, 9*). On chromosome A, V gene T1 has been joined to J1. On chromosome B, V gene T2 has been joined to J2; however, a copy of VT1 is still present. This V gene was cloned in phage and sequenced. Both V genes T1 and T2, which have undergone rearrangement, have been shown to have undergone somatic mutation (*2*).