Effect of Aromatic Retinoids on Rat Chondrosarcoma Glycosaminoglycan Biosynthesis

Stanley S. Shapiro,† Melanie Bishop, James P. Poon, and Patrick W. Trown

Departments of Biochemical Nutrition [S. S. S., M. B., J. P. P.] and Chemotherapy [P. W. T.], Roche Research Center, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

SUMMARY

Synthetic aromatic analogs of retinoic acid were administered i.p. and p.o. to Fischer F344 rats bearing a transplantable chondrosarcoma. 35CO4 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 of retinoid for 4 weeks, 35SO4 incorporated into glycosaminoglycan by treated tumor explants was reduced as much as 95%. There was no reduction 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% reduction in glycosaminoglycan per mg of chondrosarcoma; there was no reduction in costal cartilage glycosaminoglycan. Retinoid (10- to 20-mg/kg/day doses) elevated collagen 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 neoplastic 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 metaplasias, tumors, and carcinomas (1-3, 6, 25). In addition to the described prophylactic effect, vitamin A as well as vitamin 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 disappearance of established carcinogen-induced skin papillomas in mice (2). Therapeutic results have also been reported in man for actinic keratoses and basal cell carcinomas (11). Recently, Heilman and Swarm (14) reported the activity of other synthetic retinoids in inhibiting growth and achieving regression of the chondrosarcoma.

We have recently reported on the inhibition of glycosaminoglycan biosynthesis by retinoic acid in rat costal cartilage in vitro (28). We have now extended those studies and have examined the effect of synthetic retinoids on glycosaminoglycan and collagen biosynthesis in explants of rat chondrosarcoma 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 chondrosarcoma 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 preparations of synthetic retinoids were prepared by sonically disrupting 500 mg of compound for 2 min in 25 ml of deionized water containing 0.01% Triton X-100 and 0.1% carboxymethylcellulose. Preparations were stored at 4° under argon gas. Compounds were administered i.p. daily Monday to Friday. Experiments were terminated on Mondays. 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 Cartilage. Cartilage was removed from the animal and carefully trimmed free of adherent connecting tissue under a dissecting 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 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 observation and reported the activity of other synthetic retinoids in inhibiting growth and achieving regression of the chondrosarcoma.

Materials and Methods

The Effect of 13-cis-Retinoic Acid on the Growth of Rat Chondrosarcoma

This group of compounds caused regression and disappearance of established carcinogen-induced skin papillomas in mice (2). Therapeutic results have also been reported in man for actinic keratoses and basal cell carcinomas (11). Recently, Heilman and Swarm (14) reported the activity of other synthetic retinoids in inhibiting growth and achieving regression of the chondrosarcoma.

We have recently reported on the inhibition of glycosaminoglycan biosynthesis by retinoic acid in rat costal cartilage in vitro (28). We have now extended those studies and have examined the effect of synthetic retinoids on glycosaminoglycan and collagen biosynthesis in explants of rat chondrosarcoma 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 chondrosarcoma 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 preparations of synthetic retinoids were prepared by sonically disrupting 500 mg of compound for 2 min in 25 ml of deionized water containing 0.01% Triton X-100 and 0.1% carboxymethylcellulose. Preparations were stored at 4° under argon gas. Compounds were administered i.p. daily Monday to Friday. Experiments were terminated on Mondays. 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 Cartilage. Cartilage was removed from the animal and carefully trimmed free of adherent connecting tissue under a dissecting 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 50 x 10⁴ cpm of carrier free H₂³⁵S⁰₄. 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 0.15% 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
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-

**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 H₂[35S]SO₄ 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 [35S]SO₄ 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 [35S]SO₄ 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.

**Effect of Retinoids on [35S]Glycosaminoglycan Produced by Costal Cartilage Explants.** The incorporation of [35S]SO₄ 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 [35S]SO₄ 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 [35S]SO₄ into glycosaminoglycan. This observation differs sharply from that described for neoplastic cartilage (Chart 2B).

**Correlation between Tumor Weight and Sulfate Incorporo-
ration. The relationship between tumor weight (g) after retinoid treatment and the ability of the explants to incorporate $^{35}$SO$_4$ 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 $^{35}$SO$_4$ in glycosaminoglycan per mg of explant. In the i.p. experiment represented in Chart 4A, 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 $^{35}$SO$_4$ incorporated into glycosaminoglycan after a 2-week period. However, there was no effect of Ro 11-1430 on $^{35}$SO$_4$ 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). $^{35}$SO$_4$ 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 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 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.
Retinoids and Chondrosarcoma Glycosaminoglycan

Table 1

<table>
<thead>
<tr>
<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>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>0</td>
<td>21.0</td>
<td>22.5</td>
</tr>
<tr>
<td>Ro 11-1430</td>
<td>p.o.</td>
<td>20</td>
<td>2.9</td>
<td>104.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>4.0</td>
<td>77.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>6.6</td>
<td>73.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>18.1</td>
<td>20.4</td>
</tr>
<tr>
<td>Ro 10-9359</td>
<td>p.o.</td>
<td>10</td>
<td>3.2</td>
<td>71.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>10.6</td>
<td>55.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>18.0</td>
<td>33.2</td>
</tr>
<tr>
<td>Ro 11-1430</td>
<td>i.p.</td>
<td>20</td>
<td>2.3</td>
<td>86.0</td>
</tr>
<tr>
<td>Ro 10-9359</td>
<td>i.p.</td>
<td>10</td>
<td>2.5</td>
<td>108.0</td>
</tr>
</tbody>
</table>

* 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 35SO4 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 35SO4 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. Chytil, personal communication). Thus, retinoids may be operative in 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.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent assistance of Margaret Buck and Rosanne Hansen for transplanting the chondrosarcoma and administering the drugs. We also thank Alex Georgiadis for carrying out the hydroxylproline determinations. We also express our gratitude to Dr. Myron Brin for his valuable suggestions in preparing the manuscript.

REFERENCES

12. Felix, E. L., Loyd, B., and Cohen, M. H. Inhibition of Growth and Differentiation as in the case of epithelial lesions. The possible role of vitamin A as an immunoenhancer has yet to be explained. Metzler and Cohen (20), Seifert 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.

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 35SO4 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 35SO4 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. Chytil, 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. Metzler and Cohen (20), Seifert 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.
S. S. Shapiro et al.

Effect of Aromatic Retinoids on Rat Chondrosarcoma Glycosaminoglycan Biosynthesis

Stanley S. Shapiro, Melanie Bishop, James P. Poon, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/36/10/3702

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.