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Immunohistological Study on Collagen in Cartilage-Bone Metamorphosis and Degenerative Osteoarthrosis*

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Immunhistologische Untersuchungen an Kollagen bei der Knorpel-, Knochenumwandlung und in der degenerativen Osteoarthrose

Zusammenfassung. Unter Verwendung von spezifischen Antikörpern ist es möglich, mit Hilfe immunhistologischer Methoden die Verteilung der verschiedenen Kollagentypen und auch die Kollagensynthese einzelner Zellen zu verfolgen. Diese Methode wurde angewendet, um die Kollagensynthese von Chondrocyten im normalen Gelenkknorpel, Epiphysenknorpel der Wachstumsplatte, osteoarthrotischen Gelenkknorpel, sowie unter in vitro Kulturbedingungen zu verfolgen. Während im normalen Knorpel nur Typ II Kollagen synthetisiert wird, wurde festgestellt, daß die hypertrophierenden großen Chondrocyten an der Basis des Säulenknorpels auf die Synthese des Typ I Kollagens umschalten. Ähnliches gilt auch für die arthrotischen Zellen des Gelenkknorpels. Hier führt die Degeneration zu einer Umschaltung der Kollagensynthese von Typ II auf Typ I Kollagen. Dieses Phänomen kann auch in vitro nachvollzogen werden. In Suspensionskulturen, in denen Chondrocyten in Aggregaten eine knorpelähnliche Matrix aufzubauen vermögen, vollzieht sich der Umschaltungsprozeß langsamer als in Monolayerkulturen.

Diese Beobachtungen zeigen, daß Chondrocyten zur Aufrechterhaltung ihrer Typ II Kollagensynthese eine spezielle Zell-Matrix-Wechselwirkung benötigen. Wird durch eine Veränderung der Knorpelmatrix (z.B. bei dem Degenerationsprozeß der Osteoarthrose) diese Wechselbeziehung gestört, kommt es zu einer Neuorientierung der Kollagensynthese und – in deren Folge – zur Umschaltung auf Typ I Kollagen.

Schlüsselwörter: Knorpel-Knochenumwandlung – Osteoarthrose – Chondrocyten – Kollagensynthese – Immunfluorescenz.

Summary. Synthesis of collagen by chondrocytes was studied by immunofluorescence using antibodies specific for type I, II and III collagen. The following tissues and culture conditions were chosen for this immunohistological study: normal articular cartilage, epiphyseal growth cartilage, cartilage undergoing osteoarthrotic degeneration, suspension culture and monolayer culture. While type II collagen is the unique collagen all over hyaline cartilage, type I collagen is produced by hypertrophic chondrocytes in the growth plate. In addition, chondrocytes in osteoarthrotic areas of articular cartilage synthesize type I collagen. Under in vitro culture conditions, chondrocytes initially produce type II collagen and synthesize later on type I collagen. The change of synthesis from type II to type I collagen is more rapid in monolayer than in suspension culture. It is concluded that the presence of matrix compounds and the cellmatrix interaction as well are necessary to maintain synthesis of type II collagen in chondrocytes. Alterations in the cell-matrix interactions are shown to occur in the hypertrophic zone of the epiphyseal growth plate, in cartilage undergoing osteoarthrotic degeneration as well as in chondrocytes grown in culture. Thus, change in the control of gene activity may subsequently lead to change in collagen synthesis. It is possible that the synthesis of type I collagen, which cannot fulfil the physiological function of a structural element in cartilageneous tissue, is a crucial factor in the process of osteoarthrosis.

Key words: Cartilage-bone metamorphosis – Osteoarthrosis – Chondrocytes – Collagen-synthesis – Immunofluorescence.

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1. Introduction

Collagen fibrous network represents an essential structural element of the connective tissue. It is responsible for the functional integrity of skin, tendon, cartilage, bone, blood vessels and the thin reticulin meshwork, which penetrates and stabilizes most organs. New aspects of the macromolecular organization became prominent by the demonstration of genetically and chemically different types of collagen (collagen polymorphism) which were found in various connective tissues (Chung and Miller, 1975; Epstein, 1974; Gay et al., 1976a). The predominant collagen in skin, tendon and bone is termed type I. Hyaline cartilage consists of type II collagen. Type III collagen has been found in skin and arterial walls and is present as thin network in many other tissues (e.g. liver, spleen, lymph nodes, kidney, etc.). Finally, the collagenous moiety of basement membranes is known as type IV collagen. Type I collagen is unique in so far that its molecule is composed of two $\alpha 1$(I) and one $\alpha 2$ chain. The other collagen types contain three identical polypeptide chains in their molecule: type II: $(\alpha 1(II))_3$, type III: $\alpha 1(III)_3$ and type IV: $\alpha 1(IV)_3$. The various α chains are homologous polypeptide chains which differ in their amino acid composition and sequence as well as in their carbohydrate content. The ability of the individual collagen types to form specific macromolecular structures is of particular importance for the biomechanical properties of the different kinds of connective tissue. Apparently, different types of collagens are necessary to adapt the tissues to their distinct physiological functions. A change in relative proportion of one of the collagen types may result in an alteration of the properties of the particular connective tissue and lead to a pathological condition. It has been reported that in some instances of osteogenesis imperfecta an excess of type III collagen was present (Penttinen et al., 1975; Müller et al., 1975a), while in the Ehlers-Danlos-Syndrom IV no type III collagen was found in skin and arterial walls (Pope et al., 1975). Acquired diseases with disturbance of collagen syntheses are also known. Nimni and Deshmukh (1973) have made the interesting observation that type I collagen is synthesized in culture by pieces of cartilaginous tissue taken from areas undergoing osteoarthrotic degeneration, whereas pieces of cartilage from nondegenerated areas synthesize only type II collagen. This change in collagen synthesis as it is observed in osteoarthrotic tissue could have something in common with similar observation in chondrocyte cultures. Under certain experimental conditions, such as extended culture in vitro, chondrocytes assume a more fibroblastic appearance and synthesize type I collagen or a molecule composed solely of $\alpha 1$(I) chains (Holtzer and Abbot, 1968; Layman et al., 1972; Levitt and Dorfmann, 1974; Mayne et al., 1975; Müller et al., 1975b; Schlitz et al., 1973).

Since type I, II and III collagens are chemically different, it is possible to prepare specific antibodies against each of these proteins (Furthmayr and Timpl, 1976; Hahn et al., 1974; Nowack et al., 1976; Wick et al., 1975). These antibodies have been used for estimating the relative content of different collagen tissues by immunofluorescence techniques (Gay et al., 1975a, b, c; Wick et al., 1975; Gay et al., 1976a; von der Mark et al., 1976) as well as to study collagen synthesis by cultured cells (Müller et al., 1975b; Gay et al., 1976b). In the present study, we investigated by immunofluorescence the distribution of type I and type II collagen in the epiphyseal cartilage growth plate, in the normal articular cartilage and in the cartilage undergoing osteoarthrotic degeneration. In addition, we have studied the types of collagen synthesized by chondrocytes grown under in vitro conditions.

2. Materials and Methods

2.1. Source of Tissues

Samples of epiphyseal cartilage were taken from tibia and femure of new-born infants. In addition, samples of articular cartilage were obtained from the femoral head, femoral condyles and tibia head from persons of various ages with or without degenerative osteoarthrosis. Tissue fragments were removed from patients during corrective surgery and were stored frozen until used. Frozen sections of 4 to 6 µ thickness were cut using a cryotom.

2.2. Cell Cultures

Chondrocytes from sternal cartilage of embryonic chicken and from articular cartilage of calf and human were isolated by limited enzymatic digestion of tissue with collagenase and trypsin (Dehm and Prockop, 1973). Freshly liberated chondrocytes were plated at an initial density of 1×10^5 cells/75 cm^2 plastic flask (Falcon) or at a low density (10^3/flask) which allowed clone-like development. All cells were fed with Ham's F12 medium (Biocult Lab.) supplemented with 10% fetal calf serum, sodium ascorbate (50 ug/ml), penicillin (400 units/ml) and stroptomycin (50 ug/ml). Each flask was flushed with a mixture of CO_2/air (5%/95%) and kept at 37° C.

2.3. Properties of Antibodies Specific for Distinct Types of Collagen

Purified antibodies were prepared from rabbit antisera to bovine type I, II and III collagen and rendered specific for the immunizing antigen by appropriate immunoadsorption procedures (Becker et al., 1976; Nowack et al., 1976). These antibodies showed a high specificity for a distinct type of collagen and negligible cross-reactions with the other types of collagen in hemagglutina-

tion and radioimmune assays. Serological and immunofluorescence studies demonstrated a strong cross-reaction of these antibodies with human collagens (Wick et al., 1975). Antibodies to bovine type I collagen were also produced in rats (Hahn et al., 1974). Cross-reacting antibodies in the rat antisera were removed on a type II collagen adsorbens prior to the isolation of the bulk of the antibodies from a type I collagen adsorbent (Nowack et al., 1976). Purified rabbit antibodies to chicken type I and II collagens were prepared by the same immunoadsorption procedures (von der Mark et al., 1976).

2.4. Indirect Immunofluorescence Technique

Frozen tissue sections (4–6 μ) or air-dried monolayer cultures in plastic plates were used as antigenic substrates. In some experiments these samples were pretreated with hyaluronidase prior to exposure to antibodies (von der Mark et al., 1976). Bone samples were demineralized by treatment with 0.3 M EDTA for several days.

Tissue sections or dried cell cultures were exposed to purified antibodies (50 ug/ml) for 30 min at room temperature. After washing with phosphate buffered saline pH 7.2 (2 times) the samples were treated with FITC conjugated goat antibodies to rabbit IgG (diluted 1:32) or rhodamine-conjugated goat antibodies to rat IgG (purchased from Behringwerke, Marburg). Further details have been described elsewhere (Gay et al., 1975a, b). Antibodies to bovine fibrinogen were used to assess nonspecific staining (Nowack et al., 1976) and gave essentially negative results.

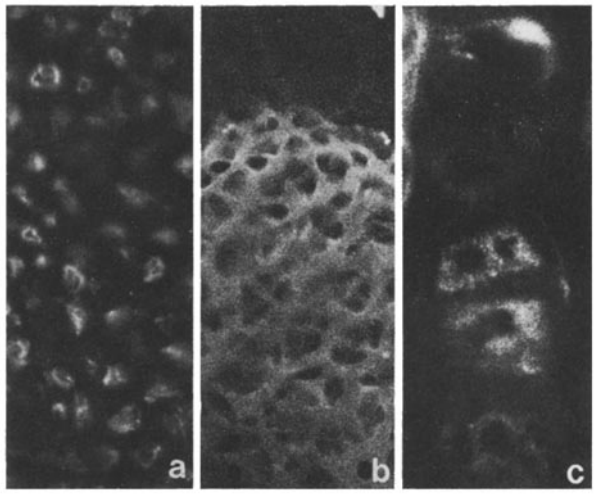

Fig. 1a–c. Articular cartilage without (**a**) and after (**b**) hyaluronidase treatment, stained with anti-type II antibodies. Epiphyseal cartilage (**c**) from the column zone of the growth plate stained with anti-type I antibodies (×200). Tissues not treated with hyaluronidase show fluorescence with anti-type II collagen on chondrocytes and lacunar surface only (**a**). Following treatment with hyaluronidase also the cartilaginous matrix is labeled with anti-type II collagen antibodies (**b**). In addition to type II collagen, enlarged cells of the hypertrophic column zone show weak fluorescence with anti-type I antibodies (**c**)

3. Results

3.1. The Distribution of Collagen Types in Normal Human Cartilage

In sections of articular cartilage chondrocytes and lacunar surfaces only show fluorescence when stained with antibodies directed against type II collagen (Fig. 1a). The cartilage matrix itself, although it is known to contain type II collagen shows little or no fluorescence. However, following treatment with hyaluronidase, the intercellular cartilaginous matrix of these sections also react with the antibodies against type II colagen (Fig. 1b) (von der Mark et al., 1976). This is probably due to an unmasking effect which improves the access of antibodies to the antigen. After staining with antibodies against type I collagen no fluorescence could be observed.

In the developing and growing bone, chondrocytes are arranged in three distinct zones of the epiphyseal growth plate: the reserve zone, the zone of proliferation and the column zone. Using collagen type specific antibodies the state of synthesis of different collagen types in the three distinct zones was investigated: In the reserve zone as well as in the zone of proliferation staining was observed only with type II collagen antibodies. In the column zone, however, the small less mature cells were found to synthesize type II collagen, while in areas of the largest hypertrophic cells, the presence of both type I and type II collagen was demonstrated (Fig. 1c).

Figure 2, a section through the epyphyseal growth plate, depicts the column zone of calcified cartilage and the bone spicules in the zone of first mineralization. After double staining with antibodies specific for type I and type II collagen (Fig. 3a, b) the spicules show a central core stained with anti type II collagen covered by a type I positive tissue layer. This is in agreement with the fact that the calcified cartilage matrix is not completely removed before bone formation. Remnants of this calcified cartilage are used as a temporary scaffolding to lay down the type I collagen containing osteoid matrix. The function of type I collagen which has been synthesized by the enlarged chondrocytes of the column zone before bone formation is not yet clear and will be discussed later on.

3.2. Alterations in the Osteoarthrotic Cartilage

The different stages of osteoarthrotic degeneration are shown schematically in Figure 4. Osteoarthrosis starts as a focal process which is called fibrillation and characterized by the fraying and splitting of the

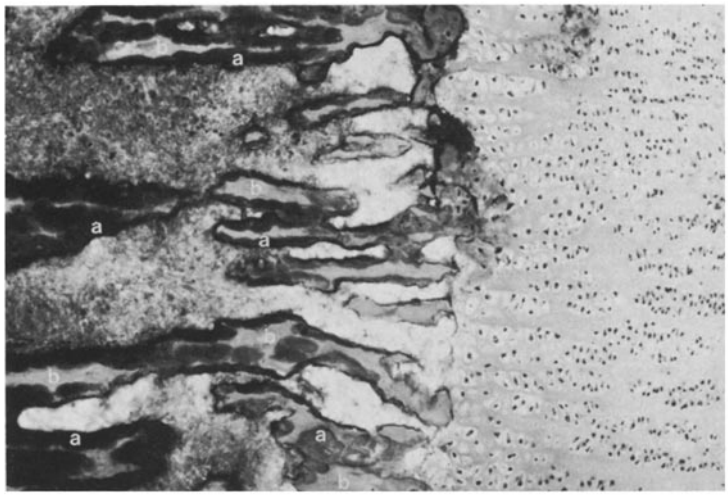

Fig. 2. Epiphyseal growth plate treated with EDTA and hyaluronidase and subsequently stained with hematoxine eosine (×75), **a** bone matrix, osteoid, **b** hyaline calcified cartilage

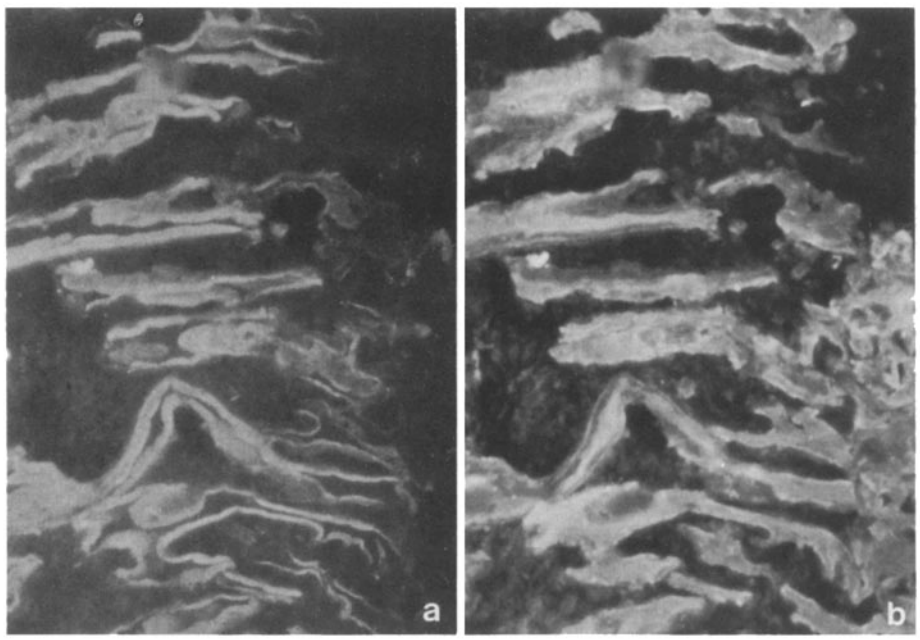

Fig. 3a and b. Bone spicules of the epiphyseal growth plate treated with EDTA and hyaluronidase. Double staining with rat anti-type I antibodies (rhodamine) (**a**) and with rabbit anti-type II antibodies (FITC) (**b**) Bone matrix stains with anti-type I antibodies (**a**). Remnants of hyaline calcified cartilage show fluorescence with anti-type II antibodies (**b**)

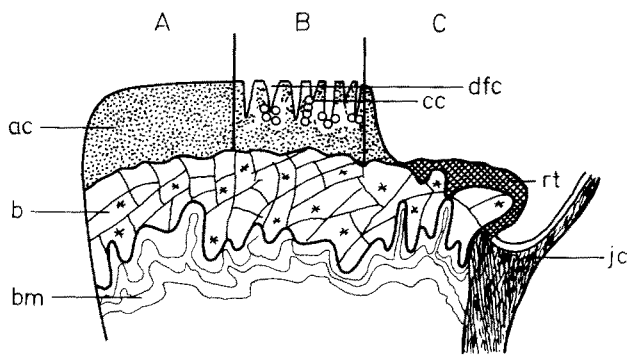

Fig. 4. Schematic representation of the different stages of degenerative osteoarthrosis. *A* Normal articular cartilage, ac articular cartilage, type II collagen, b bone matrix, type I collagen, bm bone marrow with type I and type III collagen. *B* Fibrillated osteoarthrotic cartilage, dfc demasked articular cartilage, type II collagen, cc chondrocyte clusters, type I collagen. *C* Osteoarthrotic "repair tissue", jc joint capsule, type I and type III collagen, rt repair tissue from subarticular bone plate and from juxta-articular or synovial soft tissue, type I and type III collagen

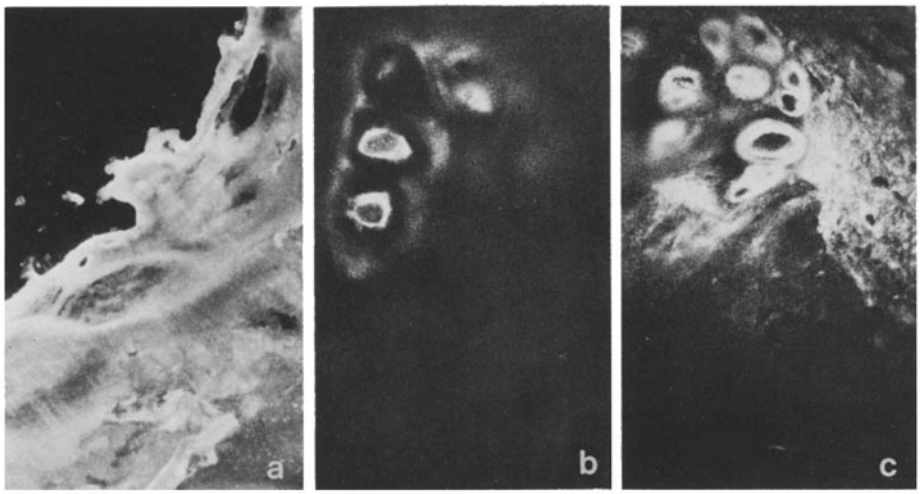

Fig. 5a–c. Cartilaginous tissue from a patient with osteoarthrosis. **a** frozen sections stained with anti-type II antibodies (×300). The unmasked matrix shows fluorescence even without prior treatment with hyaluronidase. **b** Stained with anti-type I antibodies; in areas of fibrillated cartilage the chondrocytes and the margins of the lacunar space contain type I collagen (×300). **c** Stained with anti-type III antibodies; fibrous "repair tissue" (arrow) on the margins of the damaged articular cartilage contains both type I (not shown) and type III collagen (×300)

articular cartilage. In such lesions a breakdown of the extracellular matrix takes place, which can be shown histochemically as a loss of metachromasia. In addition proliferation of chondrocytes is observed which leads to a clustering of cells in areas next to the surface.

As a consequence of the diminished or missing proteoglycans, the matrix surrounding the cell clusters generally is found to stain with anti-type II collagen antibodies even without treatment with hyaluronidase (Fig. 5a). Furthermore, the chondrocytes themselves and the margins of the lacunal spaces of the multicellular clusters in fibrillated areas were found to contain type I collagen as indicated by staining with anti-type I collagen antibodies (Fig. 5b). This change in the molecular form of collagen was produced by cells which are cartilaginous in appearance. In the disturbed tissue increasing proportions of cartilage are lost as fibrillation proceeds. The damaged cartilage has only a minor capability for self repair and to restore previous morphology and function.

The predominant "repair" mechanism occurs by a deposit of non-osseous fibrous tissues or osseous tissue in the remainder of the cartilage tissue. This "repair tissue" arises either from spaces of soft tissue of the subarticular bone plate or from periarticular or synovial tissue, which contain type I and type III collagen synthesizing cells (see scheme in Fig. 4). This newly synthesized tissue shows therefore fluorescence after staining with anti type I and anti type III collagen antibodies (Fig. 5c).

3.3. Collagen Types Synthesized by Chondrocytes in vitro

As shown in the preceding sections, certain physiological and pathological conditions favour a switch of synthesis from type II to type I collagen. The chondrocytes involved in this synthesis are clearly located in and surrounded by cartilage tissue. In both instances it can be assumed that an as yet unknown process has caused a change in the matrix-chondrocytes interaction, which subsequently induced a change in gene expression as seen by the change in the type of collagen synthesized. The influence of various environmental conditions or factors can be studied more easily in in vitro culture systems. Here, it could be shown, that cartilage explants grown in organ culture synthesized type II collagen (Müller and Jamhawi, 1974). The same was shown for desintegrated chondrocytes grown in suspension culture by which cells tend to aggregate (Müller et al., 1975b). A more detailed examination of these aggregates with immunofluorescence shows that cells in the center part stain exclusively with anti-type II collagen antibodies, whereas cells on the surface which are exposed to a different environment show with time increasing fluorescence with anti-type I collagen antibodies (Fig. 6a, b).

The biochemical data from experiments in monolayer cultures showed that after 7 days of culture, about 25% of the collagen synthesized in such cultures was type I. This increased to more than 80%

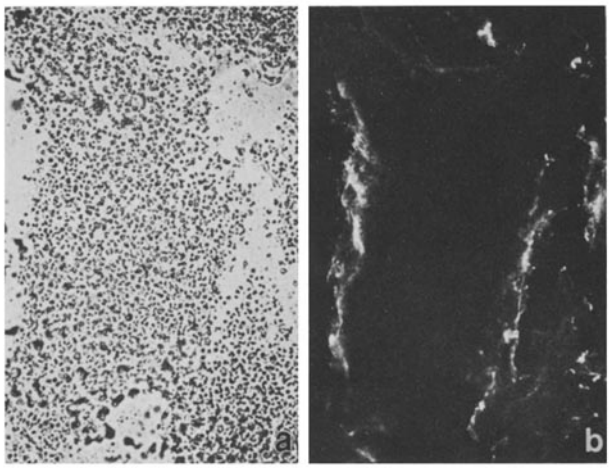

Fig. 6a and b. Typical aggregate of chondrocytes on the 7th day of culture (×75). Cells were isolated from sternal cartilage of embryonic chicken by treatment with collagenase and grown in suspension under standard conditions. **a** bright field illumination of the same field shown in b), **b** stained with anti-type I antibodies. The cells in the inner part of the aggregate continue to fluoresce with anti-type II antibodies (not seen) whereas the cells on the surface begin to show fluorescence with anti-type I antibodies

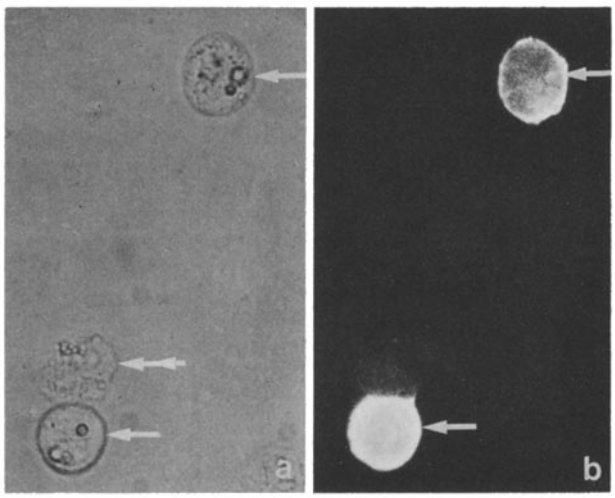

Fig. 7a and b. Freshly isolated chondrocytes from sternal cartilage of embryonic chicken (×400). The cells were plated into small petri dishes and used for studies after 6 h of culture. **a** phase contrast micrograph, **b** stained with anti-type II antibodies. Viable chondrocytes only (←) and not damaged cells (←–←) react with the antibodies

type I collagen after 14 days (Müller et al., 1975b). In the present study, chondrocytes were grown for various periods in monolayer culture under low density conditions and then tested for reaction with anti type I or II collagen antibodies. All viable chondrocytes showed labeling with anti type II collagen antibodies within 4 to 6 h after isolation and transfer in culture (Fig. 7). The number of cells attached to

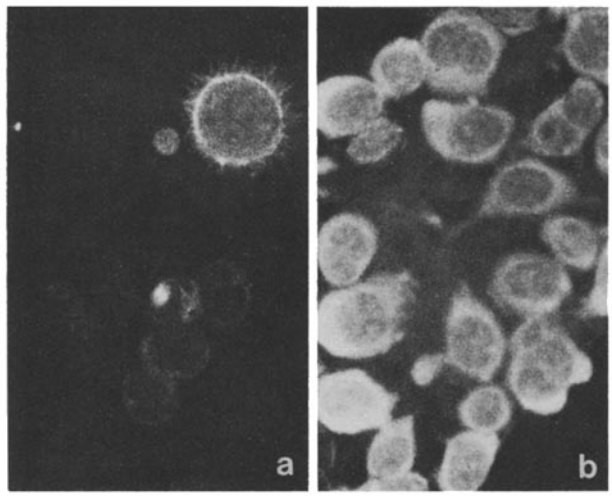

Fig. 8a and b. Chondrocytes isolated from sternal cartilage of embryonic calf (×400). Cells were plated into petri dishes and grown as monolayers under standard conditions. **a** Stained with anti-type II antibodies. A type II collagen synthesizing cell is seen besides dividing cells. The latter do not fluoresce with anti-type II antibodies. **b** Stained with anti-type I antibodies. Chondrocytes on day 7 have gone through several cell divisions. The cells are shown to synthesize type I collagen

the surface of the culture dishes increased continuously during the first 24 h and all cells labeled with anti type II but not with anti type I antibodies. After as little as two days, cells in the chondrocyte cultures started to divide and, subsequently, were labeled with anti-type I collagen antibodies. Figure 8a shows a type II collagen synthesizing chondroblast beside dividing cells which no longer fluoresce with anti-type II antibodies, but stain with anti type I collagen antibodies (not shown). After seven days in culture almost all cells stain only with anti type I collagen antibodies (Fig. 8b). At the same time they begin to change to fibroblast-like appearance. As control, mixed cultures were prepared from fibroblasts and chondrocytes. Beside the morphological appearance, one can clearly differentiate between those cells which synthesize type I and those which synthesize type II collagen by means of specific antibodies.

Discussion

Cartilage-bone metamorphosis and degenerative osteoarthrosis have been studied by morphologists ever since histochemical methods became available to distinguish between various tissue components. In our present study we attempted to view these processes by means of antibodies which were specific for the different collagen types. Since articular cartilage contains type II as an unique collagen type, it was tempting to investigate whether the change in morphology and function is also accompanied by a switch in syn-

thesis from the cartilage to the bone type of collagen. Furthermore, we extended our study to cell culture to determine how chondrocytes respond to changes in their environment.

In the epiphyseal growth plate of the developing and growing bone, cartilaginous tissue is substituted for by osseous bone. In the hypertrophic zone, the intercellular matrix undergoes degradation, chondroblasts proliferate and apparently a change in the cell matrix interaction is induced. This in turn, probably causes a change in synthesis of the chondroblasts as indicated by the appearance of type I collagen. This could mean, that factors from the environment are necessary to maintain the commitment of the chondroblasts to synthesize type II collagen and, probably, cartilage specific proteoglycans. In the hypertrophic cartilage zone in which the type I collagen, produced by enlarged chondrocytes, was found, also the first deposition of osteoid matrix takes place. The question arises, therefore, whether this type I collagen has a physiological function in the process of cartilage bone metamorphosis. Two possibilities can be discussed. First, it may facilitate the induction of the calcification of cartilage, although calcified cartilage is observed in areas where no type I collagen can be detected. Second, it may serve as a substrate for the first deposition of the type I collagen containing osteoid.

Another example for an interference in the cell matrix interaction probably represents the onset of osteoarthrosis. Cartilage destruction can begin in areas exposed to excessive mechanical stress or can follow in the aftermath of a number of inflammatory or metabolic disorders (Freeman and Meachim, 1973; Freeman, 1975; Meachim and Fergie, 1975). Subsequently, the cartilage matrix is depleted of proteoglycans, a fact which is indicated by the low metachromatic reaction as well as by the ready accessability of the antigenic sites of collagen in the matrix. In such areas of tissue the chondrocytes proliferate and form cell clusters (Meachim and Collins, 1962). Subsequently, they synthesize type I collagen. Since type I collagen forms different macromolecular structures no functional tissue can be reformed. The localization of type I collagen around the cell clusters as well as the fact that these clusters are embedded in type II collagen containing cartilage matrix clearly rules out the possibility that type I collagen was synthesized by cells invading these areas. Our results confirm the interpretation of results of Deshmukh and Nimni (1973), and Nimni (1974), who have shown that chondrocytes in the initial lesions of osteoarthrosis synthesize type I collagen.

In later stages of the progressive osteoarthrosis, loose, textured or fibrous connective tissue containing type I and type III collagen proliferates to the joint surface ("extrinsic repair"). In this process, the type I and type III producing fibroblasts and fibroblast-like cells may invade the areas of degraded cartilage in two ways (Landells, 1975). First, the cells can grow out from soft tissue spaces in the subarticular bone plate, when degradation has led to the loss of full-thickness cartilage and caused erosion of underlying bone. Second, when the lesion of the cartilage is located near an articular margin, the cells can spread onto a joint surface from periarticular or synovial tissue.

The influence of matrix-cell interactions on the control of collagen synthesis was further substantiated by our in vitro cell culture studies on isolated chondrocytes. Both mechanisms, proliferation and changing environmental conditions, seem to induce change of collagen synthesis. The results obtained from the suspension cultures suggest, that chondrocytes remain only functional chondrocytes in vitro as long as they form cartilage-like aggregates. In the centre part, the micro environment of each chondrocyte appears to be comparable to the natural condition of intact organs. Therefore, these cells continue to synthesize type II collagen. Cells on the surface, however, are exposed to a different environment, and the synthesis of type I collagen is observed. This means, that cell matrix and cell-cell interaction as well as the presence of matrix components are necessary to maintain cell specificity. In an artificial environment, as it is provided by the culture conditions, the chondrocytes switch from synthesis of type II collagen to synthesis of type I collagen. Similar conclusions can be drawn from the monolayer cultures, the synthesis of which seems to depend on the initial cell density of the culture plates.

These in vitro studies as well as the observations made during the cartilage bone metamorphosis in the growth plate and during the degenerative process of osteoarthrosis suggest that the gene activity of chondrocytes is modulated by cell matrix interaction. Whether the gen activity is controlled at the level of transcription or translation is unclear.

Abbreviations. EDTA = Ethylendiaminetetraacetate, FITC = Fluoresceine isothiocyanate.

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