Vital staining indicating cell migration towards the periphery in the growth plate

Studies of fibular heads in rabbits

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In 38 fibular heads of young rabbits the germinal layer of the growth plate was traversed perpendicularly to the axis of the bone with an injection needle. The resulting channel was packed with pieces of raw agar-agar carrying Nile blue sulfate dye. The rabbits were killed after 1–8 days. In frozen sections of the fibular heads, stained cells were scattered over the germinal layer; in 19 of the plates a row of distinctly stained cells reached from the periphery of the germinal layer to the borderline of the innermost corner of the ossification groove of Ranvier (1889). Our results strengthen the view that the cells forming the periosteal bone originate in the growth plate, with implications for the pathogenesis of diseases of the growing skeleton.

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Submitted 92-12-30. Accepted 93-06-17

Although the question as to the mechanism of growth in width of the growth plate seems to have been settled, the question as to the histogenesis of the cells in the ossification groove of Ranvier is still unanswered (Shapiro et al. 1977). Direct evidence of cellular migration in or out of the growth plate in the transverse direction is not possible to achieve—for example, by injection of ^{3}H-thymidine—because cell labeling occurs simultaneously in the cartilage and in the ossification groove.

Experiments on localized roentgen ray injury of parts of growth plates led Langenskiöld and Edgren (1950) to the following conclusion: "In normal epiphyseal cartilage the interstitial growth of the layer generally called 'the layer of the reserve cells' causes an expansion of this layer and a successive displacement of its cells to the periphery in relation to the bony epiphysis and the cartilage cell columns." The function of the peripheral part of the growth plate seems not to have been the subject of special research in recent years (Robertson 1990).

We report further evidence of migration of cells of the germinal layer of the normal growth plate towards the periphery, based on labeling of cells by vital staining.

Animals and methods

In New Zealand white rabbits 26–46 days old, 38 heads of the fibula were exposed under local anesthesia (Lidocain-Adrenalin, Orion). Holes were made with a 0.8-mm thick injection needle traversing the germinal layer of the growth plate close to the bone tissue of the epiphysis, perpendicular to the axis of the bone some distance from the center of the plate (Figure 1). The head of the fibula was chosen because the ossification groove of Ranvier is well marked in this site. The channel produced in the plate was somewhat widened with a broken 0.8-mm needle and then packed with pieces of raw agar-agar carrying Nile blue sulfate (Fluka). The size of the pieces was about 7 × 1 × 0.5 mm. The pieces were kept for 6–10 days in 1 percent or 5 percent solution of Nile blue sulfate powder in distilled water and then dried. In addition, the dried pieces were dipped once more in the solution of dye and dried again. After introducing the agar, the wound was closed with resorbable sutures.

The animals were killed after 1–8 days by injecting 1 cc Hypnorm® (Janssen) intramuscularly and 2 cc Mebumat® (Orion) intravenously. The fibular heads were removed and frozen at –20 °C, and sectioned, in a cryomicrotome in the chamber of a cryostat, perpendicularly to the channels containing the agar carrying the dye (Figure 1). Sections of 7–10 µm were made.

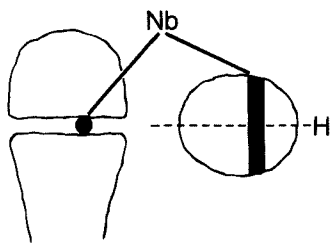

Figure 1. Schematic drawing showing in 2 projections how in the fibular heads of young rabbits the germinal layer of the growth plate was traversed perpendicularly to the axis of the bone with a 0.8 mm thick injection needle. The resulting channel was packed with pieces of raw agar-agar carrying Nile blue dye (Nb). H shows the plane of histologic sections.

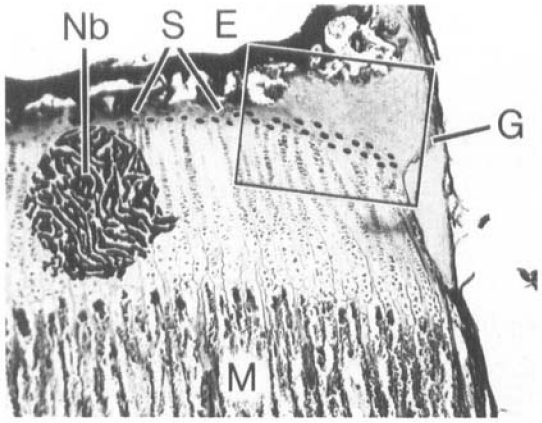

Figure 2. Photomicrograph of a stained paraffin section of a part of the fibular head of a 1 month old rabbit. By schematic drawing on this photomicrograph the location of the agar carrying Nile blue dye (Nb) is seen. The stained cells visible in frozen sections 2–6 days after application of the agar are schematically marked as dots. S are stained cells in the germinal layer, G is the borderline between the cartilage and the ossification groove, E is the epiphysis, M is the metaphysis. The square area corresponds schematically to Figure 4 in which the finding in a frozen section from a fibular head of a rabbit killed 3 days after application of the agar is documented. Note the location of the row of stained cells.

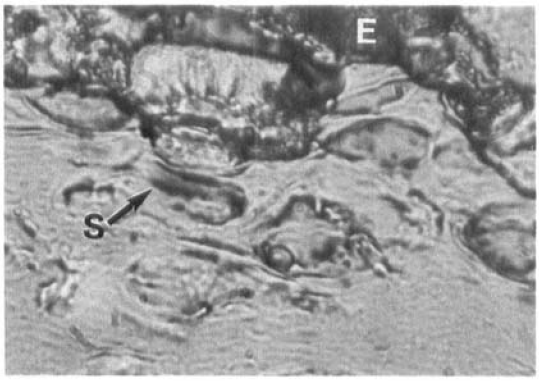

Figure 3. Photomicrograph of a frozen section of a part of a fibular head of a rabbit killed 3 days after application of agar carrying vital staining dye, as shown in Figure 1. The picture shows stained cells in the germinal layer close to the bone tissue of the epiphysis marked in Figure 2 (S), ×180. E is epiphyseal bone.

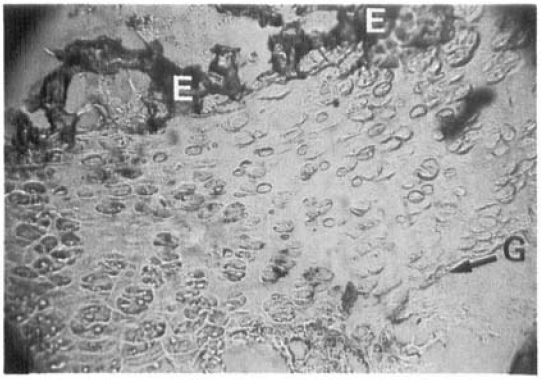

Figure 4. Photomicrograph of a frozen section of the same bone as seen in Figure 3. A row of Nile blue stained cells is seen reaching from the peripheral part of the germinal layer of the plate to the borderline of the inner corner of the ossification groove, ×60. The site of the area is shown schematically in Figure 2. G is the borderline between the cartilage and the ossification groove. E is epiphyseal bone.

The sections were taken up on unprepared slides or on slides prepared with Poly-L-Lysine® (Sigma Diagnostics) solution. They were studied and photographed in a microscope without fixation.

Results

In 21 growth plates, cells of the germinal layer were found to be stained. In the parts of the plates in which the agar was closer to the periphery, stained cells were seen scattered throughout the germinal layer (Figures 2 and 3). Especially marked was the regular appearance of a row of distinctly stained cells reaching in 19 plates from the peripheral edge of the germinal layer of the plate to the borderline of the innermost corner of the ossification groove (Figures 2 and 4). In 2 bones from a rabbit killed 1 day after implantation of the dye, the row of distinctly stained cells emerging from the germinal layer in the direction of the groove was seen, but it did not reach the borderline. In most bones a few cell columns in varying parts of the proliferation zone were also stained.

Of the 19 bones showing staining of the germinal layer (Figure 3) and a row of stained cells reaching to the groove (Figure 4), 16 were in animals killed after 3 days, and 1 each in animals killed after 2, 4, and 6 days, respectively. In a rabbit killed after 8 days, no stained cells were seen in either fibula. In 1 of the bones showing a positive result, Neutral red (Merck) was used as a vital staining dye instead of Nile blue sulfate.

In the 17 bones giving negative results, the needle may have traversed the plate too far from the bone tissue of the epiphysis in some bones. Traumatic physeolysis had disturbed growth and sectioning in several fibulae. A possible toxic effect of the dye could not be ruled out as a factor causing a negative result (Vogt 1925). In 12 bones, application of the dye in 1 percent or 5 percent solution of Nile blue dye in a gel of 2 percent purified Agar-Agar powder (Merck) in distilled water did not stain the germinal layer.

Discussion

Ranvier wrote in 1889: "The cells forming the periosteal bone probably originate in cells of the cartilage which are released with their fibers and follow them". His views were supported by studies by Carey (1922), Dahl (1936), Policard (1941) and Langenskiöld and Edgren (1950). Lacroix (1951) concluded from his experiments that the growth plate grows in width by apposition of cells from the periphery. Studies by means of ^{3}H-thymidine (Rigal 1962, 1969, Meikle 1975, Shapiro et al. 1977) and colchicine (Hert 1972) have indicated that the growth plate grows in width by interstitial proliferation of cartilage cells.

Concerning the growth in width of the growth plate, Hert (1972) wrote: "The results of the study provide grounds for rejecting the hypothesis of appositional growth of the epiphyseal plate from perichondrium". Meikle (1975) wrote: "One must agree therefore with the conclusions of Rigal ('62) and Hert ('72) that epiphyseal cartilage increases in transverse diameter by interstitial growth." Concerning the innermost cell layer in the ossification groove, Shapiro et al. (1977) wrote: "... the cells of the region of densely packed cells do not differentiate to chondroblasts ..."

The possible use of other vital staining methods such as injection of horseradish peroxidase, fluoresceinated dextran or fluorescent carbocyanine was investigated but, for different reasons, the substances were not found suitable for the purpose in question.

In 1967, Langenskiöld et al. showed that ^{35}S taken up by the germinal layer of a growth plate within a few days is retained throughout this layer after a growth in diameter of 70 percent in 3 weeks. The phenomenon is difficult to explain without the assumption of the occurrence of interstitial growth and expansion transversely to the long axis of the bone in the germinal layer. However, as far as migration of cells is concerned, this evidence is considered indirect.

Labeling of cells with Nile blue in order to study migration of cells or cell layers has been used extensively in embryology since the time Vogt (1925) published his classic work, which led to decisive progress in this field. He used raw agar as a carrier of vital staining dye.

Lin et al. (1991) reported studies of the mechanism of uptake of Nile blue derivatives in tumor cells, and pointed out that the cellular uptake of these dyes is directly proportional to the extracellular dye concentration. They also suggested that the lysosome is the main site of localization of the dye and ion trapping is probably the process by which Nile blue dyes are accumulated in cells. In their opinion the dyes cannot diffuse out across the membrane of the lysosome because of its acidic environment.

It is obvious that diffusion of Nile blue dye from the agar implant in our experiments caused staining of a part of the cells of the germinal layer close to the implant. Although Vogt (1925) did count on a possible use of his method in studying growing mammals, this seems not to have been done using vital staining. Thus definite opinions concerning the spread from and in the germinal layer of the growth plate cannot be expressed. However, once accumulated in the cells of this layer the dye seems to remain in them as long as their metabolism stays low and they do not differentiate. This statement is in agreement with findings reported in numerous articles on vital staining detecting cell migration in the embryo. It was already pointed out by Vogt (1925) that a low metabolism of the cells is a prerequisite of their accumulation of vital dye. As the germinal layer of the growth plate is a stem cell area (Rigal 1962) with a low metabolism it seemed probable that its cells would tend to take up and retain Nile blue stain.

It was shown by Trueta and Morgan (1960) that a rich blood supply is available "... directly in the midst of the intercellular substance surrounding the germinal cells ..." The spread of the Nile blue dye in our experiments to a large area of the germinal layer within 24 hours suggests that it may have taken place partly through the epiphyseal blood vessels rather than only through cell migration. However, the regular appearance of a row of distinctly stained cells reaching from the periphery of the germinal layer to the inner corner of the ossification groove of Ranvier (Figures 2 and 4) suggests a migration of cells from the germinal layer

to the borderline of the groove. This area lies outside the region of the epiphyseal vessels and it is not penetrated by the vessels of the ossification groove (Rigal 1962). The stained cell layer reached to the borderline of the groove, but the fate of the cells could not be judged in studying frozen sections. The tissue in the ossification groove is well vascularized (Rigal 1962) and it is known that vital stain disappears from cells when their metabolism increases (Vogt 1925). It should be noted that the fibula of a rabbit aged 30 days grows 0.95 mm in length in 2 days from its proximal end (Hansson 1967). This corresponds to the thickness of the growth plate.

Assuming that the views of Ranvier on normal bone growth are true, it could be expected that a layer of cells reaching from the germinal layer of the growth plate to the inner corner of the ossification groove would be stained by Nile blue dye in our experiments. The results corresponded to this working hypothesis.

From the inner corner of the groove the layer of the "densely packed cells" (Shapiro et al. 1977) continues towards the periosteum and merges in its osteogenic cambium layer. We know no facts which speak in favour of the assumption that the stained cells lying between the germinal layer and the ossification groove in our experiments had taken up the dye within that otherwise unstained area which is not vascularized.

Studying bones of human fetuses, Sandberg and Vuorio (1987) found that the cambium layer of the periosteum adjacent to the ossification groove contains collagen type II mRNA. This means that the cells of this layer have, on the molecular level, a property characteristic of cartilage. Holtrop (1965) wrote "... to the list of possible precursors of bone cells the cartilage cell should be added".

The findings of Sandberg and Vuorio (1987) were later confirmed in experiments on young rabbits by Langenskiöld et al. (1993). Both the experiments with collagen mRNA and the predicted results of studies on vital staining reported above bring further support to Ranvier's view that the cells forming the periosteal bone originate in the growth plate. They also support the views on the pathogenesis of multiple exostoses, enchondromas and achondroplasia put forward by Langenskiöld (1947, 1967a, 1967b) and Langenskiöld and Edgren (1950).

Acknowledgements

The authors would like to thank laboratory assistants Ritva Sohlman and Marja Heino at the Research Laboratory of the Invalid Foundation and Taina Hutro at the Department of Orthopedics and Traumatology, Helsinki University Central Hospital, for invaluable help.

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