THE VASCULAR CONTRIBUTION TO OSTEOGENESIS

V. The Vasculature Supplying the Epiphysial Cartilage in Rachitic Rats

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In previous papers of this series (Trueta and Amato 1960, Trueta and Trias 1961) experiments were described in which the effect of a decrease or total suppression of the blood to either one or other side of the epiphysial cartilage was investigated. These experiments showed that the only source of nourishment of the growth cartilage was the blood of the epiphysial vessels. It was also demonstrated that reducing the epiphysial blood supply led sometimes to a slight and transient thickening of the growth cartilage in areas made experimentally hypoxic, whereas in those parts persistently deprived of blood flow, first the cells of the germinal and proliferative segments, and ultimately the whole of the cell columns, became seriously damaged and finally disintegrated (Trueta and Amato 1960). When the role of the metaphysial vessels was studied it was found that they played little or no part in the nourishment of the cartilage cells but that, on the contrary, the presence of these vessels was necessary to enable the hypertrophic cells of the columns to be removed in the normal process of growth. Consequently, when the metaphysial vessels were interrupted, no calcification occurred and the cartilage cells continued in their hypertrophic phase and did not disintegrate. With this cell preservation the cartilage thickened, taking on histological characteristics resembling those of rickets; if revascularisation was allowed the rachitic picture was rapidly reversed.

These findings suggested a rigid sequence of events in the process of epiphysial growth following cell division and cell hypertrophy thus: pericellular calcification, vascular invasion and bone formation. It was thus of interest to investigate the part played by calcification in the preparation of the vascular advance. Calcification was prevented by withholding the necessary fat-soluble vitamins.

MATERIAL

Eighteen black hooded Norwegian strain rats of both sexes were used. They had been separated from their mother after twenty-one days and then fed on Steenbeck's rachitogenic diet for twenty-eight days to induce rickets. The rachitogenic diet had the following composition: maize meal 76 per cent; wheat gluten 20 per cent; calcium carbonate 3 per cent; and salt 1 per cent. The diet was free from vitamins A and D.

Five normal rats were used as controls. They were of the same breed and age as the rachitic rats. The control rats were fed on a diet of the following composition: wheat meal 47 per cent; sussex ground oats 40 per cent; white fish meal 8 per cent; dried skimmed milk 3 per cent; dried yeast 1 per cent; and salt 1 per cent. Stabilised vitamin supplement (Alpha Beta N.8) was added in the proportion of two and a half pounds to the ton.

METHOD

All the animals were seven weeks old when the studies began and they were killed before they were nine weeks old. In each animal vascularity and histology were studied in the right hind leg and the alkaline-phosphatase determination was done in the left hind leg.

Under ether anaesthesia a 20-gauge needle was inserted into the left ventricle and a contrast medium was perfused before the animals were killed; in two of the control rats perfusion was through the abdominal aorta instead of the left ventricle.
As in previous work the most useful perfusion medium was a warmed mixture of equal parts of 2 per cent solution of Berlin blue and a 10 per cent barium sulphate suspension (Micropaque). Some of the animals were perfused with Berlin blue (2 and 4 per cent only). The amount of the perfusion mass was approximately 10 millilitres and was injected very slowly; no attempt was made to estimate the injection pressure. After perfusion the specimens were fixed in 10 per cent formol saline. They were decalcified in formic acid and mounted in celloidin. Thin sections (7 μ) and moderately thick sections (30 μ) were cut and stained with haematoxylin and eosin. Microradiographs were made using the technique described by Barclay (1951). Photographs of the specimens prepared by the Spalteholz method were also obtained in a number of experiments using the technique of Trueta and Harrison (1953); the sections in these cases were as thick as 300 μ. The alkaline phosphatase was demonstrated by Azo dye coupling.

Five animals with advanced rickets were killed and injected. The remainder of the rachitic animals were then placed on the normal diet. Two animals died after being on the normal diet for six days and were injected as described above. Five animals were perfused and killed after being on the normal diet for ten days and five more were treated similarly after being on the normal diet for fourteen days.

RESULTS

Untreated rickets—In the control rats (Fig. 1) the arrangement of the perfused vessels adjacent to the growth cartilage shows little change from the pattern described for the rabbit (Trueta and Morgan 1960).
In the rachitic animal, as is well known, striking changes occur in the epiphysial cartilage. The severity of these changes increase from the epiphysial to the metaphysial side of the cartilage. Starting at the epiphysial end, the following were the most relevant features encountered. The bone end plate limiting the growth cartilage distally showed more breaks in continuity than in the normal rat and the calcified zone below the bone plate was narrower than normal. The proliferative zone of the columns was normal but the number of empty columnar spaces was greater than normal, which suggested a somewhat reduced activity of the germinal cells in some places.

It has been shown by Rigal (1961) that the dividing activity of the cells in the proliferative segment of the columns was best assessed by their uptake of tritiated thymidine. However, an alternative, but indirect, method consists of estimating the progressive rate of lengthening of the columns once the removal of the cells at their metaphysial end has been interrupted (Trueta and Amato 1960). This method shows that in rickets there is only very slight, if any, interference with the activity of cell division (Fig. 2). The hypertrophic cells of the columns were flattened and compressed on top of each other as if no room was available to them, but despite this evident lack of space the general columnar arrangement remained, with each column still separated from its neighbours by matrix, although the distance between them was reduced (Fig. 3).

The most characteristic feature found in all the growth cartilages affected by rickets was the variety of length of the cell columns, the longest of which showed as two penetrating prongs at the junctions of the outermost two-thirds of the growth cartilage with the central portion (Fig. 4). At these sites it was a constant finding that the arborescent progression of the vessels into the metaphysial end of the columns was lacking (Fig. 5). As will be discussed later, the inhibition of penetration of the vessels was similar to that seen after heavy pressure had been exerted on the growth cartilage in rabbits (Fig. 6) (Trueta and Trias 1961).

There were, at the same time, other important vascular changes. The metaphysial vessels no longer consisted of long loops running a straight course with each loop associated with a single column of cells and with its end in contact with the most distal of the remaining hypertrophic cells, as is shown in Figure 1. Instead the vessels had an arborescent character with finger-like processes groping up into the abnormal epiphysial cartilage, as in the foetal pattern (Fig. 7).

The shape, size and number of metaphysial vessels and the way they remove the hypertrophic cells so much resembled that of the invasion of the anlage before the growth cartilage has appeared that it is of interest that any cartilage cells were left at all. Our findings
suggested that the hypertrophic cells, not weakened by the calcification of their surrounding matrix, were resistant to vascular invasion. This will be elaborated upon later.

**Relationship of calcification and blood vessels in experimental rickets**—It was thought to be of interest to investigate the area of calcification in relation to the placement of the metaphysial vessels because it had been observed previously, in the normal, that there was an almost constant distance between the two (Trueta and Morgan 1960, Trueta and Little 1960, Trueta 1962). For this investigation the method of vascular perfusion was complemented by microradiographic studies using fine-grain films. The information gathered showed that, in
its central portion, the metaphysial line of calcification was much closer to the epiphysis than the rest of the line (Fig. 8). This wide central area of more advanced calcification was seen to correspond to the position of the vessels in the central portion which, to our knowledge, has never been noted before. Pommer (1885), Schmorl (1909) and Nakahara (1938), three workers who have studied the vasculature in rickets, do not mention it. Instead of remaining in line with the rest of the metaphysial vessels, those of the central zone appear much nearer the epiphysis, reducing the length of the columns of hypertrophic cells in that area (Fig. 9). These findings give further support to the view that the proximity of blood vessels is an essential for calcification. The reason for this peculiar vascular distribution will be discussed later, but it may be said here that excessive pressures seem to be responsible for most of the
great inhibition of vascular penetration once the initial lack of calcification has prevented the normal lengthening of the calcified tubes from occurring.

The way the hypertrophic cells were removed by the wandering vessels was examined closely. After shortening many columns from their ends the vessels moved sideways and attacked the neighbouring columns at their middle, driving deep wedges into them (Fig. 10). This prepared the disconnected metaphysial portion of the columns for removal piecemeal. Nevertheless, the disorganised fragments of the uncalkified columns were visible deep down the metaphysis for a long time; this gives further evidence of the important role that calcification plays in preparing the removal of the hypertrophic cells of the growth cartilage, for once calcification has been prevented the cartilage cells remain unaltered for a long time (Fig. 11).

Vascular and calcification in healing rickets—Few, if any, changes were seen during the first two days of healing. After the third day the first signs of calcification appeared in the radiographs, not, as would have been expected, at the metaphysical border of calcification but well up towards the middle of the uncalkified area, near the position where calcification would have occurred in the normal animal. This phenomenon has been known for many years (Harris 1933). By the fifth day the columns had shortened and the whole of the metaphysical vasculature had increased. By the tenth day radiographs showed a considerable narrowing of the previously wide area occupied by the growth cartilage, but the line of calcification was still at the normal level with incomplete calcification underneath it (Fig. 12). It is interesting that at this stage of healing some large portions of the growth cartilage still had not been invaded by the vessels, even though a much increased vascular activity had cut across the middle of the columns of hypertrophic cells, not far from the area occupied in the normal by the vascular loops (Fig. 13). This area of upper vascular invasion was characterised by an increase in size and number of the vessels (Fig. 14). Nevertheless, this vascular profusion was not uniform at the tenth day (Fig. 15) and there was a large area of less intense vascularity in the remainder of the metaphysis (Fig. 16).

When the radiograph in Figure 12 and the angiograph in Figure 16 are compared it is evident that the zone of more advanced calcification corresponds almost exactly with that of the more distally situated vascularity.

At the fourteenth day dramatic changes had taken place in the region of the growth cartilage, to such an extent that the rickets appeared virtually healed although the metaphysical side of the cartilage had not yet fully reverted to normal in all places. While most cell columns had already been shortened to their normal length, some portions corresponding to the areas of greatest pressure still showed evidence of their recent enormous enlargement (Fig. 17). The vasculature at this stage was almost back to normal. The upper area of high vascularity between the end of the vessels and the metaphysis had disappeared (Fig. 18). The looping system of end vessels, characteristic of the normal, was found here and there, but their final re-establishment did not occur until later, probably about the twentieth day. This may be considered the final stage of recovery in experimental rickets.
The tenth day of healing rickets in the rat. Figure 12—A radiograph showing that the line of calcification of the growth cartilage has reached its normal level, but beneath it calcification is still very incomplete. Figure 13—The columns of hypertrophic cells are being cut across their middle by the growing blood vessels, near the area occupied in the normal by the vascular loops. (Haematoxylin and eosin, x 75.)

Microradiographs of the tenth day of healing rickets in the rat. Figure 14—There is a considerable increase in the size and number of the vessels at the upper end of the vascular invasion. (x 15.) Figure 15—Despite the progress of healing there are many vascular gaps breaking the continuity of the metaphysial invasion. (x 15.)
Alkaline phosphatase was present in the hypertrophic cells along the whole of the long columns and particularly round the epiphysial and metaphysial vessels adjacent to the growth cartilage.

**DISCUSSION**

In two previous articles in this series the normal process of growth was shown to be altered by interference with the flow of blood at the metaphysial side of the growth cartilage. Both by cutting the vessels (Trueta and Amato 1960) or by compressing them to the point of preventing the blood from circulating (Trueta and Trias 1961) the hypertrophic cells of the columns were preserved until their excessive accumulation interfered with their vitality. Both experiments also showed that calcification ceased from the moment the blood flow was discontinued. The suspicion that calcification played a greater part than had been suspected in the mechanism of vascular progression and cell removal suggested the present investigation of the prevention of calcification without interfering with the blood flow, as had been done in the two previous experiments. Since the classical work of Mellanby (1925, 1950) it is known that, in the group of fat-soluble vitamins responsible for the prevention of rickets, vitamin A is associated with vitamin D. In experimental rickets it is usual to withdraw both, as was done here, even if the withdrawal of vitamin D alone appears to be more radical.

There is a vast collection of literature on every aspect of rickets but we could only find three studies on the behaviour of the vasculature...
in experimental rickets, by Pommer (1885), by Schmorl (1909) and particularly by Nakahara (1938).

The changes we observed after withholding vitamins A and D were as follows: the last two or three cells of the hypertrophic segment of the columns remained surrounded by uncalcified matrix while the rate of cell reproduction in the proliferative segment of the column appeared normal. This initial lack of calcification at the metaphysial side of the growth plate inhibited vascular penetration and consequently prevented the removal of the last hypertrophic cells of each column. Despite the fact that the suppression of the fat-soluble vitamins caused changes in the ground substance, they were not severe enough to enhance the rate of degeneration and death of the hypertrophic cells, which, with no calcification in the altered matrix, remained as though hibernating.

Because the lack of vascular invasion at the metaphysial side of the growth cartilage was not accompanied by a similar vascular inhibition at the epiphysial side, cell proliferation continued unabated and new cells were repeatedly added to the columns to produce the widening of the growth plate so characteristic of rickets.

In a previous paper (Trueta and Trias 1961) it was shown that when heavy pressure was placed upon the growth cartilage the areas where the pressure was greatest had the longest columns, and it was suggested that this was because the vessels were unable to grow into cartilage so greatly compressed. There were two areas of extremely high pressure in all the growth cartilages which corresponded to the so-called cones of maximum pressure. In the present work the longest columns were in the same position as those obtained with compression. This strongly suggests that, at least in experimental rickets, the initial inhibition of vascular penetration at the metaphysial side of the columns, caused by the lack of calcification, is further inhibited by the enormous pressure which causes the remarkable lengthening of the columns until they contain 120 cells or more. In these experiments, as in those with compression, vascular inhibition was the only common factor found responsible for the peculiar preservation of the growth cartilage.

This is supported by the changes seen in healing, because recovery is directly related to the rate of vascular penetration and usually starts and progresses from the middle of the metaphysial side of the plate, the area least subjected to pressure. Soon, recovery reaches the place where, in the normal, calcification would have occurred; this suggests that the cells of those segments of the columns have already prepared the matrix for calcification, and that the proximity of the metaphysial vessels is unrelated to the changes which prepare the matrix for calcification. This cannot be said of the epiphysial vessels, which seem responsible for the changes in the cells and matrix which make the latter calcify, even though the hypertrophic segments of the columns are pushed farther and farther away by the addition of new cells.

Administration of the fat-soluble vitamins allowed calcification to occur in the susceptible matrix and the metaphysial vessels were then able to bring normal blood to a convenient distance from the matrix. Consequently, in withholding calcification the whole process of growth and bone formation was made to break down, confirming that calcification is the prerequisite for the progress of the vessels and that, in its absence, the decisive role of the vessels as blood conductors, cell removers and active agents of ossification was lacking.

After the hypertrophic cartilage cells have matured and have laid down the appropriate matrix—and perhaps alkaline phosphatase—calcification is necessary to allow the mechanism of growth to proceed by the active intervention of the blood vessels.

CONCLUSIONS AND SUMMARY

1. It has been shown that in experimental rickets the well known changes in the epiphysial cartilage which so seriously affect growth are accompanied by severe interference with the progress of the metaphysial vessels into the growth cartilage.
2. Further evidence has been found that, by the repeated increase in their number, the cartilage cells occupying the more distal part of the proliferative segment become more and more affected by their remoteness from the epiphysial vessels, which supply the transudates to these cells. At a given distance these cells are affected and change, becoming hypertrophic, with increasingly large vacuolae, and are rich in glycogen and alkaline phosphatase.

3. The hypertrophic cells alter the nature of the intercellular substance they deposit and this becomes calcifiable. Provided that the metaphysial vessels are situated at an appropriate distance—about three cell capsules away—and that the blood has its necessary components, calcification occurs.

4. Calcification produces the advancing, rigid multitubular structure within which the progressing metaphysial vessels are protected.

5. The interruption of calcification by the withdrawal of fat-soluble vitamins breaks down the whole mechanism of growth and stops the vessels growing into their proper position. The administration of the required vitamins re-establishes the normal sequence of events and allows the vessels to play their decisive role in osteogenesis.

6. Any mechanism which causes the interruption of the vascular progression, whether from metaphysial ischaemia (Trueta and Amato 1960), from severe pressure (Trueta and Trias 1961) or from lack of calcification by withdrawing the fat-soluble vitamins, equally interrupts growth.

We would like to express our gratitude to Dr G. A. Stewart and Mr John V. Smart of the Biological Testing Laboratories, Burroughs Wellcome and Co., for supplying the experimental animals and for the detailed instructions which they made available. Our special thanks are due to Mr D. W. Charles, Miss A. Craib and Miss M. Litchfield for their technical assistance.

REFERENCES


