Biochemical Pharmacology, Vol. 33, No. 20, pp. 3263–3267, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

EFFECT OF RETINOIDS ON COLLAGEN PRODUCTION BY CHONDROCYTES IN CULTURE

RUEDIGER HEIN,* THOMAS KRIEG,†‡ PETER K. MUELLER* and OTTO BRAUN-FALCO†
*Max-Planck-Institut fuer Biochemie, Martinsried, Federal Republic of Germany and †Dermatologische Klinik und Poliklinik der LMU Muenchen, Federal Republic of Germany

(*Received* 2 *March* 1984; *accepted* 24 *May* 1984)

Abstract—The effect of vitamin A and of some of its derivatives on chondrocytes in culture has been studied. In the presence of retinoids the proliferation of the cells decreased and they lost their characteristic polygonal shape and assumed a fibroblast-like morphology. All retinoids also caused dedifferentiation of chondrocytes as indicated by the induction of types I and III collagen. 13-*cis* retinoic acid (= isotretinoin) was the most active derivative in this aspect. Since appropriate control of the synthesis of extracellular matrix proteins is a prerequisite for their normal physiological function, alterations such as those observed here may be involved in the pathogenesis of side effects which are observed during the treatment of dermatological disorders with retinoic acid derivatives.

Vitamin A is known to interfere with the process of chondrogenesis in the developing embryo [1] and in cartilage rudiments maintained in organ culture [2, 3]. Vitamin A has also been found to alter the morphology [4] and glycosaminoglycan metabolism [4–6] of chondrocytes in culture. Furthermore, the action of retinoids on ectodermal cells, which are considered the prime target tissue has been investigated extensively [7].

In the past few years, interest in vitamin A has grown enormously since it was found to decrease cell proliferation in experimental neoplasias [8, 9]. Indeed, several synthetic analogs of vitamin A have been developed which are even more active than vitamin A (for review see [7]). These chemically modified derivatives have reduced toxicity and could be used in the treatment of cancers and various dermatological disorders [10–13].

However, results of studies with some retinoids in various biological systems showed teratogenic effects [14, 15] and certain side effects in patients treated with retinoids [16] indicate that the vitamin A derivatives may have a similar action on cartilage as found for the parent compound itself. One of the major biosynthetic products of chondrocytes is type III collagen (for review see [17]). However, certain culture conditions and various chemicals can induce a switch from the synthesis of type II to type I and type III collagen [18].

The aim of our study was to determine if vitamin A and its derivatives promote a switch in the type of collagen produced, which would indicate dedifferentiation of chondrocytes.

‡ Address for correspondence: Dr. Thomas Krieg, Max-Planck-Institut fuer Biochemie, D-8033 Martinsried b. Muenchen, Federal Republic of Germany.

MATERIALS AND METHODS

Materials. Ham's F10 medium, Dulbecco's modified Eagle's medium, penicillin/streptomycin (1000 U/10,000 μg/ml), EDTA-versene (1%), L-glutamine (200 mM), trypsin (2.5%) and fetal calf serum were obtained from L + S, Laborservice (Muenchen).

Collagenase (EC 3.4.4.19, Worthington CLS) was purchased from Roth (Karlsruhe). Pepsin (EC 3.4.23.1, 2 × crystallized) was obtained from Serva (Heidelberg). Falcon plastic ware for tissue culture was purchased from L + S, Laborservice (Muenchen). L-(2,3-)[^{3}H]proline was a product of New England Nuclear Corporation (Dreieichenhain). Sodium ascorbate was obtained from Merck (Darmstadt).

Retinoids. Vitamin A and various retinoids (isotretinoin = 13-*cis* retinoic acid, etretinate = Ro 10-9359, free acid of etretinate = Ro 10-1670) were obtained as a gift from Hoffmann La Roche, Basle. They were dissolved in 94% ethanol and added to the cell culture medium. The same amount of pure ethanol (10^{-5} M) alone had no obvious effect on the morphology, proliferation or the type of collagen synthesized by chondrocytes.

Chondrocyte cultures. Chondrocytes were isolated from chick sterna following an established procedure [19] and then seeded at high cell density in Ham's F12 medium containing streptomycin (50 μg/ml), penicillin (400 U/ml), ascorbate (100 μg/ml) and 10% dialyzed fetal bovine serum. Cultures were examined by phase contrast microscopy and changes in morphology were evaluated. After 3 and 5 days, cell numbers were determined in triplicate cultures. As reported by Gauss [20], in culture the freshly isolated cells when attached to the plastic surface revealed the typical polygonal morphology of chondrocytes (Fig. 3). No fibroblast-like cells could be observed in any cultures.

Radioactive labeling and measurement of the synthesis of collagen and non-collagenous proteins. Radioactive labeling of cells was performed on 5-day-old cultures with L-(2,3-)[^{3}H]proline (10 μCi/ml) in Dulbecco's MEM [18] for 24 hr. Retinoids were added as indicated previously. The medium and cell layer were first dialyzed against 1 M $CaCl_2$, 0.05 M Tris, pH 7.4, then against 0.5% acetic acid and finally lyophilized. After hydrolysis (6 M HCl, 120°, 24 hr) the samples were analyzed for proline and hydroxyproline content with an automated amino acid analyzer (Multichrom, Beckmann). Radioactivity of the hydroxyproline and proline peaks was measured and the proportion of collagen to non-collagenous protein was calculated as described in [21].

Analysis of collagen types. Newly synthesized collagen was extracted from the medium and cell layer by pepsin treatment [22], precipitated with 17.5% KCl [18] and then analyzed by slab gel electrophoresis according to Laemmli [23]. Radioactively labeled material was visualized by fluorography [24] and the intensity of individual bands (increase of alpha 2(I) band) was quantitated by densitometry using a Zeiss scanner (KM3). For measurement of the hydroxylation degree of collagen chains the isolated alpha-chains were collected and hydrolyzed as described in [21]. The samples were subsequently chromatographed on an automated amino acid analyzer, and the radioactivity eluting in the position of proline and hydroxyproline was determined.

RESULTS

Proliferation of chondrocytes

In the first set of experiments, chondrocytes were plated at an initial density of 2.5×10^5 cells/ml and their proliferation was followed for the next 5 days. As shown in Fig. 1, vitamin A reduced the rate of proliferation to about 50% of the control. Isotretinoin and free acid of etretinate were less active. None of the compounds inhibited proliferation at concentrations below 10^{-9} M.

Quantitative aspects of collagen synthesis

Chondrocytes were incubated for 5 days with or without a retinoid (10^{-5}–10^{-9} M) and subsequently labelled with L-(2,3)[^{3}H]proline for 24 hr. As summarized in Table 1, the collagen synthesis relative to total protein synthesis decreased to approximately 30 ± 5% of control. All retinoids tested showed approximately the same inhibitory effect (Fig. 2).

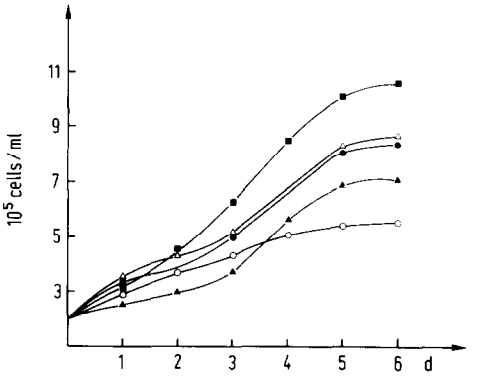

Fig. 1. Inhibition of proliferation of chondrocyte cultures by various retinoids (10^{-5} M). The cells were seeded at low density and triplicate cultures were counted daily. ■ Control, ○ vitamin A, △ isotretinoin, ▲ etretinate, ● free acid of etretinate.

Reduced collagen synthesis was even evident at concentrations of 10^{-9} M, where cell proliferation was not significantly affected. The degree of hydroxylation of proline was not altered, as calculated from the ratio of hydroxyproline to proline in isolated collagen chains (data not shown).

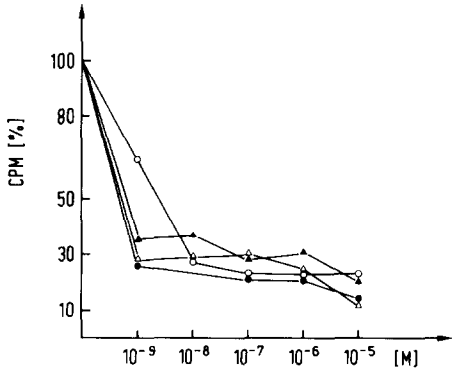

Fig. 2. Influence of retinoids (10^{-5}–10^{-9} M) on synthesis of collagen measured as protein bound hydroxyproline (5 days). The values are expressed as a percentage of untreated cultures. ○ Vitamin A, △ isotretinoin, ▲ etretinate, ● free acid of etretinate.

Table 1. Influence of retinoids on collagen synthesis in chondrocytes. Collagen production was measured as protein bound hydroxyproline and the percentage collagen vs non-collagenous proteins was calculated (see Material and Methods)

Samples	Concentrations (M)	Percentage of collagen from total protein production: 3 days incubation, Experiment 1	3 days incubation, Experiment 2	5 days incubation, Experiment 1	5 days incubation, Experiment 2
Controls	0	13.8	13.4	15.2	14.9
Vitamin A	10^{-5}	2.5	2.7	2.2	2.2
	10^{-9}	12.2	10.9	12.6	12.1
13-*cis* retinoic	10^{-5}	2.2	2.3	1.4	1.6
acid = isotretinoin	10^{-9}	5.8	5.5	3.7	3.7
Etretinate =	10^{-5}	4.3	4.6	3.0	3.3
Ro 10-9359	10^{-9}	6.9	6.3	4.8	5.8
Free acid of etretinate =	10^{-5}	2.3	2.9	2.0	2.1
Ro 10-1670	10^{-9}	5.8	5.5	5.5	5.3

The values represent the means of triplicate determinations.

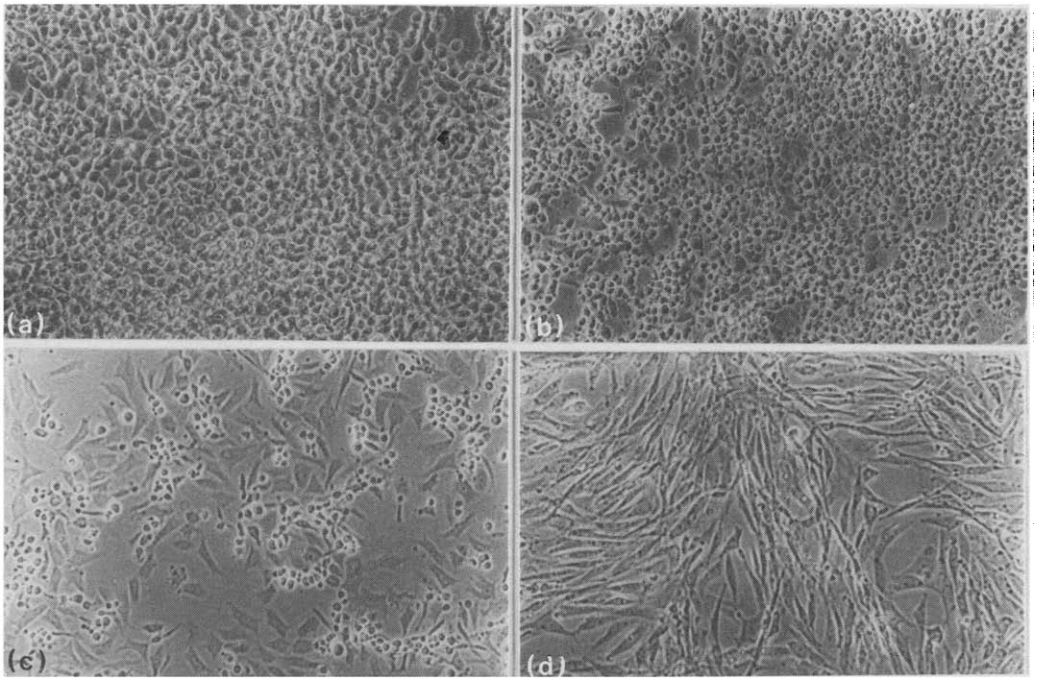

Fig. 3. Phase contrast microphotography of chondrocytes after 3 (a) and 5 days (b) in culture. Parallel cultures have been treated for 3 (c) or 5 days (d) with free acid of etretinate (10^{-7} M). Similar pictures were obtained with all other retinoids.

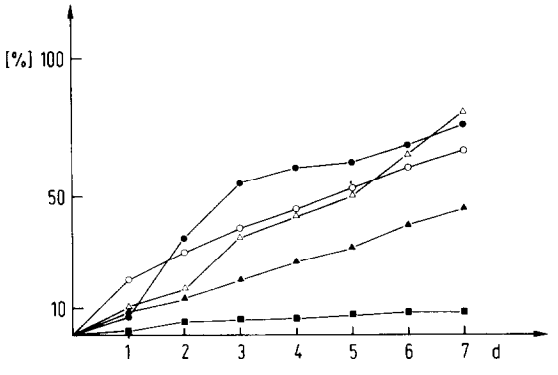

Fig. 4. Switch of chondrocytes to fibroblast-like cells measured by alteration of morphology in percentage of total cells. Several retinoids have been added to the culture medium (10^{-7} M). ■ Controls, ○ vitamin A, △ isotretinoin, ▲ etretinate, ● free acid of etretinate.

Dedifferentiation of chondrocytes

Chondrocytes in long term culture dedifferentiate and switch from type II collagen production to the synthesis of type I and III collagen [25]. They also change their morphology to a more fibroblastic shape. This is an easy means to continuously monitor the state of dedifferentiation. In the presence of retinoids this process is accelerated (Fig. 3c, d). The morphology of cells in control cultures did not significantly change after 3 or 5 days (Fig. 3a, b). Such a change was mostly seen in the presence of isotretinoin, where about 85% of the cells became fibroblastic (Fig. 4). Labeled proteins from control and retinoid treated cultures were characterized by slab gel electrophoresis (Fig. 5). Whereas only minute amounts of type I collagen were found in control cultures, retinoids caused a dose dependent increase in the amount of alpha 2(I) chains. When the amount of type I collagen production was calculated (Table 2), isotretinoin was found to be most active in inducing modulation of collagen type synthesis in chondrocytes. The other retinoids, including vitamin A, were far less effective.

Table 2. Relative amount of type I collagen (percentage of type I from type I and type II) in chondrocyte cultures after incubation with retinoids during 5 days

Concentration (M)	% (I)	Vitamin A % (I)	13-*cis* retinoic acid = isotretinoin % (I)	Free acid of etretinate = Ro 10-1670 % (I)	Etretinate = Ro 10-9359 % (I)
Controls (without retinoids)	5				
10^{-6}		32	38	23	37
10^{-7}		31	41	28	5
10^{-8}		22	29	17	5
10^{-9}		7	23	13	5

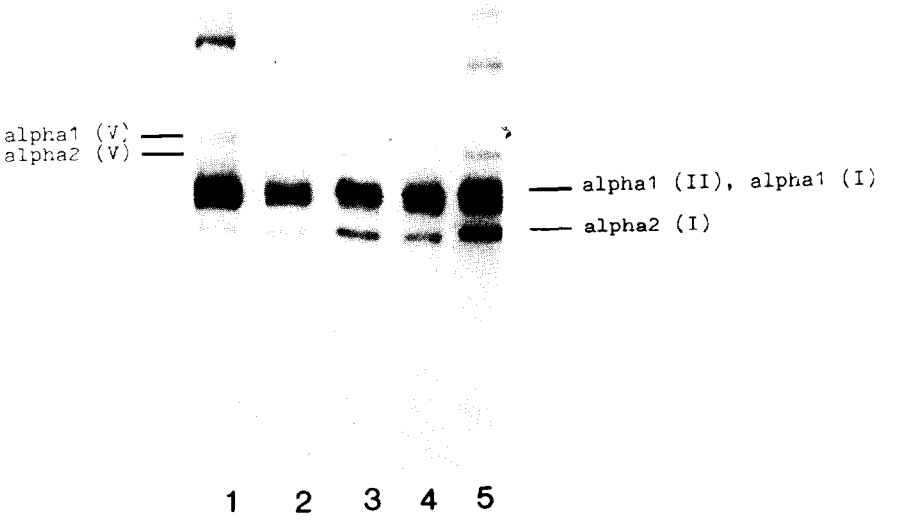

Fig. 5. Slab gel electrophoresis of collagens synthesized by chondrocytes in culture. Lane 1: control (5 days); lane 2–5: increasing concentrations of free acid of etretinate (10^{-9}–10^{-6} M).

DISCUSSION

Under certain culture conditions chondrocytes are known to retain their differentiated phenotype *in vitro* and to express similar characteristics as found *in vivo* [19, 24]. Such cultures therefore provide a useful system to study the influence on various factors of the state of differentiation of these particular mesenchymal cells. Since vitamin A has been reported to alter the metabolism of chondrocytes [28], it was of interest to determine which effects various synthetic retinoids widely used in the therapy of human diseases might have on chondrocytes.

Examination of newly established chondrocyte cultures by phase contrast and immunofluorescence microscopy using antibodies against type I collagen indicates that there were no contaminating fibroblasts. The concentrations of retinoids used in our experiments were similar to serum levels of retinoids found in treated patients (1.6×10^{-6} M). Recently, we have shown that retinoids have a rather general impact on fibroblasts by lowering most significantly synthesis of total protein [29], while collagen synthesis is much less affected. This indicates to us that chondrocytes in culture maintain a rather delicate balance of the amount and the types of collagen synthesized in contrast to fibroblasts.

Since the drastic reduction of collagen synthesis was observed at concentrations as low as 10^{-9} M, with a minor decrease thereafter, it indicates to us that there is no dose-dependent effect on collagen synthesis using our experimental conditions. Hydroxylation of isolated alpha chains was normal, and therefore the compounds under investigation do not obviously inhibit prolyl and/or lysylhydroxylase. Whether the decrease of protein bound hydroxyproline is due to reduced synthesis or an enhanced breakdown of newly synthesized collagens [4, 5] is not known. However, enhanced degradation seems unlikely since it has been reported that collagenase activity in synovial fibroblasts was inhibited by retinoids [14]. Furthermore, as the synthesis of noncollagenous proteins was much less affected, the inhibition of collagen production appears to be a highly specific event.

In addition to these alterations in the quantity of collagen production, major changes in the regulation of collagen types synthesized were induced by various retinoids. We have demonstrated that the type of collagen produced switched from type II to type I and that this switch is accompanied by a change in cell morphology from a polygonal to a fibroblastic shape [18]. In all aspects examined, isotretinoin (13-*cis* retinoic acid) induced the dedifferentiation of chondrocytes more actively than the other retinoids. It still remains unclear whether the drastic decrease of collagen synthesis and the modulation of chondrocyte dedifferentiation have a common cause or whether the two effects of retinoids are independent processes. However, the switch to fibroblast-like morphology and type I collagen synthesis requires higher concentrations of retinoids than the inhibition of collagen production.

The switch of chondrocytes from the synthesis of type II to type I collagen has been postulated to play an important part in the pathogenesis of arthrosis [27]. Although data derived from cell cultures, whether from avian sources as we used or mammalian, do not necessarily reflect *in vivo* conditions. Our results suggest that the effects of various retinoids on chondrocytes may possibly indicate similar roles for these compounds in patients under treatment with retinoids. This may be of clinical importance, since alterations in joint and bone metabolism have been occasionally reported in patients during treatment with retinoids [30]. Thus, cell culture systems, like confluent chondrocytes, which maintain some of their differentiated features in culture, may be useful to investigate the influence of new compounds on cell physiology.

Acknowledgements—We thank Professor K. Kuehn for helpful discussions. The technical assistance of D. Veranic and A. Pavlovic is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft Kr.558/4 and Mu.378/13.

REFERENCES

1. D. M. Kochhar, *Teratology* **7**, 289 (1973).
2. H. B. Fell and J. T. Dingle, *Biochem. J.* **87**, 403 (1963).
3. J. R. Hassell, J. P. Pennypacker and C. A. Lewis, *Expl Cell Res.* **112**, 409 (1978).
4. M. Solursh, *Calc. Tiss. Res.* **13**, 131 (1973).
5. S. S. Shapiro and J. P. Poon, *Archs Biochem. Biophys.* **174**, 74 (1976).
6. S. S. Shapiro and D. J. Mott, *Ann. N.Y. Acad. Sci.* **359**, 306 (1981).
7. R. Lotan, *Biochim. biophys. Acta* **605**, 33 (1980).
8. M. B. Sporn, N. M. Dunlop, D. L. Newton and W. R. Henderson, *Nature, Lond.* **263**, 110 (1976).
9. M. B. Sporn and D. L. Newton, *Fedn Proc. Fedn Am. Socs exp. Biol.* **38**, 2528 (1979).
10. B. Dahl, K. Møllenbach and F. Reymann, *Dermatology* **154**, 261 (1977).
11. G. L. Peck, T. G. Olsen, F. W. Yoder, J. S. Strauss, D. T. Downing, M. Pandya, D. Butkus and J. Arnaud-Battandier, *New Engl. J. Med.* **300**, 329 (1979).
12. G. L. Peck, F. W. Yoder, T. G. Olsen, M. D. Pandya and D. Butkus, *Dermatology* **157** (suppl. 1), 11 (1978).
13. J. S. Strauss, D. B. Windhorst and G. D. Weinstein, *J. Am. Acad. Derm.* **6**, Part 2, 573 (1982).
14. C. E. Brinckerhoff, C. A. Vater and E. D. Harris, *Effects of Retinoids on Rabbit Synovial Fibroblasts and Chondrocytes*, Ch. 17, pp. 215–230. Elsevier North Holland, Amsterdam (1981).
15. J. A. Geelen, *Crit. Rev. Toxicol.* **6**, 351 (1979).
16. M. Herold, J. Cahn and P. Gomont, *Acta Derm. Vener. Stockh.* **55** (suppl. 74), 29 (1975).
17. P. K. Müller, C. Lemmen, S. Gay, K. von der Mark and K. Kühn, *Extracellular Matrix Influences on Gene Expression* (eds. H. C. Slavkin and R. C. Greulich), pp. 293–302. Academic Press, New York (1975).
18. V. Gauss and P. K. Müller, *Biochim. biophys. Acta* **652**, 39 (1981).
19. P. Dehm and D. J. Prockop, *Biochim. biophys. Acta* **240**, 358 (1971).
20. V. Gauss, Kollagensynthese von Chondrocyten—Ein System zum Studium der Genexpression in Eukaryonten. Ph.D. Thesis (1978).
21. M. Wiestner, Th. Krieg, D. Hörlein, R. W. Glanville, P. Fietzek and P. K. Müller, *J. biol. Chem.* **254**, 7016 (1979).
22. B. F. Pontz, P. K. Müller and W. N. Meigel, *J. biol. Chem.* **248**, 7558 (1973).
23. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
24. W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46**, 83 (1974).
25. M. Duchene, S. Wiedebusch, K. Kühn and P. K. Müller, *FEBS Lett.* **135**, n.1, 119 (1981).
26. P. K. Müller, C. Lemmen, S. Gay, V. Gauss and K. Kühn, *Expl Cell. Res.* **108**, 47 (1977).
27. S. Gay, P. K. Müller, C. Lemmen, K. Remberger, K. Matzen and K. Kühn, *Klin. Wschr.* **54**, 969 (1976).
28. N. Vasar and J. W. Lash, *Calif. Tiss. Res.* **19**, 99 (1975).
29. R. Hein, Th. Kreig, P. K. Müller and O. Braun-Falco, *Collagen Res.* **3**, No. 2, 159 (1983).
30. R. A. Pittsley and F. W. Yoder, *New Engl. J. Med.* **308**, 1012 (1983).