The control of muscle
and cartilage development in the chick limb: the
role of differential vascularization

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SUMMARY
The results of previous studies on the effects of nicotinamide levels on chick limb mesodermal cell expression have indicated that there may exist a metabolic gradient situated radially across the limb which has a controlling influence on whether a limb cell will differentiate into either a myogenic or chondrogenic phenotype. This study investigates the possible role of the vascular pattern in establishing such a metabolic gradient. Observations are reported which show that prospective myogenic and chondrogenic areas become differentially vascularized before the onset of molecular differentiation, thus indicating that the vascular pattern is capable of establishing metabolic gradients across the limb. Furthermore, predictions based on these results have been tested and verified in vitro. The hypothesis is presented that the spatial differentiation of cartilage and muscle in the developing chick limb is under the controlling influence of gradients of metabolic potential which can be established by the differential vascularization of the limb.

INTRODUCTION
Muscle and cartilage elements differentiate from a homogeneous population of chick limb mesodermal cells in a precise pattern and during a short, but specific, time period (Zwilling, 1968; Searls, 1965a, b; Searls & Janners, 1969; Saunders, Cairns & Gasseling, 1957; Finch & Zwilling, 1971). We have suggested that limb mesodermal cell expression is influenced by local concentration differences of nicotinamide and/or pyridine nucleotides (Caplan, 1970, 1971, 1972a–d). To account for such local concentration differences, it was suggested that differential vascularization of the chondrogenic and myogenic areas of the limb bud might be involved (Caplan, 1972b). Although the vascular development of the chick has been described in detail, observations have not been presented concerning the sequence and timing of the vascular pattern in the developing limb. Observations are presented here which detail the vascular changes in the developing limb; these observations show that vascular changes precede morphological and molecular changes in the limb. Furthermore, these observations can be used as the basis for suggesting that concentration dif-

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ferences of various nutrients could influence or control the cytodifferentiation of limb mesodermal cells.

Based on these observations, predictions could be made that relatively high oxygen tension would potentiate myogenic expression and that low oxygen tension would potentiate chondrogenic expression. This possibility was investigated by culturing limb mesodermal cells under different oxygen tensions. The results of these experiments further support the hypothesis that a gradient of metabolic potential across the limb can be a controlling factor in myogenic and chondrogenic expression.

**METHODS AND MATERIALS**

*The vascular system*

Two procedures were used to expose the White Leghorn chick embryos to experimental manipulation. (1) Windows were cut in most of the egg shells after 3 days of incubation at 37.5 °C (Zwilling, 1959) and sealed with 'Scotch Brand Magic Transparent Tape No. 810.' These eggs were then returned to incubation until the desired stages of development were reached (Hamburger & Hamilton, 1951). (2) Some of the embryos were exposed by breaking away the shell directly over the air space and carefully removing the shell membrane from the surface of the vitelline membrane.

To analyse the vascular system, an embryo of the desired stage of development was injected by pipetting Indian ink through a finely drawn capillary tube into the aorta or into one of the vitelline veins and allowing the heart to pump the ink through the circulatory system. In most cases the injection was made into the anterior vitelline vein. The ink was sonicated (Branson Heat Systems, Inc.) for 1 h before use in order to break up the larger carbon particles in the ink that would otherwise clog the capillaries and give an incomplete injection. The embryos were fixed with 70% ethanol, dehydrated with ethanol (three changes of 95% and three changes of absolute ethanol, 4 ml per change) and cleared with xylene. The limbs were then mounted whole in Permount.

*Cultured cells*

Stage 24 limb mesodermal cell cultures were prepared as described previously (Caplan, 1970, 1972a, c). Falcon plastic tissue culture plates of 60 and 35 mm diameter were inoculated with $12 \times 10^6$ and $3.8 \times 10^6$ cells respectively. The bicarbonate-buffered nutrient medium was replaced every 24 h. Cells were placed into one of the following environments at 0, 24, 48, 72, or 96 h after plating: 95% N₂ and 5% CO₂, 95% air and 5% CO₂, or 95% O₂ and 5% CO₂. On day 9 the plates were fixed with Bouin's. Subsequently the plates were stained with

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Footnote 1: A preliminary report of this work was presented at the Society of Cell Biology Meetings in New Orleans, Louisiana, November 1971.
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hematoxylin, toluidine blue, or Bodian's silver stain and scored for morphological features indicative of cartilage or muscle (Caplan, 1970). All gas mixtures were supplied and certified by Ohio Medical Company, Cleveland, Ohio. Eighteen separate experiments involving separate cell preparations were conducted during the period January to November of 1971. Fifteen experiments involved the extremes described above while three experiments involved gas mixtures of 5, 10, 15, 25, 30, 40, or 50%.

RESULTS

The vascular system

The injections with indian ink proved successful in exposing the capillary net of the limb buds, though the capillaries of the youngest embryos used were sometimes clogged by the larger particles of ink. In most cases the heart continued to pump the ink through the vessels for some time after the ink had circulated through the entire vascular system. One feature common to all limb buds up to stage 28 is an area of unvascularized mesoderm at the extreme periphery of the limb (Figs. 1–7). This unvascularized area includes the mesoderm that is just below the apical ectodermal ridge and is on the order of 0·10 ± 0·02 mm wide.

Examination of the circulatory pattern of the early wing buds shows that until early stage 20 many small lateral offshoots of the aorta [the primary subclavian capillary plexus and later the multiple segmental subclavian vessels (Evans, 1909a)] supply circulation to the wing (Figs. 1–3). These vessels are on the order of 10μm and are classified as capillaries. The broadening of these offshoots begins near the aorta at stage 20; this broadening is quite evident by late stage 20 (Fig. 4) and spreads into the wing at stage 21. A recognizable central artery (the primary subclavian artery) can be observed by late stage 21 or early in stage 22 (Fig. 5). In early stage 22 the broad collecting vessels begin to form at the margin of the limb. These processes occur about one stage slower in the leg buds.

As the major central and marginal vessels are being formed by the broadening of the existing vessels, the prospective cartilage-forming areas of the limb bud become less vascularized than the prospective muscle-forming areas. Thus, by stage 22 fewer capillary-sized vessels are present in the core region than in the more peripheral regions of the limb bud.

Early in stage 24 the brachial artery and the marginal vein are quite advanced in their development (Fig. 6); the core of the limb at this stage of development is essentially avascular while the prospective muscle-forming tissue is well vascularized. Later in stage 24 the capillaries of the prospective muscle-forming tissue begin to organize into a double layer of capillaries. The question of whether this segregation of various capillary networks is related to the future appearance of different muscle bundles remains for future experimentation. The
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dominance of this central artery and marginal vein separated by capillaries
does not change as development progresses (Fig. 7).

Fig. 8 is a camera-lucida drawing of a stage 24 wing bud. This drawing
illustrates the flow of blood into the limb via the subclavian and brachial arteries
into the capillary bed of the premyogenic region and finally into the marginal
vein. Fig. 9 schematically illustrates and summarizes the development of the
large central arteries and marginal veins of the chick limb from stage 18 to
stage 25. Difficult to portray in these schematic drawings is the fact that the
capillary network is established in the premyogenic areas dorsally and ventrally
to the chondrogenic area.

In summary, by stage 24 the vascular networks have, in an orderly and well
timed manner, isolated two prominent areas — an avascular area which corre-
sponds to the prechondrogenic area and a well vascularized area corresponding
to the premyogenic area. The events governing this isolation are initiated in the
developing limb at stage 20, become clearly established at early stage 22 and
become dominant at early stage 24.

Effect of various gas mixtures on limb mesodermal cell development in vitro

Because of the complexity of the tissue culture situation, the results reported
for the following experiments must be considered extremely crude. Assays were
visual and involved the scoring for obvious myogenic and chondrogenic proper-
ties. Various biochemical assays were abandoned when it became clear that
under the extremes of oxygen tension involved in these studies, varying numbers
of cells did not survive depending on when they were exposed to the high or low
oxygen tension. However, the trends reported in Table 1 were grossly apparent
and reproducible.

Exposing the cells to the extremes of oxygen tension at the time of plating
(at 0 h) prevented most of the cells from attaching to the Petri dish. Exposing
the cells to the oxygen tension extremes at 72 h or later after plating produced

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Figures 1–5

Fig. 1. Wing buds of an early stage 18 embryo. The arrow points out one of the
subclavian capillaries extending to the capillary plexus of the limb bud. \( A = \) aorta;
\( C = \) capillaries. (Mag. \( \times 35 \).)

Fig. 2. Wing buds of a stage 19 embryo. The arrow points out one of the subclavian
capillaries, which are now much more numerous. Note the region of unvascularized
\( (u) \) mesoderm around the periphery of the limb. \( V = \) vein. (Mag. \( \times 40 \).)

Fig. 3. Stage 20 limb bud. The limb is supplied blood uniformly by the subclavian
capillaries and capillary plexus. Note, however, that a few of the capillaries (as
indicated by the arrows) have begun to broaden. (Mag. \( \times 45 \).)

Fig. 4. Wing buds of a late stage 20 embryo. Most of the subclavian capillaries have
atrophied giving way to the primary subclavian artery (see arrows). (Mag. \( \times 45 \).)

Fig. 5. Points out the primary subclavian artery extending deep into the limb of an
early stage 22 embryo. The veins \( (v) \) have also started to form. (Mag. \( \times 40 \).)
Table 1. Effect of the extremes in oxygen tension on the expression of myogenic and chondrogenic phenotypes

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>95% O₂</th>
<th>95% air</th>
<th>95% N₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myogenic</td>
<td>+</td>
<td>+</td>
<td>- *</td>
</tr>
<tr>
<td>Chondrogenic</td>
<td>- *</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
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Stage 24 limb mesodermal cells were initially seeded at 12.5 x 10⁶ cells per 60 mm Falcon petri dish and 24 to 48 h later were placed in atmospheres of either 95% O₂ and 5% CO₂, 95% air and 5% CO₂, or 95% N₂ and 5% CO₂. On the ninth day after plating the plates were scored for morphologic features indicative of cartilage or muscle.

* Indicates completely absent.
† Indicates morphologically identical to that observed in 95% air.
‡ Indicates nodular organization was normal but mucopolysaccharide deposition was never observed.

essentially no effect on their phenotypic expression. The most reproducible and consistent results were obtained when the cells were exposed to the oxygen tension extremes at 24—48 h after plating as is exemplified in Table 1. In the presence of 95% N₂, myogenic phenotypes were never observed; instead, the cells arranged themselves in a fashion characteristic of chondrogenic cells (Caplan, 1970; Schacter, 1970) and eventually deposited acid mucopolysaccharide. The deposition of acid mucopolysaccharide was most apparent in samples which were exposed to small amounts of O₂. It is clear that in absolutely anaerobic systems, mucopolysaccharide is not deposited. Since normal procedures involved completely replacing the nutrient medium, the cells routinely received brief exposure to oxygen which allowed for some mucopolysaccharide.

**Figures 6-9**

Fig. 6. An early stage 24 right wing bud. The subclavian artery is extending distally in the limb to form the brachial artery. The marginal vein (v) continues to develop around the limb. C = capillaries. (Mag. x 65.)

Fig. 7. The right wing bud of a stage 26 embryo. The brachial artery extends deep into the limb and the marginal vein is well developed. Note that the unvascularized mesoderm (u) at the periphery of the limb still persists. (Mag. x 43.)

Fig. 8. A camera-lucida drawing of a stage 24 right wing bud illustrating the flow of blood into a central artery to a peripheral capillary and finally draining into a marginal vein.

Fig. 9. A schematic representation of the development of the vascular pattern in the limb.
deposition; in the few cases where attempts were made to completely eliminate oxygen, mucopolysaccharide deposition was coordinately eliminated although the cells were observed to group in a fashion characteristic of cartilage secreting cells (i.e. grouped into a cartilage secreting nodule). In a series of studies in which the oxygen tension was varied from 0 to 35 %, the ideal system for both phenotypic expression and deposition of copious amounts of mucopolysaccharide employed 5 % O₂. This is in agreement with Nevo, Horwitz & Dorfmann (1972) who report that in vitro mucopolysaccharide biosynthesis is maximal in 5 % oxygen. Multinucleate, cross-striated cells could be seen in those systems exposed to 95 % oxygen, while the characteristic grouping of chondrogenic cells or cells producing extracellular matrix material was completely absent.

Thus extremes of oxygen tension effectively eliminated one or the other of the prominent limb phenotypes. High oxygen tension allowed myogenic expression while low oxygen tension allowed chondrogenic expression.

**DISCUSSION**

These studies clearly establish the temporal landmarks involved in the differential vascularization of the developing chick limb: (A) prior to stage 20, blood is distributed uniformly in the wing bud by the capillary network; (B) from stage 20 to late stage 21 or early stage 22, a central artery is established in each wing; (C) concurrent with the formation of the central artery is the establishment of differential vascularization in the limb, i.e. the prospective chondrogenic areas of the limb become less vascularized than the prospective myogenic areas; (D) the establishment of the major venous vessels begins at early stage 22; and (E) by stage 24 the vascular pattern is essentially established with a centrally located artery, peripherally located capillaries, marginally located veins, and an avascular core. Furthermore, predictions based on this pattern have been tested in vitro where stage 24 limb mesodermal cells exposed to extremes of oxygen tension showed that high oxygen tension potentiates myogenic expression while low oxygen tension potentiates chondrogenic expression.

Relative to the timing and sequence involved in the cytodifferentiation of muscle and cartilage other workers have shown that before reaching stage 25 (Hamburger & Hamilton, 1951), the mesodermal cells of the developing chick limb seem to be able to differentiate into either chondrogenic or myogenic phenotypes (Zwilling, 1968; Searls & Janners, 1969). At stage 25, the developmental fate of these cells becomes established with the prospective chondrogenic cells located at a central or core position in the limb and the prospective myogenic cells occupying a more peripheral position. This process of differential cell expression seems to be initiated at stage 22, as marked by the protraction of the average cell cycle (Janners & Searls, 1970) and by the increased fixation of ³⁵S by the core cells, and finalized by stage 25, when 75 % of the core cells have
effectively withdrawn from the cell cycle and the core cells seem to fix $^{35}$S primarily into acid mucopolysaccharide (Searls, 1965a). It is also at stage 25 when the limb mesodermal cells fail to exhibit ‘morphogenic properties’ as defined by Zwilling (1968) as evidenced by their inability to respond to apical ectodermal ridge in the bioassay system which employs the reassociation of limb mesoderm with isolated ectodermal jackets (Finch & Zwilling, 1971).

Prior to this study of the development of the vascular pattern in the limb bud, other studies reported similar findings (Evans, 1909a, b; Sabin, 1905; Romanoff, 1960; Bakst & Chafee, 1928). Evans (1909a) reported that numerous capillaries grow laterally at irregular points from the aorta. In the wing bud these capillaries give rise to the subclavian artery; in the leg bud they give rise to the femoral artery. The subclavian capillaries anastomose with part of the capillary plexus that condenses to form the umbilical vein to establish the circulation of the wing bud. Thus, at the stage of 32 somites (stage 17), blood flows into the limb through the primary subclavian plexus and is drained by the umbilical vein. Those capillaries that are not at segmental points atrophy, and the remaining vessels give rise to the multiple segmental subclavians at about the stage of 34–35 somites (stage 18). The multiple segmental subclavians then give way to the establishment of the primary subclavian artery from that pair of segmental subclavians at the 18th segment before the stage of 45 somites (stage 22). The secondary subclavian artery first appears at some time between the 3rd and 6th day of development (stage 18–28) (Sabin, 1905; Ramanoff, 1960; Bakst & Chafee, 1928). Both subclavian arteries supply blood to the wing until the primary subclavian atrophies on the 8th day of development (stage 33) (Sabin, 1905; Bakst & Chafee, 1928). The marginal vein has been reported to first appear at stage 24 (Romanoff, 1960). Our studies, however, indicate that it first appears at early stage 22. This observation is supported by the recent work of Seichert & Rychter (1971) on the establishment of the marginal plexus of the developing chick limb.

Several studies have also alluded to the differential vascularization of the early limb bud. Evans (1909b) reported that though the limb is at first uniformly supplied blood by capillaries, avascular areas corresponding to cartilage or muscle groups develop at some later time. Saunders (1948) reported that the central core of the wing is delimited by blood vessels at the stage which he designated as stage 8 [Hamburger & Hamilton’s (1951) early stage 22]. In more recent studies blood vessels have been used to mark the boundaries between prospective cartilage and prospective muscle (Gould, Day & Wolpert, 1972).

The temporal events involved in the establishment of differential vascularization in the limb have not, however, been reported previous to the present study especially with regard to muscle and cartilage differentiation. The data reported here indicate that the initiation of the process of differential vascularization is concurrent with the establishment of the primary subclavian artery (stage 20 to late stage 21).
Our interest in the establishment of the vascular pattern arose from predictions based upon the results of studies investigating the role of nicotinamide in mesodermal cell expression (Landauer, 1957; Landauer & Clark, 1962; Tanaka, Yamamoto & Hayashi, 1967; Caplan, Zwilling & Kaplan, 1968; Caplan, 1970, 1971, 1972a–d). The hypothesis has been presented that shifts in concentrations of essential and low molecular weight compounds, especially nicotinamide, DPN, TPN (i.e. NAD, NADP), and oxygen, play a central or controlling role in chick limb mesodermal cell expression (Caplan, 1970, 1972d). One possible mechanism of establishing metabolic gradients which control or influence phenotypic expression is differential vascularization of the chondrogenic and myogenic areas in the limb bud (Caplan, 1972b).

The present study has shown that the establishment of the differential vascular pattern is concurrent with the establishment of the primary subclavian artery (stage 20 to late stage 21). Thus the differential vascularization of prospective cartilage- and muscle-forming areas immediately precedes the differential uptake of $^{35}$S in the limb as reported by Searls (1965a) and the protraction of the average cell cycle. Furthermore, the process of differential vascularization slightly precedes the condensation of prospective muscle cells in the proximal region of the limb (Gould et al. 1972). This supports the hypothesis that differential vascularization, or its consequences, is a factor in controlling the initial stages of differential cell expression in the developing chick limb. In this regard, Ede (1971) has suggested that the connection between low oxygen tension and chondrogenesis as observed by Pawelek (1969) may be an important factor in determining the site and boundary of cartilage regions in the developing limb.

The model presented here is also consistent with the analysis of positional information put forth by Wolpert (1969) and with the phase-shift model of Goodwin & Cohen (1969). Wolpert (1969) has pointed out that those processes that control spatial differentiation, or the process by which a population of cells within a defined spatial pattern undergo molecular differentiation, probably do not rest in the process of molecular differentiation. A gradient of metabolic potential like that proposed in the present study could provide a coordinate system to relate the positional information necessary for spatial differentiation. The metabolic potential of a population of cells could also affect the periodic activities of the cells, such as the cell cycle, producing ‘phase-differences of periodic events’ and thus altering the ‘cell state’ and providing positional and temporal information to the cells according to the phase-shift model of Goodwin & Cohen (1969).

In regard to the observations and interpretations presented above, the report that the vascular pattern does not play a vital role in limb development (Searls, 1968) deserves comment. Searls (1968) used an organ culture system for limb buds in which the limbs of stage 18–24 embryos were placed on a Gelman filter on the chorioallantoic membrane of 8–10 day embryos. This culture was assumed to be avascular. Many of the limbs that were placed under these con-
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Fig. 10 schematically summarizes the role of the vascular pattern in the spatial differentiation of cartilage and muscle in the developing chick limb. It is pro-

ditions developed distorted digits. Well developed cartilage was evident in the cultures after 7 days; muscle, however, developed poorly, and cross-striations did not appear. These results were interpreted as indicating that cellular differentiation in the limb bud is independent of the vascular pattern; however, these results are also consistent with the hypothesis that differential vascularization influences mesodermal cell expression. Searls’ (1968) interpretation was based upon the assumptions that (1) the filter prevented cellular contact between the cultured tissue and the chorioallantoic membrane, (2) the cultured tissue was avascular, and (3) the flow of nutrients, metabolic products, and gases was independent of any directing influences by either the host or the limb tissue itself. The validity of these three assumptions has not been demonstrated. For the purposes of this discussion, the important assumption is the third. The present study indicates that the nutrient flow in the limbs is established very early in their development; thus in these cultures the pre-existing vascular pattern of the limbs themselves may have provided a directing influence on the nutrient distribution. If, for the sake of argument, we assume that these organ cultures were essentially avascular, we would predict a low oxygen and nutrient supply to the mesodermal cells in Searls’ organ culture. Under these conditions, we would expect good cartilage differentiation but poor muscle development. This is, indeed, what Searls observed. Thus, depriving mesodermal cells of oxygen and other nutrients could be expected to enhance or at least permit chondrogenic expression while being detrimental to myogenic expression. We believe that Searls’ observations can be used to argue for a controlling role of the vascular pattern (or nutrient supply) in limb development.

Fig. 10 schematically summarizes the role of the vascular pattern in the spatial differentiation of cartilage and muscle in the developing chick limb. It is pro-
posed that the developing vascular system establishes a nutrient gradient and thus a metabolic gradient radially across the limb from core to periphery. This gradient enhances chondrogenic expression while being detrimental to myogenic expression at the core of the limb. At a position of maximum nutrient supply in the peripheral zones, myogenic expression is enhanced while chondrogenic expression is prevented. Studies to further test these suggestions are in progress.

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