Effect of Aromatic Retinoids on Rat Chondrosarcoma Glycosaminoglycan Biosynthesis

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SUMMARY

Synthetic aromatic analogs of retinoic acid were administered i.p. and p.o. to Fischer F344 rats bearing a transplantable chondrosarcoma. 

13CO2 incorporation into glycosaminoglycans were compared for neoplastic and normal cartilage explants after removal from animals given various analogs. There was a direct relationship between [35S]glycosaminoglycan synthesis by chondrosarcoma chondrocytes and inhibition of tumor growth. The degree of inhibition of [35S]glycosaminoglycan synthesis in the neoplastic cartilage was dependent on the dose of the retinoid administered. At 20-mg/kg/day doses for 4 weeks, 

35SO4 incorporation into glycosaminoglycan by treated tumor explants was reduced as much as 95%. There was no re-

duced i.p. and p.o. to Fischer F344 rats bearing a transplan-

table chondrosarcoma. 

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duction of [35S]glycosaminoglycan produced in normal 

costal cartilage of the same animals. Retinoid treatment 

of 20-mg/kg/day doses for 4 weeks resulted in a 75% re-

duction in glycosaminoglycan per mg of chondrosarcoma; 

there was no reduction in cartilage glycosaminogly-

can. Retinoid (10- to 20-mg/kg/day doses) elevated col-

lagen levels per mg of chondrosarcoma but had no effect 

on costal cartilage collagen. Combined in vitro and in vivo 

studies showed that retinoid administration modified neo-

plastic chondrocyte function but had no measurable effect 

on normal chondrocyte function.

INTRODUCTION

Vitamin A (retinol) and vitamin A acid (retinoic acid) have been shown to prevent chemically induced epithelial meta-

plasias, tumors, and carcinomas (1–3, 6, 25). In addition to 

the described prophylactic effect, vitamin A as well as vita-

min A acid and its derivatives have also been reported to 

have a therapeutic effect in precancerous epithelial lesions. 

This group of compounds caused regression and disap-

pearance of established carcinogen-induced skin papil-

lomas in mice (2). Therapeutic results have also been re-

ported in man for actinic keratoses and basal cell carci-

nomas (4), leukoplakias of the oral cavity (24), and urinary 

papillomas (11).

Recently, Heilman and Swarm (14) reported the regres-

sion of an established transplantable rat chondrosarcoma 

by treatment with 13-cis-retinoic acid. Trown2 extended this 

observation and reported the activity of other synthetic reti-

noids in inhibiting growth and achieving regression of the 

chondrosarcoma.

We have recently reported on the inhibition of glycosami-

noglycan biosynthesis by retinoic acid in rat costal carti-

lage in vitro (28). We have now extended those studies and have 

examined the effect of synthetic retinoids on glycosamin-

glycan and collagen biosynthesis in explants of rat chondro-

sarcoma and costal cartilage removed from animals that 

have been treated with these analogs for periods of 2 or 4 

weeks.

MATERIALS AND METHODS

Tumor Transplantation. The description of the rat chon-

drosarcoma used in this study has been previously reported 

(5, 19). The tumor was transplanted by a s.c. trocar into the 

right inguinal region of Fischer F344 rats. Tumors were 

palpable 3 to 5 weeks after transplantation. Drug therapy 

was initiated 5 to 7 weeks after transplantation. Stock prepa-

rations of synthetic retinoids were prepared by sonically 

disrupting 500 mg of compound for 2 mm in 25 ml of 

deonized water containing 0.01% Triton X-100 and 0.1% 

carboxymethylcellulose. Preparations were stored at 4° un-

der argon gas. Compounds were administered i.p. daily 

Monday to Friday. Experiments were terminated on Mon-

days. Alternatively, retinoids were administered as a dietary 

additive. Gelatin beadlets containing 10% drug (w/w) were 

mixed with Purina rat chow. Mixtures were prepared to give 

the indicated amount of drug assuming that a rat ate 10 g of 

chow per 100 g of body weight.

35S Incorporation Into Chondrosarcoma and Costal Car-

tilage. Cartilage was removed from the animal and carefully 

trimmed free of adherent connecting tissue under a dissect-

ing microscope. The cartilage was minced and incubated 

in 0.5 ml of Krebs-Ringer bicarbonate for 2.5 hr at 37° under a 

mixture of O2:CO2 (95:5, v/v). The incubation contained 50 

to 70 mg of cartilage (wet weight) and 5 × 104 cpm of 

carrier free H235SO4. MgCl2 was substituted for MgSO4 in the 

Krebs-Ringer buffer to avoid isotope dilution of the added 

radiolabeled material. The incubation was terminated after 

2.5 hr by rapidly withdrawing the media and by washing the 

cartilage twice with 3 ml of 1.15% KCl. A 1.0-ml solution of 

0.1 M acetate buffer (pH 5.5) containing 0.75 mg papain, 5 

mm EDTA, and 5 mm cysteine was added to the washed 

cartilage. The papain digestion was maintained at 57°. After 

18 hr, 50% trichloroacetic acid was added to give a final

1 To whom requests for reprints should be addressed.

2 P. Trown. The Effect of Retinoids on Skin Papillomas in Mice and 

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trichloroacetic acid concentration of 10%. After dialysis overnight, a 25-μl aliquot of the trichloroacetic acid supernatant solution containing the glycosaminoglycans was chromatographed on Whatman No. 3 paper in isobutyric acid:0.5 M NH₄OH (5:3, v/v) overnight (30). The origins containing the radioactive glycosaminoglycans were cut out, placed in 10 ml of Aquasol, and counted in a liquid scintillation spectrophotometer.

**Glycosaminoglycan Determination.** The total glycosaminoglycan content of the cartilage was determined by withdrawing a 25-μl aliquot of the papain digestion mixture after trichloroacetic acid treatment. The aliquot was reacted quantitatively with Alcian Blue according to the method of White- man (32).

**Collagen Determination.** Cartilage (50 to 60 mg, wet weight) was hydrolyzed in 6 n HCl in a vacuum for 20 hr. The hydrolysate was dried free of HCl, reconstituted with buffer, and applied to a Durham amino acid analyzer. The amount of collagen was calculated by multiplying the ng of hydroxyproline per mg of cartilage by a factor of 13.

**RESULTS**

**Effect of Retinoids on [35S]Glycosaminoglycan Produced by Chondrosarcoma Explants.** Rats were given 3 synthetic retinoids (Chart 1) for 2 weeks (Chart 2A) and 4 weeks (Chart 2B) i.p. as described in "Materials and Methods." At the end of that period, the animals were sacrificed and the chondrosarcoma and costal cartilage were removed. The size and weight of the tumors were recorded. Explants of neoplastic and costal cartilage were prepared and incubated with H₂¹⁵SO₄ as described in "Materials and Methods." The chondrosarcoma explants removed from animals given retinoids for 2 weeks (Chart 2A) showed a decreased ability to fix ³⁵SO₄ into glycosaminoglycan with increasing dose of drug. With Ro 10-1670 and Ro 11-1430 (For explanation of Ro numbers, see legend to Chart 1), there was a large decrease in [³⁵S]glycosaminoglycan production at 10- and 20-mg/kg/day doses. After 4 weeks of drug treatment (Chart 2B), the ability of the neoplastic cartilage to fix ³⁵SO₄ into glycosaminoglycan was dramatically reduced. At drug levels of 10-mg/kg/day doses the explants produced only 10 to 15% of [³⁵S]glycosaminoglycan relative to the control explants. At levels of 20-mg/kg/day doses of Ro 10-1670 and Ro 11-1430, the amounts of [³⁵S]glycosaminoglycan produced by the explants were only 3 to 5% that of the control tumor explants.

![Chart 1](image)

**Effect of Retinoids on [³⁵S]Glycosaminoglycan Produced by Costal Cartilage Explants.** In A, explants were prepared from animals receiving the indicated drug for 2 weeks i.p. as described in "Materials and Methods." Each explant incubation contained 50 to 60 mg of tumor. The [³⁵S]glycosaminoglycan produced has been normalized to total glycosaminoglycan per explant. The results are a combination of 2 separate experiments with 2 animals per group per experiment. The control tumors fixed 7600 cpm [³⁵S]glycosaminoglycan per μg glycosaminoglycan per 2.5 hr of incubation. Control tumors weighed 4.3 g (wet weight). In B, animals received drug for 4 weeks i.p. The experimental conditions were identical to those of the 2-week study. Control tumors fixed 3480 cpm [³⁵S]glycosaminoglycan per μg glycosaminoglycan per 2.5 hr of incubation. Control tumors weighed 24.0 g (wet weight).

![Chart 2](image)

**Effect of Retinoids on [³⁵S]Glycosaminoglycan Produced by Costal Cartilage Explants.** The incorporation of ³⁵SO₄ into costal cartilage glycosaminoglycan was measured in the tumor-bearing animals after retinoid treatment (Chart 3). After 2 weeks of drug treatment (Chart 3A), there was no inhibition of ³⁵SO₄ incorporation into costal cartilage explants unlike that of neoplastic cartilage explants (Chart 2A). After 4 weeks of drug treatment (Chart 3B), there was no decrease in the ability of costal cartilage to fix ³⁵SO₄ into glycosaminoglycan. This observation differs sharply from that described for neoplastic cartilage (Chart 2B).

**Correlation between Tumor Weight and Sulfate Incorporation**

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Chart 2. Effect of retinoids on [³⁵S]glycosaminoglycan production by chondrosarcoma explants. In A, explants were prepared from animals receiving the indicated drug for 2 weeks i.p. as described in "Materials and Methods." Each explant incubation contained 50 to 60 mg of tumor. The [³⁵S]glycosaminoglycan produced has been normalized to total glycosaminoglycan per explant. The results are a combination of 2 separate experiments with 2 animals per group per experiment. The control tumors fixed 7600 cpm [³⁵S]glycosaminoglycan per μg glycosaminoglycan per 2.5 hr of incubation. Control tumors weighed 4.3 g (wet weight). In B, animals received drug for 4 weeks i.p. The experimental conditions were identical to those of the 2-week study. Control tumors fixed 3480 cpm [³⁵S]glycosaminoglycan per μg glycosaminoglycan per 2.5 hr of incubation. Control tumors weighed 24.0 g (wet weight).
The relationship between tumor weight (g) after retinoid treatment and the ability of the explants to incorporate $^{35}$S sulfate into glycosaminoglycan was examined. As seen in Chart 4, there was a direct relationship between relative tumor weight (expressed as weight of treated tumor per weight of control tumor) and the amount of $^{35}$S glycosaminoglycan produced (expressed as treated explant/control explant). For any given drug (Chart 4A), there was a simultaneous decrease in tumor size and ability of the tumor explant to fix $^{35}$S sulfate in glycosaminoglycan per mg of explant. In the i.p. experiment represented in Chart 4B, this relationship was linear over the entire range of tumor weights. In the p.o. experiment represented in Chart 4B, this relationship existed for treated tumors that were less than 50% of the control tumor weights.

**Effects of Retinoids on $^{35}$S Glycosaminoglycan Production.** The effect of p.o. administration of Ro 11-1430 on tumor growth and $^{35}$S glycosaminoglycan production was investigated (Chart 5A). At levels of 40 and 80 mg/kg p.o. (intubation), Ro 11-1430 significantly reduced the levels of $^{35}$S sulfate incorporated into glycosaminoglycan after a 2-week period. However, there was no effect of Ro 11-1430 on $^{35}$S sulfate fixation into costal cartilage explants from animals bearing the chondrosarcoma. Drugs were also administered as a dietary additive over a 4-week period (Chart 5B). $^{35}$S sulfate incorporation into glycosaminoglycan of neoplastic explants was inhibited over 95% by 10 and 20 mg/kg of drug.

**Total Cartilage Glycosaminoglycan after Retinoid Treatment.** The levels of glycosaminoglycan per mg of cartilage were determined in the neoplastic and costal cartilage after drug treatment (Chart 6). After 2 weeks of drug treatment (i.p.), total glycosaminoglycan per mg of cartilage was reduced in the chondrosarcoma by 20-mg/kg/day doses of retinoid (Chart 6A). There was no effect of drug on costal cartilage glycosaminoglycan (Chart 6B). After 4 weeks of drug treatment (Chart 6A), the level of glycosaminoglycan per mg tissue was greatly reduced in the chondrosarcomas of animals receiving 10- and 20-mg/kg/day doses. There was no reduction in the glycosaminoglycan per mg of costal cartilage of the same animals.

**Collagen Determinations.** The amount of collagen was calculated from the hydroxyproline content of an acid hydrolysate of the cartilage (Table 1). The levels of collagen (per mg of tissue) actually increased with retinoid treatment. The levels of glycosaminoglycan decreased in a predictable manner with retinoid treatment.
Table 1

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<th>Retinoid</th>
<th>Administration (wk)</th>
<th>Dosage (mg/kg)</th>
<th>Glycosaminoglycan (μg/mg tissue, wet wt)</th>
<th>Collagen (μg/mg tissue, wet wt)</th>
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<td>0</td>
<td>21.0</td>
<td>22.5</td>
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<tr>
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<td>2.9</td>
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<td>77.0</td>
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<td></td>
<td></td>
<td>5</td>
<td>6.6</td>
<td>73.6</td>
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<td></td>
<td></td>
<td>2.5</td>
<td>18.1</td>
<td>20.4</td>
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<tr>
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<tr>
<td></td>
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<td>5</td>
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<td>10</td>
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* The determination of glycosaminoglycan and collagen levels are described in "Materials and Methods."

DISCUSSION

The involvement of retinol in differentiation and maintenance of epithelial cells is well established (7, 10, 13, 16, 21, 33). Vitamin A, vitamin A acid, and its derivatives have been reported to reverse carcinogen-induced epithelial lesion in vivo (1–4, 6, 9, 11, 23, 25) and in vitro (7, 8, 18, 29, 31). To date, these compounds have been reported to be effective chemotherapeutics in only 2 transplantable tumors, a highly immunogenic murine melanoma (13) and the rat chondrosarcoma used in this study (14).

We have shown that retinoids given p.o. or i.p. to rats bearing chondrosarcoma will result in: (a) tumor regression; and (b) an inhibition of the neoplastic cartilage explants to fix $3^5$SO$_4^-$ into glycosaminoglycan per mg of tissue (Chart 2). There was also a reduction in the total glycosaminoglycan per mg of tissue (Chart 6). There was no effect on the ability of cartilage explants to fix $3^5$SO$_4^-$ into glycosaminoglycan or in the total glycosaminoglycan per mg cartilage (Charts 3 and 6). Collagen levels per mg tissue actually increased in tumors where growth had been greatly reduced due to retinoid treatment (Table 1). Under the described experimental conditions, we could not detect any costal cartilage chondrocyte alterations, while the neoplastic chondrocyte was greatly influenced by retinoid administration. The fact that the 2 types of chondrocytes exhibit different drug sensitivity may be explained by the bioavailability of the analogs for the 2 types of cartilage. Recently, a specific cellular retinoic acid-binding protein has been described (22, 26). Cellular retinoic acid-binding protein has been detected in the chondrosarcoma and was not measurable in normal cartilage (F. Chytii, personal communication). Thus, retinoids may be concentrated in the neoplastic cartilage.

The unique response of this nonepithelial transplantable tumor to vitamin A acid chemotherapy cannot be explained. Recently, Langer and Gross (17) showed that intact cartilage explants were not immunogenic. Free chondrocytes or shavings of cartilage were immunogenic when transplanted between inbred strains of mice. Heyner (15) has also concluded that the matrix of cartilage prevents rejection of chondrocytes in allografts of intact cartilage. It is conceivable that the reduction of glycosaminoglycan (loss of matrix) in the tumor due to retinoid treatment resulted in chondrocyte recognition and immunological rejection. Alternatively, retinoids might be operative at the level of cellular differentiation as in the case of epithelial lesions. The possible role of vitamin A as an immunoenhancer has yet to be explained. Meltzer and Cohen (20), Seifter et al. (27), and Felix et al. (12) have reported that vitamin A enhanced antitumor immunity. Thus, retinoids may be operative in inhibiting chondrosarcoma growth by any 1 or a combination of these possible mechanisms. Of course, a property of retinoic acid and its analogs yet to be described may be the mechanistic explanation for inhibition of this unique transplantable tumor. At the present time no conclusions can be drawn concerning the mechanism by which vitamin A acid and vitamin A acid analogs prevent chondrosarcoma growth.

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