Hiroshima Journal of Medical Sciences
Vol. 32, No. 4, 485～500, December, 1983
HIJM 32-72

Vascular Pattern and Limb Development

1. Normal development of the hindlimb vasculature in the mouse and its aberrations induced by 5-fluorouracil*)

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(Received September 19, 1983)

Key words: Limb, Malformation, 5-fluorouracil, Vasculature

ABSTRACT

The role of the vascular system in normal and abnormal limb morphogenesis is still a controversial subject. The present study was designed to examine the normal development of the mouse hindlimb vasculature, including the arterial pattern of the adult mouse, and the relationship between the time of teratogen administration and the resulting alteration in vascular and skeletal patterns. Polydactylies were induced in developing mouse hindlimbs by a single intraperitoneal injection of 5-fluorouracil (5-FU) in a dose of 30 mg/kg of maternal body weight at day 10.5 of pregnancy (vaginal plug=day 0; Theiler's stage T. S. 18), and reductive malformations were induced in developing mouse hindlimb in same way at day 11.5 of pregnancy (T. S. 20). For observation of gross vascular patterns, India ink was injected into the vitelline vein of embryos and fetuses at various stages between day 11.0 (T. S. 19) and day 15.5 (T. S. late 24) of pregnancy.

In normal development, the main vessels of the leg are formed by day 13.5 (T. S. early 23) of pregnancy and the vessels of the toes become evident by day 15.5. In the polydactylous limbs resulting from treatment with 5-FU at day 10.5, vascular changes were observed mainly in the foot and not in the leg. In the limbs with reductive malformations induced by treatment at day 11.5, aberrant vascular patterns were noted in the leg as well as in the foot.

In man, many cases of arterial anomalies in the malformed upper limbs have been described and interpreted as indicator of the time of teratogenic event by a few investigators. They assumed that the aberrant vessel had been just forming at the time of teratogenic event. However, the results of the present experiment clearly demonstrated that there were some gaps between the time estimated from the altered arterial pattern and the real time of teratogen administration. It is concluded that abnormal arterial pattern in a malformed limb does not directly indicate the time of teratogenic event, but reflects an abnormal patterning of mesenchymal condensations.

INTRODUCTION

Various processes in the pathogenesis of congenital limb malformations have been suggested (Yasuda and Ueba[32], 1980). Aberrant vasculogenesis is one of the suggested pathogenetic events leading to abnormal skeletogenesis in the limb (Fraser[12], 1982). It has been postulated that the developing vascular system in the limb bud may have a role in determining the concurrent differentiation of the skeletal system (Seichert and Rychter[24], 1972). Caplan and Koutroupas[4] (1973) have hypothesized from both in vivo and in vitro observations on chick limb buds that the developing vasculature may have a biochemical influence on skeletal devel-

*) 片桐則明：先天性奇形肢の血管系に関する臨床的実験的研究　1. マウス実験モデルにおける血管系の発達とその異常について

opment. Hence, it is reasonable to assume that abnormal vasculogenesis is a pathogenetic process causing limb malformations.

In the human upper limb, developmental changes in the arterial pattern have been well documented (Senior[26], 1926; Singer[27], 1933). Arterial anomalies observed in malformed upper limbs have been described and interpreted as an indicator of the time of teratogenic event (Sudo[28], 1979; Inoue[16], 1981). They concluded that malformations belonging to the categories of failure of formation of parts, failure of differentiation of parts and duplication in Swanson's classification (Swanson[29], 1976) resulted from injurious events around the embryonic age of 48 days postovulation. However, their hypotheses based on clinical observations were short of experimental examinations.

Several experimental teratological studies have indicated vascular abnormalities in the early stages of limb teratogenesis. Jurand[19] (1966) showed that thalidomide administration caused dilatation of vessels in chick limb buds and suggested that this primary effect resulted in skeletal malformations. Fraser[12] (1982) described that the blood vessels in limbs from retinoic acid-treated hamster fetuses were found scattered in the limb bud mesenchyme in areas where chondrogenic skeletal condensations normally appeared, and hypothesized that these abnormally placed blood vessels could physically interfere with the process of mesenchymal condensation and contribute to dysmorphogenesis of the skeletal anlagen. However, the relationship between the time of teratogen administration and the resulting abnormal vascular pattern has not been well documented. For the analysis of this relationship, descriptions of normal vascular development in experimental animals are indispensable.

The main purpose of the present study is to analyze the relationship between the time of teratogen administration and the resulting alteration in vascular and skeletal patterns. As bases of pursuing this purpose, the normal development of mouse hindlimb vasculature and the arterial pattern of the adult mouse hindlimb are described. Five-fluorouracil (5-FU) was used as the teratogen, because pathogenesis of limb malformations induced with 5-FU has been extensively studied in our laboratory.

MATERIALS AND METHODS

Jcl:ICR mice, obtained from Japan CLEA Co., Ltd., were used. Mature females were placed with males of the same colony overnight. Copulation was ascertained by the presence of a vaginal plug on the following morning, and 0 : 00 a. m. of that day was denoted as day 0 of pregnancy. The mice were killed by cervical dislocation at various stages of pregnancy between day 11. 0 and day 15. 5 of pregnancy. Fetuses with membranes intact were dissected from the uterus and immersed in Hanks' balanced salt solution adjusted to pH 7. 2. For observation of gross vascular pattern, India ink (Rotring Art 591017 Black, diluted ten times with Hanks' solution and filtered once through No. 2 filter paper made by Toyo Roshi, Co., Ltd.) was injected into the vitelline vein with a peristaltic pump (LKB-produkter AB).

After injection of ink, the fetuses were freed of membranes, staged according to Theiler[30] (1972; Thiler's stage=T. S.), fixed in 10% formalin, cleared in methylsalicylate, and the hindlimbs were observed under a dissecting microscope. For observation of the adult vascular pattern, 2 month old mice were anesthesized with nembutal, the thoracic region was opened and the aorta thoracica was cannulated with catheter (diameter 1. 5 mm). The mice were irrigated first with physiological saline and then with 2. 5% glutaraldehyde in Hanks' solution for fixation, and injected with a mixture of Mercox CL and MA (DAINIHON INK industrial Co., Ltd). After curing the mixture, the hindlimbs of the mice were amputated at the hip joint level. The hindlimb was immersed in 1%-0. 5% KOH for several days and rinsed in water. By treatment with this concentration of KOH, the soft tissue became transparent and remained in place without complete corrosion. The cast of the vessels was observed under a dissecting microscope. Nomenclature of rat limb arteries described by Greene[13] (1963) was utilized.

For induction of limb malformations in fetuses, some dams received a single intraperitoneal (ip) injection of 5-FU (supplied by Nakarai Chemicals Ltd.) in physiological saline at a dose level of 30 mg/kg maternal body weight at day 10. 5 or day 11. 5 of pregnancy. Controls received saline alone. Fetuses were harvested at

various intervals after teratogen injection, and their gross vascular pattern was observed with the India ink injection method. For observation of gross malformations, hindlimbs of fetuses of day 15.5 were examined under a dissecting microscope. For observation of cartilage elements of the limb the specimens were fixed in 95% ethanol, stained in 70% ethanol containing 0.015% alcian blue and 5% acetic acid at 37°C for 2-3 days, cleared through 1% aqueous KOH, and stored in glycerine (modified after Inouye[17], 1976). For histological observation, the dissected hindlimbs were fixed in Bouin's fluid, embedded in paraffin, and serially sectioned perpendicular to the long axis of the limb at thickness of 7 μm. The sections were stained with hematoxylin and eosin, and observed under a light microscope.

RESULTS

I. The normal vascular development in the mouse hindlimb

a) Day 11.0 (T. S. 19)

The hindlimb bud at this stage is a fin-like projection, consisting of morphologically uniform mesenchyme covered with ectoderm. A foot plate has not yet been formed (Fig. 1a). A single axial artery traverses the mesenchymal core proximo-distally and breaks up into a capillary plexus connected to the marginal venous sinus. The axial artery is the primordial arteria (a.) ischiadica (Fig. 1b). The capillary plexus appears uniformly distributed throughout the mesenchyme.

b) Day 11.5 (T. S. 20)

By this stage the distal part of the hindlimb bud has widened and formed a foot plate (Fig. 2a). Mesenchymal condensations appear in the proximal core of the limb. In the foot plate the mesenchyme is still uniform. The axial artery, or the a. ischiadica, runs on the medio-posterior aspect of the proximal skeletal primordia. Near the junction of the proximal part and the foot plate, the artery divides into a dorsal capillary plexus and a plantar plexus in the foot plate (Fig. 2b). These plexuses have no anastomosis between them. A recurrent branch develops from the dorsal aspect of the distal end of the a. ischiadica. This branch will be the ramus (r.) perforans cruris.

From the lateral aspect of the a. umbilicalis proximal to the origin of the a. ischiadica, a new vessel, the a. iliaca externa, arises. The place of origin of the a. iliaca externa marks the permanent subdivision of the dorsal root of the a. umbilicalis into two parts. The proximal part becomes the adult a. iliaca communis, while the distal part represents the a. hypogastrica and a short proximal section of its umbilical branch. At this stage the marginal vein becomes most distinct.

c) Day 12.5 (T. S. late 21)

By this stage digital rays have appeared in the foot plate. The dorsal and plantar plexuses in the foot plate become denser than in the preceding stage (Fig. 3a). In specimens at T. S. early 21, there are several connections between the dorsal and platar plexuses. In specimens of the standard developmental features, a single vessel of a large size connects the dorsal and plantar plexuses. This connection is the r. perforans tarsi (Fig. 3b). The a. iliaca externa has bifurcated into the a. epigastrica inferior and the a. femoralis. The a. femoralis is united with the a. ischiadica at the distal end of the femur and then bifurcates into the a. saphena and the a. poplitea. The union between the a. femoralis and the a. ischiadica is the r. communicans superius. The a. poplitea has two branches which run longitudinally through the posterior crural region. These branches will develop into the a. surale superficialis and a. tibialis posterior.

d) Day 13.5 (T. S. early 23)

By this stage distinct interdigital notches have appeared in the foot plate (Fig. 4a). The presence of the knee is now identifiable. From the time of formation of the r. communicans superius, the caliber of the a. femoralis gradually exceeds that of the a. ischiadica, which becomes slender. At this stage, a. tibialis anterior springs from the a. poplitea and the a. tibialis anterior has one branch which passes to the dorsal surface of the leg (Fig. 4b). This branch will be the a. peronea. The part of the a. saphena which enters the sole becomes the a. plantaris medialis. The a. plantaris medialis divides into four aa. metatarseae plantares. The aa. digitales propriae are not definite arteries but are still networks of several small vessels.

e) Day 14.5 (T. S. late 23)

The toes elongate and the interdigital notches deepen as compared with the previous stage (Fig. 5a). The ankle is discernible.

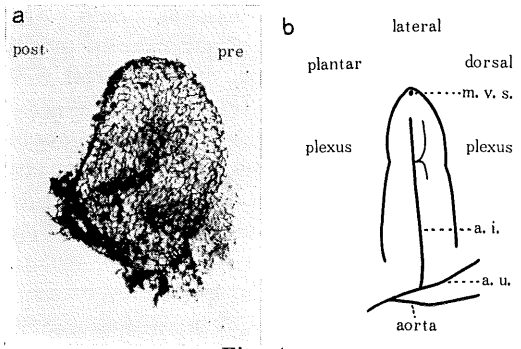


Fig. 1

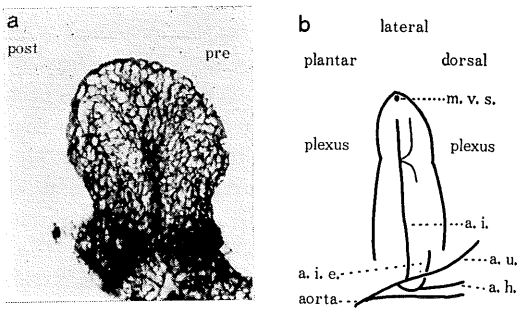
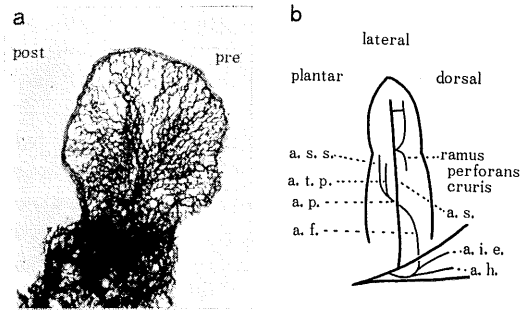


Fig. 2



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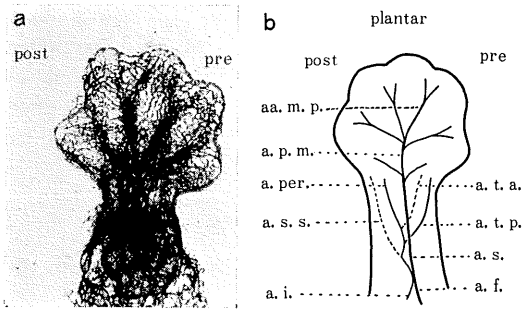

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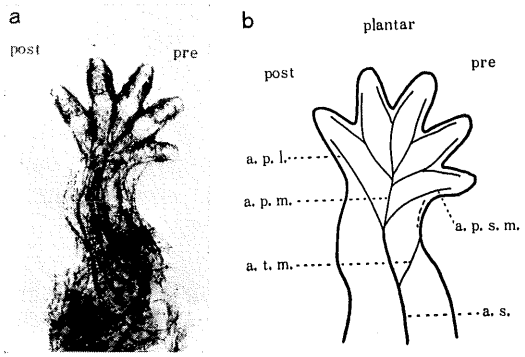

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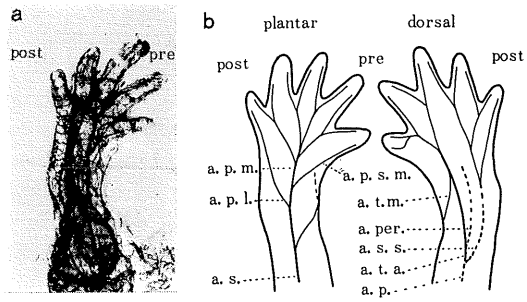

Fig. 6

Figs. 1-6. Normal vascular development in the mouse hindlimb.

Abbreviations (Figs. 1-6, 19)

a. u.	:	a. umbilicalis
a. f.	:	a. femoralis
a. i.	:	a. ischiadica
a. i. e.	:	a. iliaca externa
a. h.	:	a. hypogastrica
a. p.	:	a. poplitea
a. s.	:	a. saphena
a. t. p.	:	a. tibialis posterior
a. s. s.	:	a. suralis superficialis
a. t. a.	:	a. tibialis anterior
a. per.	:	a. peronea
a. p. m.	:	a. plantaris medialis
aa. m. p.	:	aa. metatarseae plantares
a. p. s. m.	:	a. plantaris superficia medialis
a. t. m.	:	a. tarsea medialis
a. p. l.	:	a. plantaris lateralis
m. v. s.	:	marginal venous sinus
pre	:	preaxial
post	:	postaxial

Fig. 1. a : A cleared specimen of day 11.0
b : A diagram of its lateral aspect

Fig. 2. a : A cleared specimen of day 11.5
b : A diagram of its lateral aspect

Fig. 3. a : A cleared specimen of day 12.5
b : A diagram of its lateral aspect

Fig. 4. a : A cleared specimen of day 13.5
b : A diagram of its plantar aspect

Fig. 5. a : A cleared specimen of day 14.5
b : A diagram of its plantar aspect

Fig. 6. a : A cleared specimen of day 15.5
b : Diagrams of its plantar and dorsal aspects

By this stage a part of the a. ischiadica close to the r. communicans superius has disappeared, and the a. femoralis alone conveys blood to the region beyond the knee. The a. femoralis passes a more direct course than before. The a. saphena has four branches (Fig. 5b), one of those branches runs towards the dorsal region of the foot and is called the a. tarsea medialis. The other branches enter the sole and become the a. plantaris superficia medialis, the a. plantaris medialis, and the a. plantaris lateralis. The a. plantaris medialis gives off four aa. metatarseae plantares, and each of these bifurcates into tibial and fibular branches. These branches become the aa. digitales propriae. At this stage, some of the aa. digitales are still not so distinct.

f) Day 15.5 (T. S. late 24)

The separation of toes is most conspicuous at this stage (Fig. 6a).

The main vascular pattern in the hindlimb is completed during the period between day 14.5 and day 15.5 of pregnancy and closely resembles that of the 2 month old mouse (compare Fig. 6b and Figs. 7, 8).

In the present observation, some differences in vascular development among litters were noted. However, the completed vascular pattern was rather constant.

As compared with man, the mouse showed less variations in the vascular pattern. To interpret this difference, one should note the following difference in the vascular development of the hindlimb. In man, a part of the a. interossea extending from the termination of the a. poplitea profunda to the distal end of the r. communicans inferius disappears between Carnegie stage (C. S.) 19 (about 45 days postovulation) and C. S. 21 (about 48 days) (Senior[25], 1919). In mice, the a. saphena which arises from the a. femoralis directly continues to the four branches in the leg and reaches the foot without involution. Various degrees of involution of the a. interossea result in varieties of the vascular patterns in human legs.

II. Arteries of the hindlimb of the 2 month old mouse

Results of observations of Mercox casts are schematically illustrated in Figs. 7–8. The vascular pattern of the mouse closely resembles that of the rat. Hence the descriptions given below mostly follow those in the rat given by Greene[13] (1963).

The a. femoralis is the continuation of the a. iliaca externa down the medial side of the thigh. It gives off the following branches.

1. A. genu suprema. It leaves the a. femoralis just above the knee and appears at a deep level running downward along the shaft of the femur. Around the knee it gives off several branches, which anastomose with other genicular vessels.

2. A. saphena. It is the most superficial and the most extensive branch of the a. femoralis. It leaves that vessel just below the a. epigastrica superficialis as the a. femoralis takes a deeper course to the popliteal space. It extends along the crest and medial surface of the tibia, and communicates with the superficial plexus of the knee formed by the genicular arteries around the patella.

The a. saphena divides into four terminal branches, one of which runs anterior to the leg to the extensor surface of the foot as the a. tarsea medialis. The other three pass behind the medial malleolus as the a. plantaris lateralis, and the r. superficialis and the r. profundus of the a. plantaris medialis. The r. communicans (communicating branch) running between the musculus tibialis posticus and the tibia described by Greene in the rat is not conspicuous in the mouse.

3. A. poplitea. It is very short and a continuation of the a. femoralis from the point where it enters the popliteal fossa just above the medial epicondyle of the femur to its division into the a. tibialis anterior and the a. tibialis posterior. Several genicular branches arise from the a. poplitea.

The a. tibialis anterior runs deep to the popliteal region, coming to lie on the lateral surface of the interosseous membrane.

The a. peronea leaves the a. tibialis anterior slightly distal to the a. recurrens tibialis anterior. Turning toward the fibula it gives off the r. fibularis which runs between peroneus longus and brevis, to the peroneus digiti quarti and quinti and soleus muscles. The a. peronea becomes quite seperficial above the lateral malleolus and passes behind the lateral malleolus to be distributed to the lateral surface of the heel and foot.

Beyond the origin of the a. peronea, the a. tibialis anterior runs along the lateral surface

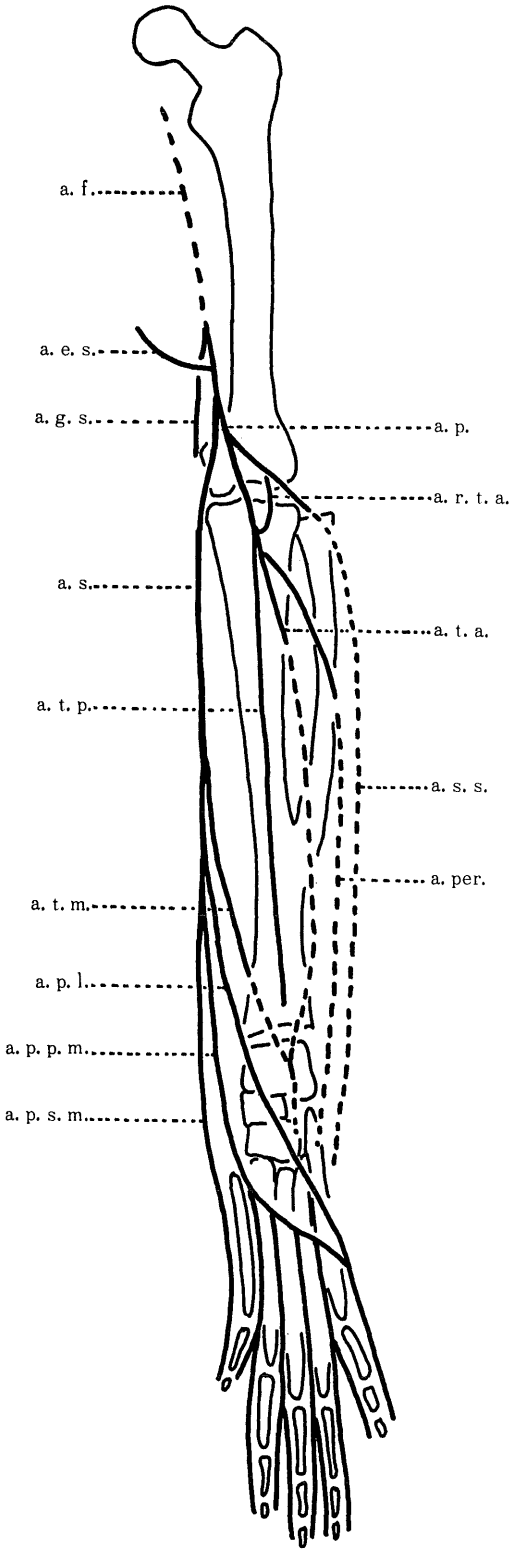

Fig. 7

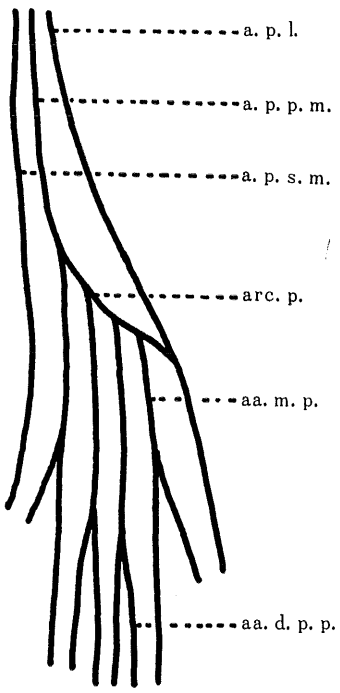

Fig. 8

Fig. 7 and 8. Diagrams of the arteries of hind-limb

Abbreviations

a. f.	: a. femoralis
a. s.	: a. saphena
a. g. s.	: a. genu superior
a. e. s.	: a. epigastrica superficialis
a. p.	: a. poplitea
a. per.	: a. peronea
a. r. t. a.	: a. recurrens tibialis anterior
a. t. a.	: a. tibialis anterior
a. t. p.	: a. tibialis posterior
a. s. s.	: a. suralis superficialis
a. t. m.	: a. tarsea medialis
a. p. l.	: a. plantaris lateralis
a. p. s. m.	: a. plantaris superficia medialis
a. p. p. m.	: a. plantaris profunda medialis
arc. p.	: arcus plantaris
aa. d. p. p.	: aa. digitales plantares propriae
aa. m. p.	: aa. metatarseae plantares

Fig. 7. Posterior view

Fig. 8. Plantar view

of the shaft of the tibia on the interosseous membrane.

The a. dorsalis pedis is a continuation of the a. tibialis anterior and begins where the latter passes under the annular ligament to the tarsal region. At a short distance below this level it is joined by an arcuate branch from the a. tarsea medialis, one of the terminal branches of the a. saphena.

The rr. superficialis and profundus of the a. plantaris medialis are continuations of the a. saphena. They pass behind the medial malleolus to reach the plantar surface of the foot. The r. superficialis runs along the medial border of the foot and supplies an a. digitalis plantaris to the medial side of the first digit, several superficial branches to the skin, and anastomotic vessels to the r. profundus of the a. plantaris medialis. The r. profundus of the a. plantaris medialis extends distally parallel with and medial to flexor digitorum brevis. Where this muscle breaks up into tendons, the artery turns laterally and unites with the lateral plantar artery to form the arcus plantaris from which the aa. metatarseae plantares arise.

In addition to the aa. metatarseae plantares, several small twigs are given off from the arcus to anastomose with the superficial cutaneous branches of the lateral and medial plantar vessels. Five aa. metatarseae plantares spring from the arcus plantaris. The first-fourth divide in their four respective interdigital spaces, send an anterior perforating branch to the corresponding a. metatarsea dorsalis, and then divide into the aa. digitales plantares propriae to supply the adjacent side of the digits. The a. metatarsea plantaris V supplies only the lateral side of the fifth digit. The medial side of the first digit is supplied by the r. superficialis of the a. plantaris medialis.

III. Gross observation of hindlimbs treated with 5-FU

1. The group treated at day 10.5 of pregnancy

Table 1 shows findings in individual litters at day 15.5.

The survival rate of fetuses treated with 5-FU was slightly lower than that of controls. Nevertheless, a sufficient number of surviving fetuses

Table 1. Survival and gross hindlimb malformations of day 15.5 fetuses from dams treated with 5-FU at day 10.5

No. of litters	1	2	3	4	5	6	7	8	9	10	Total (%)
No. of implantations	13	15	12	10	9	12	13	13	16	12	125
No. of live fetuses	12	11	10	7	8	10	9	10	12	10	99
Survival rate (%)	92	73	83	70	89	83	69	77	75	83	79
Preaxial polydactyly	12	10	10	8	8	13	9	10	10	8	98(49.5)
Axial polydactyly	9	9	8	4	6	6	6	5	8	8	69(34.9)
Others	2	0	2	0	1	0	2	3	4	2	16(8.0)
Normal	1	3	0	2	1	1	1	2	2	2	15(7.6)
Total	24	22	20	14	16	20	18	20	24	20	198(100)

Table 2. Survival and gross hindlimb malformations of day 15.5 fetuses from dams treated with 5-FU at day 11.5

No. of litters	1	2	3	4	5	6	7	8	9	10	Total(%)
No. of implantations	16	11	13	14	13	13	12	9	13	10	124
No. of live fetuses	13	7	9	10	10	8	10	7	10	9	93
Survival rate (%)	81	64	69	71	77	61	83	78	77	90	82
Cleft foot	14	8	10	8	12	10	10	8	6	10	96(51.1)
Intercalary defect	5	4	9	8	4	4	6	2	6	4	49(26.4)
Two digital ray defect	5	2	0	0	2	2	4	4	8	4	31(16.7)
Others	2	0	2	4	2	0	0	0	0	0	10 (5.8)
Total	26	14	18	20	20	16	20	14	20	18	186(100)

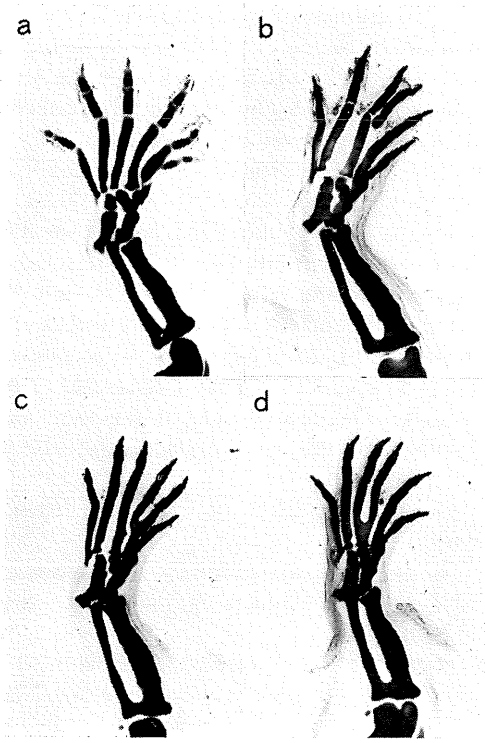

Fig. 9. Cartilage staining of the day 10.5 treated hindlimbs at day 15.5
a : preaxial polydactyly
b , c , d : axial polydactyly

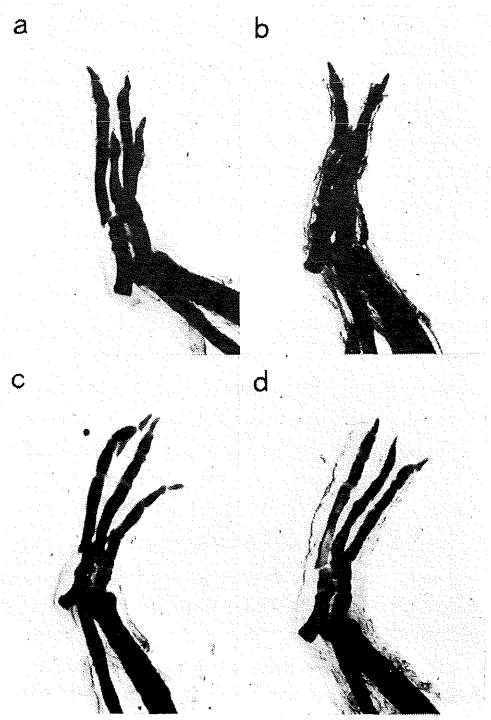

Fig. 10. Cartilage staining of the day 11.5 treated hindlimbs at day 15.5
a , b : cleft foot type
c : intercalary defect type
d : two digital ray defect type

were obtained for gross observation. There were some differences in mortality among litters.

Limb malformations were produced in all of the four limbs. While forelimbs showed reductive malformations, hindlimbs exhibited polydactyly almost exclusively. In total polydactyly was induced in 84.4% of the observed hindlimbs. Preaxial polydactyly (Fig. 9a) was observed more frequently than axial polydactyly (Fig. 9b, c, d). Both types of polydactyly occurred in each litter.

2. The group treated at day 11.5 of pregnancy

The findings at day 15.5 are summarized in Table 2.

The survival rate of fetuses was almost similar to that in the group treated at day 10.5.

Reductive malformations were observed in most hindlimbs as well as in forelimbs. The reductive malformations were subdivided into three major categories: cleft foot, intercalary defect, and two digital ray defect. The cleft foot (Fig. 10a and b) is characterized by the absence of a few toes in the axial rays. This type was most frequently seen. The intercalary defect (Fig. 10c) is characterized by the absence of the middle and proximal phalanges and/or metatarsal bones with the remaining distal phalanges. This type occurred in about a quarter of the hindlimbs. The two digital ray defect (Fig. 10d) literally has two missing digital rays with remaining three rays. The distribution of types of foot malformation was not similar among litters.

IV. The vascular development in hindlimbs treated with 5-FU

About 30 limbs were observed at each stage between day 11.0 and day 14.5. More than 60 limbs were examined at day 15.5, when the major vascular pattern was established.

1. The group treated at day 10.5 of pregnancy

At 12 hours after maternal 5-FU administration, i.e. at day 11.0, the hindlimb bud had no mesenchymal condensations. Blood vessels in the 5-FU treated limbs showed great varia-

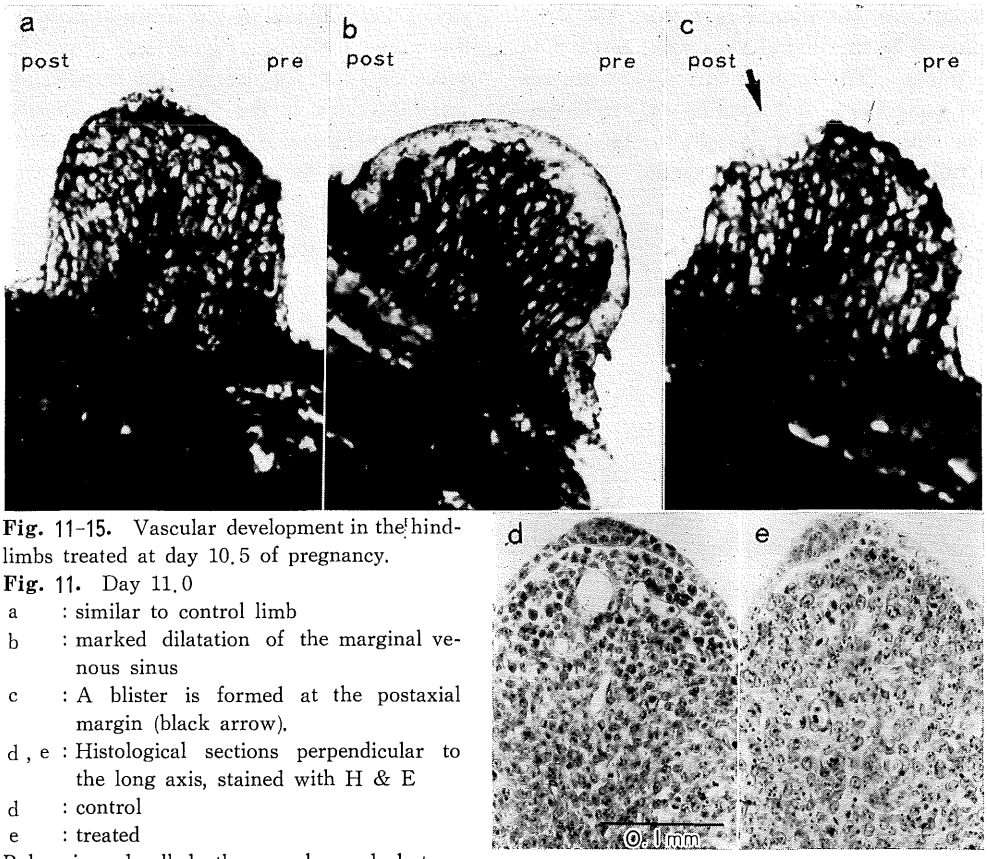

Fig. 11-15. Vascular development in the hind-limbs treated at day 10.5 of pregnancy.
Fig. 11. Day 11.0
a : similar to control limb
b : marked dilatation of the marginal venous sinus
c : A blister is formed at the postaxial margin (black arrow).
d, e : Histological sections perpendicular to the long axis, stained with H & E
d : control
e : treated
Pyknosis and cell death are observed, but no vascular damage is detected in e.

tions. In some limbs a single axial artery traversed the mesenchyme and broke up into a capillary plexus connected to the marginal sinus as in control limbs (compare Fig. 1 and Fig. 11a). An indistinct axial artery could sometimes be seen with marked dilatation of the marginal venous sinus (Fig. 11b). In some cases, a blister was formed at the postaxial margin of the limb bud (Fig. 11c). In histological sections a large number of dead cells were observed in the distal region of the limb bud mesenchyme (Fig.11e). However, numerous capillaries without apparent damages were observed even in the areas with abundant necrotic cells. The density and distribuiotn of capillaries were not much different from controls (Fig. 11d, e).

At day 11.5, most of the 5-FU treated limbs showed a vascular pattern similar to controls (Fig. 2) with a single axial artery giving origin to a capillary plexus. The vascular network was uniform in the distal part of the limb (Fig. 12a). In some cases the marginal sinus was indistinct, and capillaries to the limb margin were not numerous as in controls (compare Fig. 2 and Fig. 12b). In a small portion of the limbs, an indistinct axial artery could be seen with a postaxial blister as in the preceding stage (Figs. 11c and 12c).

At day 12.5, the vascular patterns in the foot plate could be divided into two groups. One group was characterized by an abnormally hypertrophic apical ectodermal ridge (AER) extending in the preaxial region (Fig. 13a, b). This change is an early indication of preaxial polydactyly. These cases had a uniformly distributing peripheral vascular network and a dilated marginal venous sinus. The vascular pattern resembled that of controls at younger stages (compare Fig. 2 and Fig. 13a, b). Both the axial artery and the digital mesenchymal condensations were not distinct. The other group was featured by excessive digital rays in the axial region. The capillaries were being

eliminated from the digital rays (Fig. 13c).

At day 13.5, the digital rays were distinct in all specimens. The future main vessels became clear as collections and fusions of capillaries. In cases with preaxial polydactyly, the vessels of the tibial side of the first digital ray were richer than those in the normal hindlimb at this stage (compare Fig. 4 and Fig. 14a). In cases of axial polydactyly, the third digital ray was bifurcated in the distal part. Occasionally the a. metatarsea plantaris between the duplicated digits was indistinct (Fig. 14b).

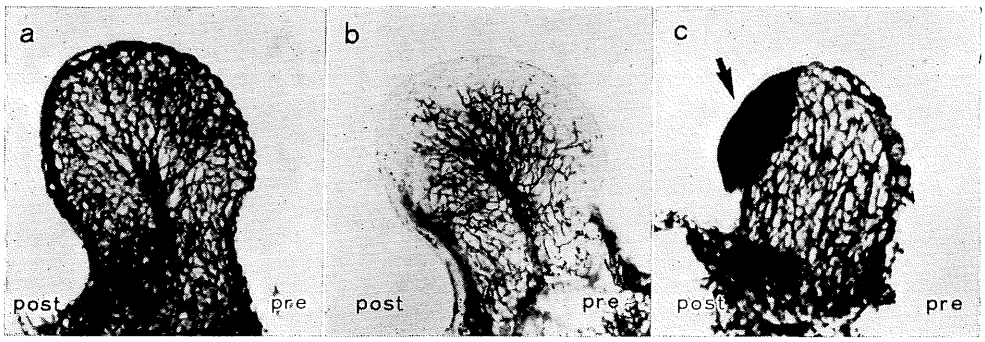

Fig. 12. Day 11.5
a : similar to control limb
b : Marginal sinus is indistinct.
c : a postaxial blister (black arrow)

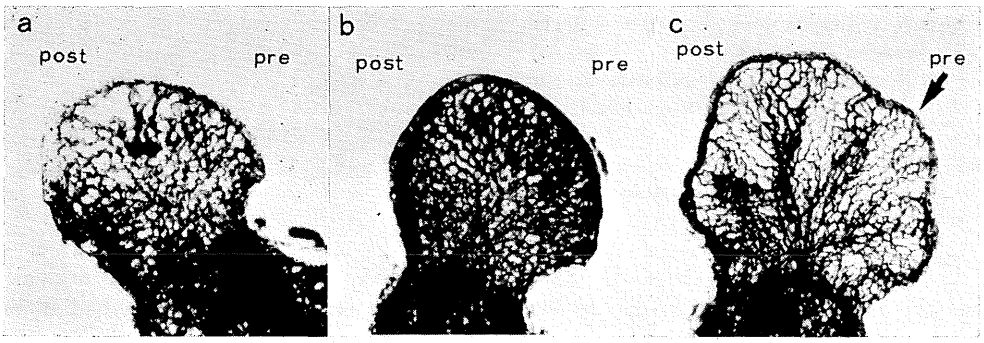

Fig. 13. Day 12.5
a, b : AER extending in the preaxial region
c : excessive digital rays in the axial region (black arrow)

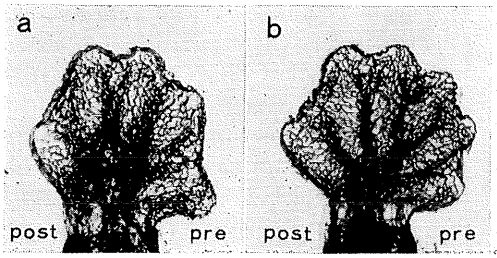

Fig. 14. Day 13.5
a : preaxial polydactyly. The vessels of the tibial side of the first digital ray are richer than the control.
b : axial polydactyly. The a. metatarsea plantaris between the duplicated digits is indistinct.

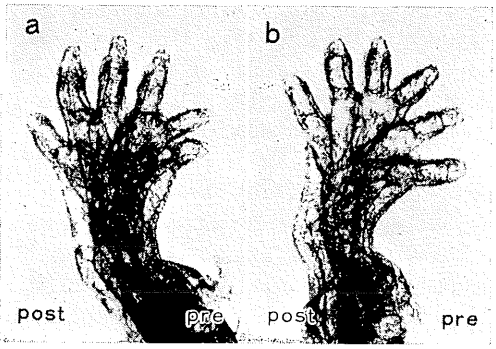

Fig. 15. Day 15.5
a : preaxial polydactyly
b : axial polydactyly

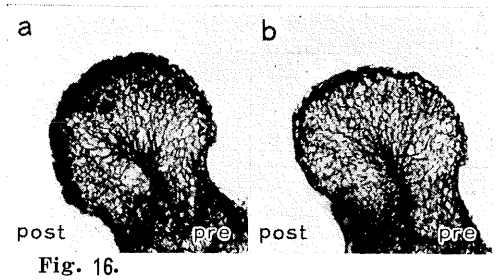

Fig. 16.

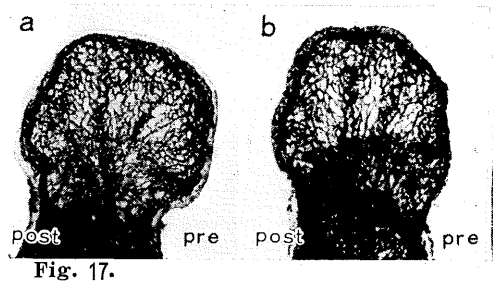

Fig. 17.

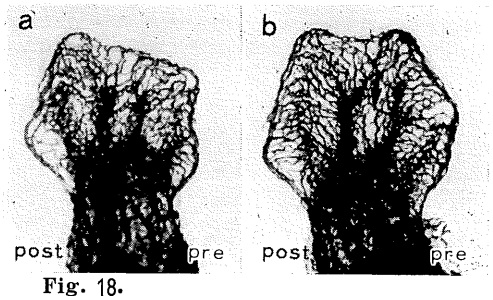

Fig. 18.

Figs. 16–19. The vascular development in the hindlimb treated at day 11.5.

Fig. 16. Day 12.0

a : The capillary network is denser in the peripheral region than in the region near the axial artery.

b : The marginal venous sinus is enlarged from the axial to postaxial margin.

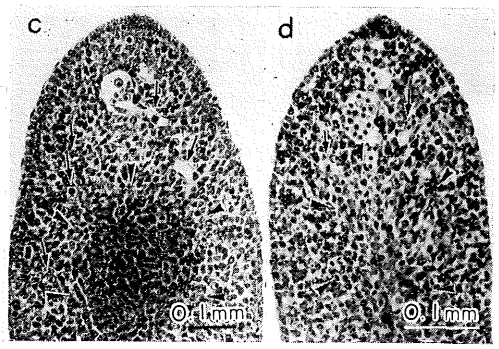

Fig. 17. Day 12.5

a : Foot plate assumes a square shape.

b : The number of digital rays is reduced.

c, d : Histological sections perpendicular to the long axis of the limb, stained with H & E. Arrows indicate vessels. The area surrounded by arrowheads shows mesenchymal condensation.

c : control

d : treated

Fig. 18. Day 13.5

a : The exclusion of capillaries from the digital condensations becomes more conspicuous.

b : Capillaries in the digital area become much less than control (black arrow).

The vascular pattern at day 14.5 was fundamentally similar to that at day 15.5.

At day 15.5, 30 limbs of preaxial polydactyly type and 36 limbs of axial polydactyly type were examined.

In both preaxial and axial polydactyly types, the vasculature of the leg was almost of the normal pattern. In the foot, aa. metatarseae increased in number according to the number of metatarsi.

In the preaxial polydactyly type, the tibial branch of the aa. digitales propriae of the tibial great toe was not so well developed (Fig. 15a). This branch is a continuation of the r. superficialis of the a. plantaris medialis. In the axial polydactyly type, the development of the individual a. digitalis propria varied according to the degree of separation of the duplicated toes. In the limb shown in Fig. 15b, the tibial branch of the aa. digitales propriae for the second toe and the fibular branch for the third toe were slender.

2. The group treated at day 11.5 of pregnancy

At 12 hours after 5-FU administration, i.e. at day 12.0, the foot plate had no distinct mesenchymal condensations. A single axial artery was distinct, which was divided into a capillary plexus. The capillary network was denser in the peripheral region close to the marginal venous sinus than in the region near the axial artery (Fig. 16a). In most cases the marginal venous sinus was enlarged from the axial to postaxial margin (Fig. 16b).

At day 12.5, the treated limb buds were smaller than controls, and some foot plate assumed a square shape (compare Fig. 3 and Fig. 17a). The digital rays were distinct, and the number of digital rays was apparently re-

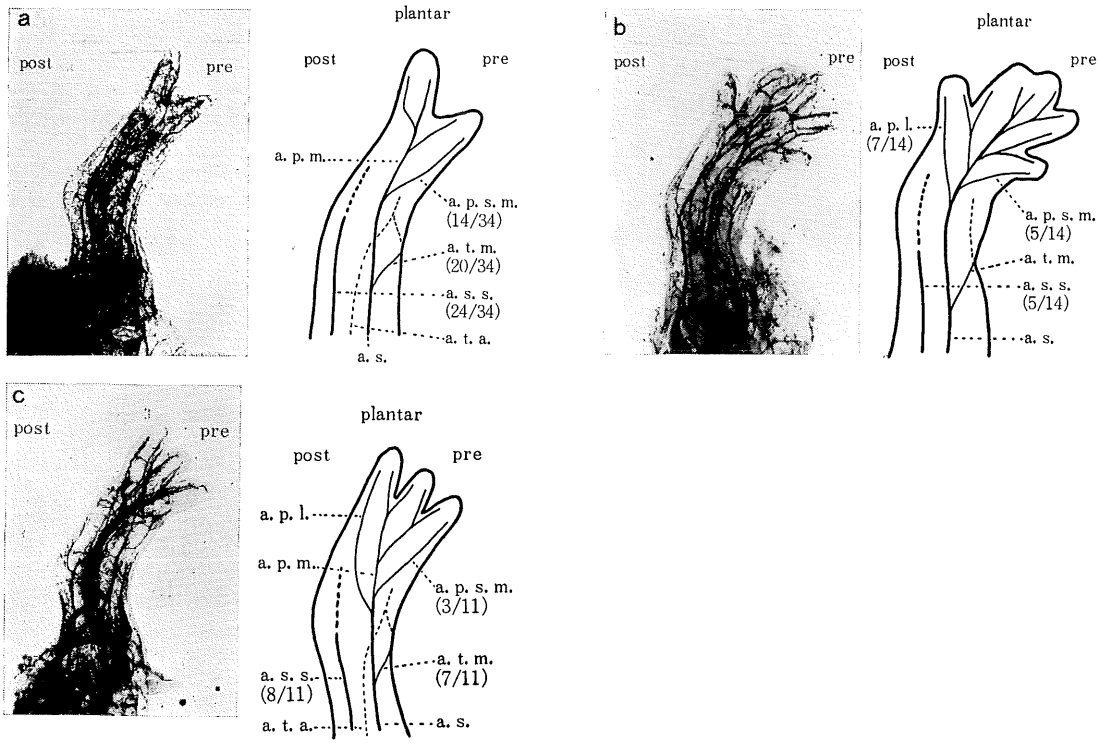

Fig. 19. Day 15.5
a : cleft foot type
b : intercalary defect type
c : two digital ray defect type
Numerators in the parentheses indicate the number of each vessel normally formed. Denominators indicate the total number of limbs examined.

duced in some specimens (Fig. 17b). Delay in involution of the AER was not so conspicuous as in the limbs treated at day 10.5. Nevertheless, the marginal venous sinus in the treated limb was dilated as compared with that in controls (Fig. 17b). The exclusion of capillaries from the digital condensations proceeded as in controls (Fig. 17c, d).

At day 13.5, the marginal venous sinus became narrower than in the previous stage. However, it was still wider than controls (Fig. 4) especially on the preaxial and postaxial borders of the foot plate. The exclusion of capillaries from the digital condensations became more conspicuous when compared with the previous stage (Fig. 18a). In some specimens capillaries in the digital area running transverse to the digital axis became much less than controls, leaving almost avascular areas (Fig. 18b). At day 13.5, classification of gross malformations was still difficult.

The vascular pattern at day 14.5 was almost similar to that at day 15.5.

At day 15.5, 34 limbs of cleft foot type, 14 limbs of intercalary defect type, and 11 limbs of two digital ray defect type were examined. In the cleft foot type, the a. plantaris superficia medialis and/or a. plantaris lateralis were missing or not well formed in 20 limbs. The a. suralis superficialis was not well developed in 10 limbs. A. tarsea medialis was missing in 14 limbs (Fig. 19a). In the intercalary defect type, the a. plantaris superficia medialis was not well formed in 9 limbs, and the a. plantaris lateralis was missing in 7 limbs. In 9 limbs, the a suralis superficialis was not formed (Fig. 19b). In the two digital ray defect type, the a. plantaris superficia medialis was not well formed in 8 limbs. The anastomosis between the a. tarsea medialis and the a. tibialis anterior was missing in 4 limbs. In 3 limbs, the a. suralis superficialis did not reach to the dorsal

region of the foot (Fig. 19c).

DISCUSSION

The main purpose of the present experiment is to analyze the relationship between the time of teratogen administration and resulting alteration in vascular and skeletal patterns. For this analysis, knowledge of developmental mechanism for determination of the arterial pattern and the role of vascular system in early limb morphogenesis is indispensable. Hence, a brief review on these subjects is presented before the discussion on the findings obtained in the present experiment.

Developmental mechanism for determination of the arterial pattern

For proper interpretation of pathogenesis of vascular anomalies in teratogen treated limbs, the mechanism of development of the arterial system should be studied. However, this mechanism has not been clarified yet (see review by Cazenave-Mahè et al.[5], 1981). Several hypotheses have been presented. While studying the development of the arterial wall of the principal vessels of the chick embryo, Hughes[14] (1943) stated that hemodynamic forces were an essential factor in vascular development.

However, the hemodynamic hypothesis gave no satisfactory explanation for the determinant of the course of the vessels. It seems unlikely that the course is randomly determined as was once thought (Baader[3], 1866; Aeby[1], 1871), nor is it probable that the vessels simply follow the nerves as suggested by De Vriese[10] (1902) and Müller[21] (1903). The first hypothesis would lead to an infinite variety of anatomical arrangements and the second is inconsistent with the studies of Detwiller[9] (1936), who showed that the vessels developed normally even when all nerve tissues had been removed. There must be some determinant in the periarterial milieu of genetic origin to explain the relative anatomical constancy of the arterial system.

The role of vascular system in early limb morphogenesis

Amprino and Malmberg[2] (1973) and Searls[23] (1968) agree that normal blood vessels probably do not determine the size and shape of the skeletal blastemata during chick limb development, and Neubert et al.[22] (1974) have shown that chondrogenesis can occur in limb buds in avascular culture. However, the possibility remains that abnormal vasculogenesis may adversely affect early skeletal development.

Caplan and Koutroupas[4] (1973) have suggested a biochemical role for the vasculature in developing limbs. They hypothesized that avascular areas of the mesenchyme are conducive to chondrogenesis, while vascular areas with a relatively high oxygen tension support myogenesis. Recent studies utilizing the quail-to-chick grafting technique have established that myogenic cells and chondrogenic cells are different in origin; the former migrate from somites into prospective limb buds and the latter originate from the somatic plate mesoderm (Chevallier et al.[6], 1977; Christ et al.[7], 1977). Thus the original hypothesis by Caplan and Koutroupas based on the assumption that the muscle and cartilage elements differentiate from a homogeneous population of limb mesodermal cells is negated. However, detailed observations of the fluid flow dynamics in the developing chick limb clearly showed that prospective morphogenetic areas of the limb are distinguished by a differential vascularization pattern prior to the overt expression of distinctive phenotypes (Jargiello and Caplan[18], 1983). Although the question of whether the differential vascularization pattern relates to patterns of muscle and cartilage condensations is not fully answered, the specific nutrient and oxygen levels determined by the vascular pattern possibly influence the muscle and cartilage development.

Pathogenesis of limb malformations induced by 5-FU and contribution of vascular aberrations

Dagg[8] (1960) published a detailed report on the teratogenicity of 5-FU in mice, and suggested that 5-FU could define the period of susceptibility of limb malformations sharply because of the prompt reaction. Limb bones are especially susceptible to 5-FU, and the variations of limb malformation thus induced can be compared with human malformations. Specific limb malformations such as polydactyly and reductive deformities can be easily obtained, depending on the dose and/or time of its administration. In our laboratory, serial experiments on limb teratogenesis using 5-FU have been conducted in mice since 1977 (Watari[31], 1978; Imagawa[15], 1980).

From the results of the previous experiments, 5-FU is considered to be a general cytotoxic

agent. The pathogenesis of 5-FU induced malformation consists of two stages (Imagawa[15], 1980). The first stage reflects the direct damage of limb bud with 5-FU, and the second stage shows various remodeling changes after the direct damage. During the first stage, 5-FU exerts mainly a cytotoxic effect on the limb mesenchyme and a slight effect on the AER. The mesenchymal cell death results in the loss of the limb tissue. During the second stage mesenchymal proliferation occurs under the effects of the AER, and the pattern of skeletal primordia is determined by the amount of the limb mesenchymal tissue; when mesenchymal tissue proliferates excessively as in the case of treatment at day 10. 5, polydactyly results; when mesenchymal proliferation is not sufficient to compensate the initial tissue loss as in the case of treatment at day 11. 5, reductive malformations occur.

In the present experiment, histological observation of the limb bud after 5-FU treatment revealed no signs of direct damage of capillaries. However, 24 hours after 5-FU injection at day 10. 5, the peripheral capillary network in some of the limbs was looser than controls (Fig. 12c). There might be damages of capillaries not revealed by light microscopy.

Injuries of capillary endothelial cells in early limb bud with teratogens have been reported. Jurand[19] (1966) described "vesicular projections" in endothelium of thalidomide treated chick limb buds 24 hours after teratogen injection. Fraser and Travill[11] (1978) showed that large, branching marginal folds and endothelial cell vesiculations protruded into the blood vessel lumina in retinoic acid treated hamster limb buds. Jurand has suggested that this primary effect results in skeletal malformation seen later in development.

In the present experiment, however, sequential observations of teratogen treated limb buds suggest no direct relationship between the early changes in the capillary network and the final abnormal arterial patterns. In some of the specimens observed 24 hours after 5-FU injection at day 10. 5, the capillary network in the limb bud was loose, the axial artery was indistinct, and a blister was noted along the postaxial border (Fig. 12c). Of the specimens observed 48 hours after 5-FU injection at day 10. 5, a larger number of limb buds showed an almost evenly distributing capillary network, although the shape of the foot plate was deformed at the preaxial margin, indicating early sign of polydactyly (Fig. 13a, b). Nevertheless, observation at 72 hours and after revealed no direct sequel of these vascular abnormalities; the arterial pattern in the proximal limb was almost normal and the branching pattern of arteries in the foot depended on the pattern of the mesenchymal condensations (Fig. 14a, b). If the presence of capillaries at the presumptive digital areas had inhibited cartilage differentiation at the sites, there would have been a smaller amount of cartilagenous tissue in the preaxial region of the limbs treated at day 10. 5. The actual findings were contrary to this expectation. Hence, the abundant capillary network at the preaxial area observed 48 hours after teratogen treatment should be interpreted as a step in the remodeling phase of 5-FU induced teratogenesis favoring excessive proliferation of undifferentiated mesenchyme at the preaxial border. After the proliferation of the mesenchyme, chondrogenesis proceeded as normal, although the pattern of digital rays was abnormal. The final arterial pattern was apparently determined according to the pattern of digital rays.

The relationship between the time of teratogen administration and the altered arterial pattern

In the human upper limb, a few investigators have claimed that abnormal arterial patterns in malformed hands could be used as indicators of the time of the limb tissue injury (Sudo[28], 1979; Inoue[16], 1981). Their estimates depend on the description of the embryonic development of the arteries of the arm given by Singer[27] (1933). The principle of their estimation method, of which details will be given in the following report (Katagiri[20], 1983), may be given in the following example. Some cases of transverse deficiency had a defective arcus palmaris superficialis. In normal development, the arcus palmaris superficialis is formed around the 48th postovulatory day. It was presumed that a teratogenic event at this stage might damage the developing arcus and lead to a defective arcus. Adequacy of this estimation method has not been tested in experimental animals.

In the present experiment the relationship

between the time of teratogen administration and resulting alteration in vascular and skeletal patterns was sequentially observed in embryonic and fetal limbs. The main findings in this respect can be summarized as follows: 1) In normal development, the main vessels of the leg (a. tibialis, a. peronea, and a. plantaris medialis) are formed by day 13.5 of pregnancy. The vessels of the toes (aa. digitales propriae) become distinct by day 15.5 of pregnancy. 2) In the group treated with 5-FU at day 10.5 of pregnancy, the vascular changes in the hindlimb with polydactyly were observed mainly in the foot (aa. metatarseae plantares and aa. digitales propriae). No vascular abnormalities were noted in the leg. In the group treated at day 11.5 of pregnancy, the vascular changes in the hindlimb with reductive malformations were observed in the leg (a. plantaris superficia medialis, a. plantaris lateralis, a. tarsea medialis, a. tibialis anterior, and a. suralis superficialis) as well as in the foot.

If the principle applied to human malformed upper limb were correct, polydactyly in the mouse foot would result from a teratogenic event during a period between day 13.5 and day 14.5 when the aa. digitales propriae are forming in normal development, and reductive deformity would be produced by teratogen treatment during a period between day 12.5 and day 13.5 when the arteries of the leg are forming in normal development. Obviously the above estimates are contrary to the real experimental results. There are some time gaps between the time estimated from the altered arterial pattern and the real time of teratogen administration. In the case of polydactyly, the time gap is longer than in the case of reductive deformity. The length of the time gap is apparently related to the duration between teratogen administration and skeletal pattern formation in the affected area. In the case of polydactyly, the interval between teratogen administration at day 10.5 and digital ray formation around day 12.5 is about two days. In the case of reductive deformity, this interval is about one day.

In considering the time of formation of an artery or an arcus, one should note that the artery or arcus does not appear suddenly as a definite structure, but is gradually formed from collection of capillaries which initially distribute randomly in the limb bud. The schematic drawings of arterial development given by Singer[27] (1933) might be misleading. In the present study, the day of formation of a certain artery is given as the time when several capillaries gather together clearly enough to be identified as a future artery. Apparently the process of reorganization of the randomly distributed capillaries into definitive arteries goes along the formation of mesenchymal condensations. From these observations and discussions, it is concluded that an abnormal arterial pattern in a malformed limb does not directly indicate the time of teratogenic event, but reflects an abnormal patterning of mesenchymal condensations.

ACKNOWLEDGMENTS

The author is indebted to Prof. Mineo Yasuda and Assoc. Prof. Harukazu Nakamura of the Department of Anatomy, Hiroshima University School of Medicine, and Prof. Kenya Tsuge of the Department of Orthopedic Surgery, Hiroshima University School of Medicine, for their critical review of the manuscript.

This study was supported by Grant-in-Aid for Scientific Research No. 57770894 from the Ministry of Education, Science and Culture, Japan.

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