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Histological Changes During Regression Induced by Retinoic Acid in a Transplantable Rat Chondrosarcoma

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Summary. Daily oral treatment with retinoic acid (100 mg/kg bodyweight) induced regression of a transplantable rat chondrosarcoma. In a previous biochemical investigation we have shown that the tissue breakdown is preceded by the loss of proteoglycan. The present study describes the histological changes induced by retinoic acid. A decrease in the intensity of metachromatic staining with toluidine blue was noted already after 1 day and the discoloration was almost complete after 4 days correlating with the loss of proteoglycan.

Especially in the perichondrium there was a rapid proliferation of fibroblasts and monocytes. Osteoclast-like cells were missing, but tumor nodules were arroded and split up by penetrating perichondrium.

After 4 days of treatment larger necrotic areas were found, initially in the center of tumor nodules only. In other areas the majority of tumorous chondroblasts survived. Tumor nodules appeared partly mesenchyma-like with some fibroblast-like cells suggesting a dedifferentiation of chondroblasts by retinoic acid. We believe that tumor regression induced by retinoic acid involved proteoglycan degradation by chondroblasts themselves and chondroclast-like activity of monocytes and fibroblasts.

Key words: Retinoic acid – Chondrosarcoma regression – Proteoglycan release – Chondroclast-like activity

Vitamin A takes a key position in bone growth and is essential for the normal differentiation of epithelial tissues (Moore 1972). Retinoids, the natural and synthetic analogs of vitamin A also possess anticarcinogenic and antitumor activities (Sporn et al. 1976; Bollag 1979; Lotan 1980). In clinical trials positive therapeutic results have been achieved in leukoplakias, actinic keratoses, basal

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cell carcinomas and urinary bladder carcinomas with various retinoids (Bollag 1979). A major handicap in using retinoids in practice is their toxicity which manifests in the so-called hypervitaminosis A-syndrome in both animal and man (Bollag 1979). This has led to a wide search for synthetic retinoids in order to detach the therapeutically useful activity from the toxic side-effects. However, up to now this has not proven possible (Bollag 1979).

It has been shown that 13-cis-retinoic acid (Heilman and Swarm 1975) and some aromatic retinoids (Trown et al. 1976) inhibit and reverse the growth of a transplantable rat chondrosarcoma. The potential therapeutic value of retinoids for the treatment of chondrosarcomas is presently investigated in clinical trials (W. Bollag, personal communication). We have recently demonstrated that the retinoic acid-induced regression of rat chondrosarcoma is preceded by the loss of proteoglycan, representing degradation of matrix (Kistler and Hartmann 1980). The release of proteoglycan is inhibited by inhibitors of RNA and protein synthesis suggesting that the catabolic effect of retinoic acid depends on continuous RNA and protein synthesis. Furthermore, ethylenediaminetetraacetic acid but not other proteinase inhibitors blocks the retinoic acid-induced proteoglycan release and Zn^{2+} restores this suppression completely. These findings indicate that the degradation of proteoglycan induced by retinoic acid may depend on newly synthesized metal-dependent proteinases (Kistler and Hartmann 1980).

Breakdown of matrix of articular cartilage also takes place in chronic arthritic disease; its extent correlates well with the resulting articular disability (Rodnan et al. 1973). The study of vitamin A action might, therefore, be a suitable approach for further understanding of the metabolism, turnover and of certain pathologic processes in cartilage and bone. Some biochemical changes in response to retinoic acid in a transplantable rat chondrosarcoma have been reported previously (Kistler and Hartmann 1980) as mentioned above. In the present study we describe histological changes induced by retinoic acid in this tumor.

Materials and Methods

The tumor used in this study originated as a spontaneous tumor in a rat and was described as a osteochondrosarcoma by Maibenco et al. (1967). By the time some elements such as bone or osteoid were lost. Recently, Breitkreuz et al. (1979) characterized the tumor as chondrosarcoma. We obtained the tumor from Dr. P.W. Trown and maintained it according to the method described by Trown et al. (1976) as a subcutaneous transplant in F344 female rats (Charles River Breeding Laboratories, North Wilmington, MA, USA). In view of our investigation 0.5 ml of a 25% (w/v) tissue homogenate in a balanced salt solution supplemented with 1.0% glucose, 2.5% streptomycin and 50,000 IU penicillin G were injected subcutaneously in the right flank of approximately 4 weeks old female rats.

Six weeks after transplantation of tumorous cells a control group of 5 rats was killed and the tumors were removed. Starting at the same time 4 groups of 5 animals were daily intubated orally with 100 mg of retinoic acid/kg bodyweight for 1, 2, 4 and 7 days, respectively. This high dose of retinoic acid resulted in a marked regression of the tumor within 1 week of treatment (Kistler and Hartmann 1980). A regression to a similar degree of the same tumor using lower doses of aromatic retinoids needs a longer treatment period (Trown et al. 1976). Retinoic acid (F. Hoffmann-La Roche & Co., Ltd., Basle, Switzerland) was suspended in rape seed oil. Animals were killed 24 h after the last dosing. Of each tumor a part was fixed in phosphate-buffered 3.5% formaldehyde and the rest was used for biochemical studies (Kistler and Hartmann 1980).

Paraffin sections (4 μm) were stained with toluidine blue at pH 3.5 and hematoxylin-eosin (HE). Trichromatic staining (Masson-Goldner) and silver-staining were done according to the techniques of Romeis (1968).

Results

Implantation of Tumor Cells and Tumor Growth

Subcutaneously implanted tumor cells grew well and rapidly in all animals. On palpation the tumor was clearly delimited and firmer than the surrounding tissue, but softer than that of normal cartilage. At the time of sacrifice of the control animals the mean tumor weight $\pm$ SD was 4.2 ± 1.2 g ($N=5$). A cross section revealed a slightly pink and opaque tissue with a fibrous capsule and fibrous septa dividing the tumor into numerous nodules of different size (Fig. 1 A).

Histology of the Untreated Tumor

The typical structure of cartilage was preserved. One or several tumorous chondroblasts surrounded by a pericellular area of matrix of a lighter colour in HE, formed a chondrone (Fig. 1 B). The latter were bigger towards the center of the nodules. Chondroblasts as a whole as well as their nuclei were polymorphous in size and shape, especially on the border of the nodules. Masson's trichrome and HE staining revealed single or multiple nucleoli of different size practically within each nucleus. Binucleated cells were found. Mitotic figures in the classic sense were hardly detectable. However, the arrangement of peripheral chondroblasts as well as the aspect of their nuclei suggested some mitotic activity. The cytoplasma was rather granular. The nucleus/cytoplasma ratio was about $^1/_2$ to $^1/_3$. Small foci of a few necrotic chondroblasts were found.

The matrix stained intensively metachromatic with toluidine blue. Double refraction was increased in the interstitial space, but only in the periphery of the nodules collagenous fibres could be detected. Silver staining did not reveal additional details.

The septa between the tumor nodules consisted of a loose connective tissue with a moderate number of fibrocytes and occasional monocytes. The perichondrium was formed by a denser connective tissue with small fibroblasts (Fig. 1 C). There were only a few blood vessels in the connective tissue.

Effect of Treatment With Retinoic Acid

During the 7 days of treatment of the rats with 100 mg of retinoic acid/kg bodyweight per day the tumor decreased to about half of its original size. It was still well delimited but slightly softer. Microscopically, the most prominent feature was a decrease in the intensity of metachromatic staining with toluidine blue. Focal discoloration of the matrix was observed already after 1 day, affecting the matrix within chondrones as much as the matrix between them. After 2 days whole nodules were involved in this process (Fig. 2 A), while others remained practically unaffected. After 4 days discoloration was almost complete

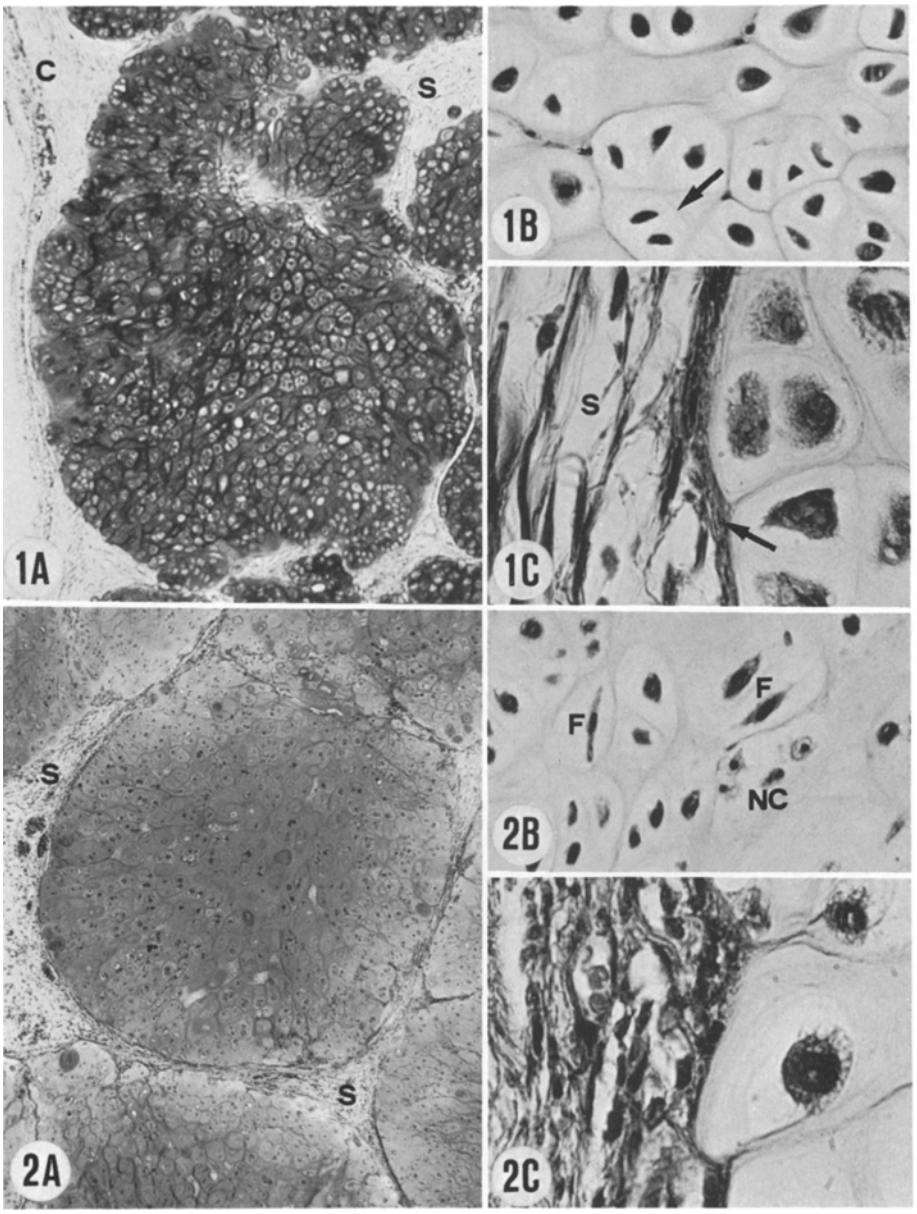

Fig. 1 A–C. Chondrosarcoma of untreated rats. **A** Tumor nodule clearly delimited by a loose connective tissue of septa (*S*) and capsule (*C*) encysting the whole tumor. The matrix stained intensively metachromatic with toluidine blue, ×30. **B** Polymorphous tumorous chondroblasts. Chondrones stained lighter (*arrow marks* border of a chondrone), HE, ×200. **C** Loose connective tissue of septum (*S*). Thin perichondrium (*arrow*) on surface of tumor nodule. HE, ×500

Fig. 2 A–C. Chondrosarcoma of rats treated with 100 mg of retinoic acid/kg bodyweight for 1 day (**C**) and 2 days (**A** and **B**). **A** Decrease of intensity of metachromatic staining with toluidine blue. Increased number of cells in septa (*S*) ×30. **B** Appearance of focally numerous fibroblast-like cells (*F*) situated predominantly within chondrones. Note some necrotic chondroblasts (*NC*). HE, ×200. **C** Increase of number of monocytes and fibroblasts in perichondrium and septum. HE, ×500

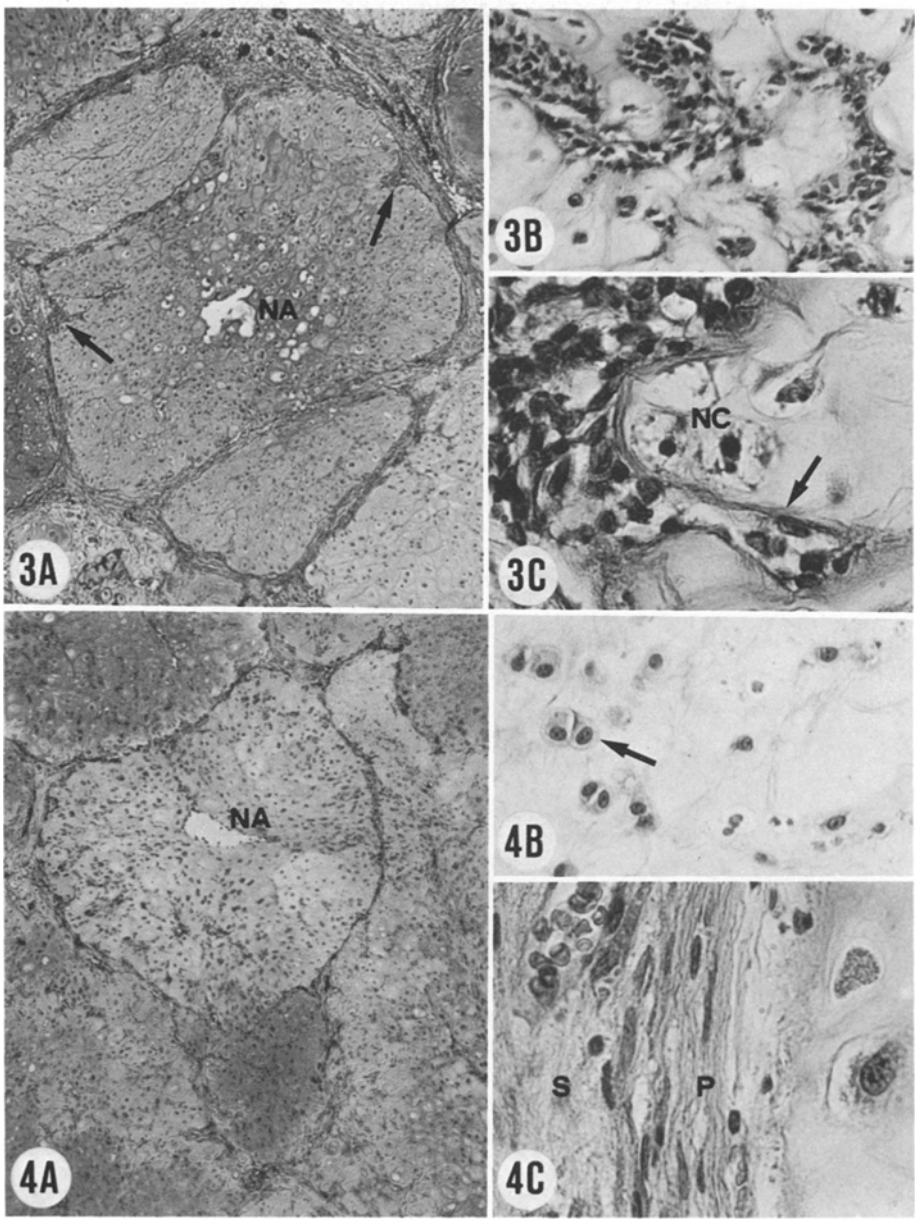

Fig. 3A–C. Chondrosarcoma of rats treated with 100 mg of retinoic acid/kg bodyweight for 4 days. **A** Further decreased intensity of staining of tumor nodules with toluidine blue. Septa penetrating into the tumor (*arrow*). Necrotic area (*NA*) in the center, ×30. **B** Central part of a tumor nodule interspersed with apparently new, cell-rich septa. HE, ×200. **C** Perichondrium penetrating into the tumor nodule (*arrow*) consisting of partly necrotic chondroblasts (*NC*). HE, ×500

Fig. 4A–C. Chondrosarcoma of rats treated with 100 mg of retinoic acid/kg bodyweight for 7 days. **A** Shrunken tumor nodules. Note central necrotic area (*NA*) and hardly detectable metachromatic staining with toluidine blue. ×30. **B** Area with different stages of necrosis of chondroblasts. Grossly unaffected chondroblasts (*arrow*), HE, ×200. **C** Fibrosis of perichondrium (*P*) and septum (*S*) HE, ×500

(Fig. 3A). At that time also the amount of collagen visible in Masson's trichrome staining was markedly increased. After 7 days most tumor nodules were smaller than those of the control (Fig. 4A).

After 2 days of treatment the chondroblasts appeared to be smaller. The number of spindle cells which were rare in untreated tumors was increased (Fig. 2B). These fibroblast-like cells also occurred in the center of tumor nodules and were often located within chondrones. With longer treatment chondrones disappeared partly and some cells tended to cluster giving so a mesenchymal appearance to the tissue.

Some spongeous areas appeared predominantly in the center of tumor nodules as a result of focal necrosis after 2 to 3 days of treatment (Fig. 3A). After 7 days of treatment the necrotic areas of some tumor nodules were very extended. However, even in these cases apparently normal chondroblasts were still found focally (Fig. 4B).

Perichondrium and septa were thickened already after 1 day of treatment. A marked proliferation of fibroblasts obviously producing collagen and of monocytes was noted (Fig. 2C). Some lymphocytes were found, however, granulocytes and osteoclast-like cells were missing. On the 2nd and especially on the 4th day of treatment the perichondrium showed a marked tendency to penetrate into the tumor nodules (Fig. 3C). Initially penetration took place predominantly in the areas between the chondrones. After 4 and 7 days of treatment the tumor nodules were interspersed with partly thin, partly large septa (Fig. 3B). Vascularisation of the tumor was remarkably increased. The cell density in the connective tissue was no longer so prominent on the 7th day of treatment and a partly marked fibrosis of perichondrium and septa was found (Fig. 4C).

Discussion

The chondrosarcoma we investigated was similar in behaviour and morphology to the one described by Breitkreuz et al. (1979). However, we found chondroblasts to be larger and more polymorphous towards the periphery of the tumor nodules than towards the center.

Growth and aspect of the chondrosarcoma was altered drastically by administration of retinoic acid. An early and prominent feature was the discoloration of the matrix observed in toluidine blue staining; this phenomenon was correlated directly with proteoglycan release as earlier demonstrated. (Goodman et al. 1974; Bard and Lasnitzki 1977; Kistler and Galli 1979).

Some authors explain the retinoic acid action as a labilization of membranes resulting in autodigestion by lysosomal enzymes (Fell and Dingle 1963). However, as we have shown, proteoglycan release depends on de novo synthesis of protein (Kistler 1978; Kistler and Hartmann 1980; Gallandre et al. 1980). Based on investigations of the mode of action of retinol carried out with a cavity organ culture method, also Dingle and Dingle (1980) suggest that matrix degradation by soluble tissue proteinases, as e.g. lysosomal enzymes, is unlikely. Proteoglycan release was also found in cultures of cubes of fetal cartilage which contained only cartilage cells (A. Kistler and B. Galli 1980, unpublished results).

Therefore, we assume that proteoglycan degrading enzymes are a product of the chondroblasts themselves. This hypothesis is also supported by the observation in this study that apparently no free cells such as leukocytes or macrophages were necessary for this step of matrix degradation.

Another distinct morphological feature occurring in the tumor after treatment with retinoic acid was the appearance of fibroblast-like cells. Those found between chondrones may have originated in the connective tissue of septa and perichondrium and penetrated through the softened matrix. However, for those spindle cells found within chondrones we have to assume a transformation of chondroblasts in situ induced by retinoic acid. Simultaneously the amount of visible collagen was also increased. Part of these fibers may have been unmasked in the course of proteoglycan release. Other fibers could have been formed by the fibroblast-like chondroblasts.

With continuation of treatment certain parts of the tumor took a mesenchymal appearance partly with cell contact between 2 or more chondroblasts. Hassell et al. (1978) found that limb bud mesenchymal cells remain in a mesenchymal state under the influence of retinoic acid. Gallandre et al. (1980) reported reversion of chondrogenesis by retinoic acid. They have also shown that staining for microsomal alkaline phosphatase in differentiating chondroblasts disappears after treatment with retinoic acid. This indicates regressive changes within cartilage cells of which the molecular mechanism is still unknown. Several findings suggest that vitamin A may control cellular differentiation by regulating gene expression (for a review see Lotan 1980).

In the course of treatment important changes occurred already early in the connective tissue of the septa and perichondrium, namely proliferation of fibroblasts and monocytes and increase of collagen. Proliferation of endost and periost cells was also reported for hypervitaminosis A in normal bone and cartilage of rats (Studer 1950) and humans (Rineberg and Gross 1951). The observed arrosion and penetration of the tumorous cartilage was suggestive for chondroclastic activity of the perichondrium. However, typical osteo-chondroclasts were missing completely. Already Heersche (1978) postulated that osteoclastic bone resorption may also involve mononuclear, fibroblast-like or monocyte-derived cells. Further evidence for osteoid resorption by mononuclear cells was given recently by Rifkin et al. (1980). These proliferative changes in the connective tissue suggest a more complex mechanism of action of retinoic acid than the cytostatic effect produced by conventional antitumor agents.

In conclusion, chondrosarcoma regression induced by retinoic acid seemed to involve (a) a generalized proteoglycan release by proteoglycan degrading enzymes probably produced by tumorous chondroblasts, (b) chondroclast-like activity of monocytes and fibroblasts especially of the proliferating perichondrium and (c) necrosis of certain parts of the tumor, probably as a secondary effect.

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