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The perichondrial ring as a reservoir for precartilaginous cells. In vivo model in young chicks' epiphysis

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Abstract *Background:* The purpose of this study is to illustrate the routes of migration of precartilaginous cells from the perichondrial ring of LaCroix, as a potential reservoir for growth-plate germ cells. *Methods:* Chondrocytes derived from the ring of LaCroix of young chicks' proximal tibia were cultured in vitro and transfected with adenovirus vector containing the gene encoding for Escherichia coli (beta)-galactosidase (lacZ) gene, which allows assessment of the migratory routes of these cells. The lacZ- transfected cells were injected back into the perichondrial ring of LaCroix of young chicks' proximal tibias. Four weeks later the migration route was assessed microscopically. *Results:* Injection of cells derived from the ring of LaCroix of neonate chicks, transfected in culture with adenoviruses containing LacZ reporter gene, allows the assessment of migratory potential of these cells. Stained cells were found at the outer layer of the epiphysis, particularly in areas adjacent to the perichondrial ring. Further longitudinal histopathological studies along the bone axis demonstrated a condensed layer of the stained cells arranged horizontally along parts of the physis.

Conclusion: The perichondrial ring of LaCroix represents a potential reservoir of growth-plate germ cells in young chicks.

Résumé *Le but* de cette étude est d'étudier la circulation des cellules souches précartilagineuses provenant de la virole péricondrale de LaCroix considérée comme un réservoir potentiel de ces cellules. *Méthode:* les chondrocytes provenant de la virole péricondrale de LaCroix prélevés au niveau de l'extrémité supérieure du tibia chez de jeunes poulets ont été cultivés in vitro et transplantés à l'aide d'un adénovirus codant le gène beta galactosidase (lacZ). Les cellules lacZ ainsi transplantées ont été injectées dans la virole péricondrale de l'extrémité supérieure du tibia des mêmes poulets. Quatre semaines après, les migrations cellulaires ont été étudiées au microscope. *Résultats:* les migrations de ces cellules ont été vérifiées sur le plan histopathologique. *Conclusion:* On peut affirmer que la virole péricondrale de LaCroix est un réservoir potentiel de cellules souches de croissance chez les jeunes poulets.

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Introduction

The embryogenesis of the germinal layer of the growth plate is still controversial. Prenatal cartilage differs significantly from postnatal cartilage with regard to its cellular elements. In postnatal cartilage, it is hard to find proliferating chondrocytes. Some researches indicate that the origin of those cells capable of becoming chondrocytes, and in turn the source of growth of the long bone, is the perichondrial ring of LaCroix, a circumferential structure in the periphery of the epiphyseal cartilage [1, 2].

The reproduction rate of these cells creating the ring is especially high, and they could represent the main source of precartilaginous cells that migrate to the growth plate and stimulate bone growth [3].

Some studies have showed that removal of the ring of LaCroix can cause a drastic growth arrest of the limbs [4, 5]. This could be explained by the fact that cells creating the

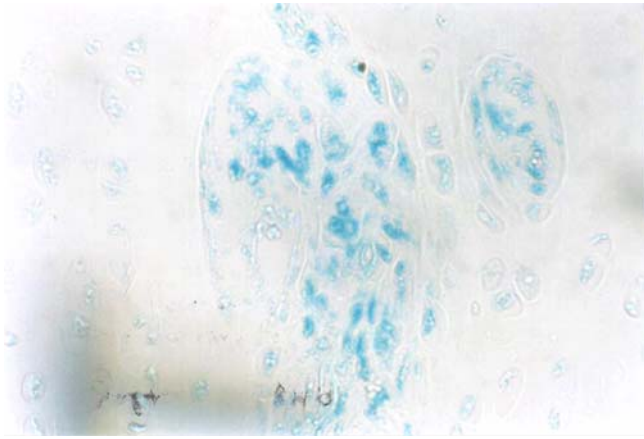


Fig. 1 Chondrocytes transfected with LacZ. (Stain, beta-galactosidase, eosin counter-stain)

ring have a high rate of proliferation and migration, as was proved in *in vivo* biopsies. This contrasts with the characteristic features of chondrocytes derived from articular cartilage or the growth plate.

This ring also contains a thin extension of metaphyseal bone and circumferential collagen fibres that provide stability to the growth plate. Rodriguez and colleagues have demonstrated the important role of the perichondrial ring in the mechanical constraint of the growth plate, which can induce bone formation at the level of an absent ring [6].

Another structure adjacent to the ring is the ossification groove of Ranvier. This groove is a wedge-shaped collection of cells pushing into the reserve and proliferative regions of the growth plate. It also appears to supply cells for the reserve layer, causing mainly an expansion of the diameter of the growth plate [7, 8].

The perichondrium, and other sources such as perivascular stem cells, supply stem cells in pre- and postnatal life. It is hard to follow those cells because of their low number and because they do not produce any specific matrix. Some antibodies are able to recognize mesenchymal precartilagenous stem cells, but they are not widely in use [9].

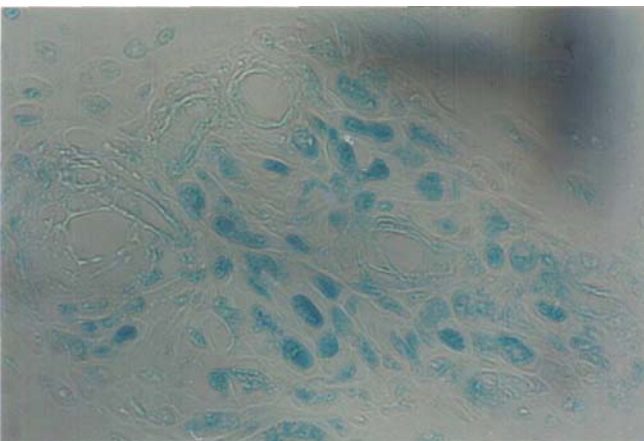


Fig. 2 Chondrocytes transfected with LacZ. (Stain, beta-galactosidase, eosin counter-stain)

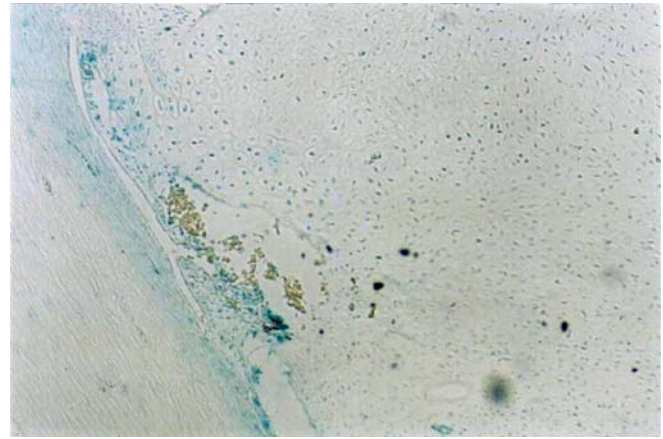


Fig. 3 A histological view of the perichondrial ring and the epiphysis, 1 month after injection of marked cells. Stained cells at the outer layer of the epiphysis, particularly in adjacent areas to the perichondrial ring (Stain, beta-galactosidase, eosin counter-stain; original magnification $\times 400$)

In this study, an attempt was made to trace the route of migration of the precartilagenous cells from the perichondrial ring towards the growth plate.

Materials and methods

As a source for cell culture we used the perichondrial ring of epiphysis from the proximal tibias of two 2-week-old Leghorn chicks. The use of animals for this study and the protocol for procedure were approved by the local ethics committee. Tissue was obtained under local anesthesia, using 2 cc of 2% Lidocaine, in a sterile manner, through an anterolateral approach to the knee. The proximal tibia was surgically removed. The ring of LaCroix was identified as a thin band which extends from the epiphysis toward the diaphysis.

Fragments of the removed ring, 2 mm in size, were cultured in the following way. The fragments were placed for



Fig. 4 A histological view of the perichondrial ring and the epiphysis, 1 month after injection of marked cells. Stained cells at the outer layer of the epiphysis, particularly in adjacent areas to the perichondrial ring (Stain, beta-galactosidase, eosin counter-stain; original magnification $\times 400$)

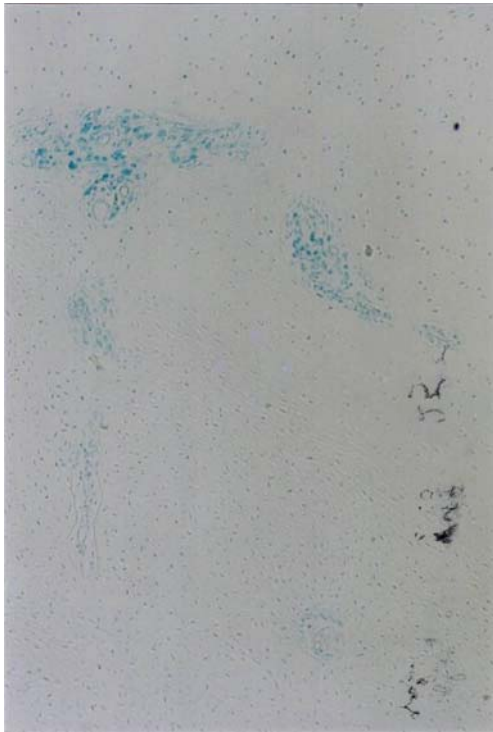


Fig. 5 A longitudinal section through the physis and epiphysis demonstrates the migration of the cells from the periphery transversely through the physis. (Stain, beta-galactosidase, eosin counter-stain; original magnification $\times 400$)

adhesion in plastic bottles for 20 minutes, then a MEM medium (Biological Industries Plant Propagation Limited, Israel) was added, containing 10% foetal calf serum (Biological Industries), L- Glutamine 1% and antibiotics (10 mg/ml streptomycin, 1000 U/ml penicillin and 1.25 U/ml nystatin).

The medium was changed every 4 days for a total of 2 weeks. Gradually the cells left the tissue and adhered to the plastic substrate until they reached confluence within 7 days. Then the cell masses were transferred to new 35 mm TC dishes, using Versene-Trypsin Solution 0.25% (Bio Lab LTD Jerusalem, Israel). To the TC dishes we added a DCCM-1 medium without serum (Biological Industries), containing L-Glutamine 1% and antibiotics—10 mg/ml streptomycin, 1000 U/ml penicillin and 1.25 u/ml nystatin. The dishes were incubated at 37° C in a humidified atmosphere of 5% carbon dioxide and 95% air. The cells propagated and reached confluence in 1 week. Microscopic examinations revealed that the cells had a polygonal morphology typical for cartilage cultures.

The cell cultures were transfected by a vector composed of a replication-defective adenovirus construction, containing the lacZ reporter gene (10:1 viruses to cells). The virus was added to sub-confluent 60-ml culture dishes containing 1 ml of medium, followed by incubation for 2 h at 37° C with 5% CO₂ in air. The virus was then washed three times with the medium and the cultures were maintained for another 48 h. They were then trypsinized. The cell pellets were prepared by centrifugation and then re-suspended.

The suspension was prepared for injection (8^7 cells per ml). Twenty ml were injected into the ring of LaCroix in a subperichondrial location in the proximal tibiae of six 4-week-old chicks. The injection was performed in a sterile manner, under local anesthesia, through an anterolateral approach to the knee and after identification of the perichondrial ring. The animals were kept for 4 more weeks and then were killed.

The migration of the transfected cells was observed histologically using a light microscope. Tissues examined included the injected knee joint. Tissue explants were fixed by paraformaldehyde 4% in 0.15 M phosphate buffered saline, PH 7.4.

Hard tissues were decalcified by ethylenediamine-tetraacetic acid (12.5%), PH 7.0. After thoroughly rinsing in running tap water, the samples were dehydrated by sequential alcohols. The samples then were embedded in paraffin, forming standard blocks, which were sectioned with a regular microtome producing 5 micrometer sections for histological staining. The paraffin was removed by xylol. Before staining, the slides were rehydrated by sequential alcohols. Slides were stained by beta-galactosidase substrate (5-bromo-4-chloro-3-indolyl-D-galactopyranoside, Sigma, St Louis, MO), and counterstained with eosin, yielding a deep blue colour in the LacZ positive cells, with a pink background.

Results

Injection of cells derived from the ring of LaCroix of neonate chicks, transfected in culture with adenoviruses containing the LacZ reporter gene, allows the assessment of the migratory potential of these cells.

The chondrocytes transfected with LacZ could be easily differentiated from the adjacent tissue by the blue colour of their nuclei (Figs. 1 and 2).

On different histological cuts of the perichondrial ring area, a large number of stained cells were identified. Whilst some cells showed invasion of the lumen of small blood vessels in this area of the ring, other stained cells were found at the outer layer of the epiphysis, particularly in areas adjacent to the perichondrial ring (Figs. 3 and 4). Only a small number of stained cells were observed in the rest of the epiphysis, close to the articular layer. So it seems that the cells appear to migrate initially into the perichondrial ring, and later deeper into the area around the physis. Further longitudinal histopathological studies along the bone axis demonstrated a condensed layer of stained cells arranged horizontally along parts of the physis (Fig. 5). In these sections, no marked cells were found in the epiphysis at all.

Discussion

Mature epiphysis structure differs from the foetal epiphysis. This has been shown previously in various studies. Buckwalter and colleagues have shown a different structure between mature and foetal epiphysis in a bovine model;

longer hyaluronic acid central filaments, a greater number of proteoglycan monomers per aggregate, and longer proteoglycan monomer core proteins [10]. Another major difference between foetal and mature epiphysis is the distribution of the mesenchymal progenitor stem cells. Whilst these cells are distributed all over the epiphysis in the foetus, in the mature organized epiphysis, the mesenchymal progenitor stem cells are from different specific reservoirs. A common site of this reservoir is the perichondrial ring of LaCroix.

The perichondrial ring is a circumferential ring in the periphery of the epiphyseal cartilage. Cell cultures derived from the ring of La Croix biopsy specimens show a high rate of cell proliferation and cell migration in vitro. These cells migrate to areas of bone and cartilage formation in the subchondral bone and on either side of the growth plate. In previous studies it was proved that operative removal of the ring of LaCroix causes growth arrest and short stature [11]. In some chondrodysplastic diseases, like achondroplasia, the perichondrium plays a major role since it contains a large amount of the affected Fibroblast Growth Factor Receptor-3(FGFR-3)-expressing cells [3]. Since those cells are responsible for physical growth, the point mutation in the FGFR-3 causes short stature.

Grade six growth-plate injuries also emphasise the importance of the perichondrium with respect to bone growth. In these injuries the main damage is caused to the perichondrial ring, resulting in growth arrest and angular deformities [12].

In this study we were able to demonstrate the route of migration of the precartilaginous cells from the perichondrial ring towards the growth plate. These cells seem to serve as cartilaginous precursor stem cells, playing a major role in endochondral ossification.

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