Retinoid modulation of collagenase production by adherent human mononuclear cells in culture*

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SUMMARY Previous observations have suggested that retinoids might be useful for the treatment of rheumatoid arthritis. In this study we examined the effects of various retinoids on collagenase production by adherent human peripheral blood mononuclear cells in culture. We have previously shown that these cells, consisting predominantly of monocyte-macrophages, actively synthesise and secrete collagenase upon stimulation with concanavalin A.¹⁶ The cells were incubated in serum free medium with all-*trans*-retinoic acid, 13-*cis*-retinoic acid, all-*trans*-retinal, or Ro 10-9359 (trimethylmethoxyphenyl retinoic acid ethyl ester) for up to 72 hours, and the collagenase activity was determined with [³H]proline labelled type I collagen as substrate. The incubation of mononuclear cells with all-*trans*-retinoic acid in the concentration range 10⁻⁷–10⁻⁵ mol/l resulted in a dose dependent inhibition of the collagenase production. All-*trans*-retinal was also a potent inhibitor, whereas 13-*cis*-retinoic acid and Ro 10-9359 in a concentration of 10⁻⁵ mol/l had a lesser effect. Control experiments indicated that the inhibition of collagenase production by all-*trans*-retinoic acid did not result from inhibition of total protein synthesis nor could it be explained by induction of an inhibitory molecule. These results indicate that retinoids with distinct structural features can inhibit collagenase production by monocyte-macrophages, and suggest a role for retinoids in the treatment of rheumatoid arthritis.

Key word: monocytes.

Retinoid therapy has recently been suggested for rheumatoid arthritis.² This suggestion was based on several experimental observations attesting to the potential efficacy of retinoids as antirheumatic agents. For example, in the adjuvant arthritis experimental model of rheumatoid arthritis the oral administration of 13-*cis*-retinoic acid (13-*cis*-RA) significantly reduced the joint inflammation in the rat.³ Also, adherent cells isolated from the inflamed joints of the rats treated with 13-*cis*-RA secreted less collagenase than the control cultures.³ Furthermore, all-*trans*-RA, 13-*cis*-RA, and 4-hydroxyphenylretinamide (4-HPR) have been shown to reduce the production of collagenase by synovial cells in culture.^{4,5} These observations

suggest that retinoids may interfere with collagenase production also in vivo and could be beneficial in the treatment of rheumatoid arthritis. On the other hand, in the collagen induced rat model of arthritis both 13-*cis*-RA and 4-HPR augmented the inflammatory component of arthritis.⁶ Thus the multiplicity of the effects of retinoids on various animal systems as models for human rheumatoid arthritis,^{3,6} and the proposed effects of retinoids on cellular metabolism,^{7,8} require additional studies to establish any validity of retinoids for treatment of rheumatoid arthritis.

Monocyte-macrophages, the hallmark of chronic inflammatory processes, have been shown to synthesise and secrete proteases, including collagenase, which are thought to mediate tissue destruction in diseases.^{9–16} We have recently shown that adherent mononuclear cells cultured from human peripheral blood actively synthesise and secrete collagenase when stimulated with concanavalin A.¹⁶ The enzyme was shown to be similar to other mammalian collagenases in that it cleaved native type I collagen

Accepted for publication 17 October 1986.

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*A preliminary report on this study was presented at the western regional meeting of the Society for Investigative Dermatology, Carmel, CA, 4–7 February 1986.¹

at a single locus three quarters of the chain length away from the amino terminus of the molecule.¹⁶ The enzyme was shown to be secreted in an active form, but the activity could be enhanced by sulphhydryl reagents such as *N*-ethylmaleimide (NEM).¹⁶ The total cumulative production in a 48 hour culture of the adherent mononuclear cells activated by concanavalin A was shown to be of the same order of magnitude as the enzyme activity present in the same number of polymorphonuclear leucocytes.¹⁶ Thus collagenase synthesised and secreted by monocyte-macrophages present in rheumatoid synovium may contribute to the connective tissue destruction in this disease. To assess the potential of monocytes as a target for retinoid therapy we have examined in this study the effects of various retinoids on collagenase production in adherent mononuclear cell cultures.

Materials and methods

RETINOIDS

All-*trans*-RA and all-*trans*-retinal were purchased from Sigma Chemical Co, St Louis, MO; 13-*cis*-RA and Ro 10-9359 (trimethylmethoxyphenyl RA ethyl ester; etretinate) were obtained from Hoffmann-La Roche, Nutley, NJ. The retinoids were dissolved in ethanol immediately before use and kept in the dark during the procedures. The final concentration of ethanol in cell cultures was 0.19% or less (except for 5×10^{-5} M all-*trans*-RA, in which case it was 1%). Parallel control cultures were incubated with ethanol alone in the corresponding concentration. Ethanol in 0.2% concentration was not toxic to the cells, as assessed by [³H]leucine incorporation, trypan blue dye exclusion test, or by release of lactate dehydrogenase (LDH) to the medium (see below). Also, 0.2% ethanol had no effect on collagenase production by these cells. One per cent ethanol inhibited collagenase production in control cultures by 19%.

MONONUCLEAR CELL CULTURES

Blood samples were obtained from healthy volunteers and from a patient with haemochromatosis whose monocytes have been previously shown to be active in synthesising and secreting collagenase even without concanavalin A activation.¹⁶ Peripheral blood mononuclear cells were isolated by density gradient centrifugation on Ficoll-Hypaque (Histopaque-1077; Sigma Chemical Co, St Louis, MO).¹⁷ The isolated mononuclear cells were pooled, washed in Hanks's balanced salt solution (HBSS), and suspended in serum free Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml

penicillin, 100 µg/ml streptomycin, and 0.2% lactalbumin hydrolysate (DMEM-LH; Sigma). To establish the monocyte cultures 2×10^6 cells in 500 µl of DMEM-LH were plated in 24-well tissue culture plates (Corning, NY). The mononuclear cells were allowed to adhere for 120 min under 5% CO₂/95% air at 37°C. The medium containing the unadhered cells was then removed, the adherent cells were washed with 500 µl of HBSS, and 500 µl of fresh, serum free DMEM-LH was added to the cultures. To enhance the production of collagenase the culture medium was supplemented with 20 µg/ml concanavalin A (Difco, Detroit, MI); the retinoids were added at the same time. The incubations were then continued for varying time periods up to 736 hours at 37°C under 5% CO₂/95% air.

COLLAGENASE ASSAY

At different times of cell incubation aliquots of the serum free media were assayed for collagenase activity by a sensitive and specific method.¹⁸ In these assays [³H]proline labelled, native, triple helical type I collagen, with a specific activity of 2.27×10^4 cpm/µg, was used as substrate. Details of this assay procedure have been reported previously.¹⁸ Briefly, in a standard assay the substrate (3×10^4 cpm in 20 µl of 0.01 M acetic acid) was mixed with 10 µl of 0.5 M TRIS (trometamol)-HCl pH 7.8, containing 2.0 M NaCl, 0.1 M CaCl₂ and 0.1 M NEM, and with 10 µl of a solution containing bovine serum albumin, 0.2 mg/ml. Aliquots (60 µl) of the tissue culture supernatant to be assayed for collagenase activity were then added, and the incubations were continued for 120 min at 37°C. The collagenase digestion was stopped by the addition of 50 µl of 0.2 M disodium ethylenediaminetetraacetate (Na₂EDTA). Unlabelled soluble type I collagen, trypsin, and α-chymotrypsin, in final concentrations of 0.2, 0.16, and 0.16 mg/ml respectively, and dissolved in 0.2 M NaCl and 50 mM TRIS-HCl, pH 7.5, were then added to give a final volume of 400 µl; the incubations were continued for another 20 minutes at 37°C. An equal volume of 20% ice cold trichloroacetic acid (TCA) was then added, and the undigested [³H]proline labelled substrate was precipitated by centrifugation. The radioactivity in the supernatant, representing collagen degradation, was counted with a liquid scintillation counter (Beckman LS-7500). In each assay a set of control tubes containing medium not incubated but containing trypsin and α-chymotrypsin was included. Collagenase activity was expressed in units (U), where 1 U equals 1 µg collagen degraded per minute.

For assay of intracellular collagenase activity the medium was removed and the adherent monocyte

were rinsed with phosphate buffered saline. DMEM-LH, 500 μ l, was added to each well, the cells were then detached by scraping with a rubber policeman and homogenised with a Teflon glass tissue homogeniser at 4°C. The homogenates were centrifuged at 18 000 g for 20 min, and the supernatants were assayed for collagenase activity as above.

OTHER ASSAYS

Total protein synthesis by the cultured mononuclear cells was assayed by incubation of the cells with 1.11 MBq of [L-4,5-³H]leucine (specific activity 5.59 TBq/mmol; Amersham) under conditions identical with those used for collagenase production. At the end of incubation protease inhibitors were added to the following final concentrations: Na₂EDTA (10 mmol/l), NEM (10 mmol/l), and phenylmethylsulphonyl fluoride (0.3 mmol/l). The adherent cells were detached by brief sonication, and the [³H]leucine labelled macromolecules in combined medium and cell fractions were precipitated by the addition of an equal volume of 20% TCA. The TCA precipitates were collected on Millipore prefilters (Millipore, AP1502500, Millipore, Bedford, MA) on a vacuum manifold. The filters were air dried and counted in a Beckman LS-7500 liquid scintillation counter, as described previously.¹⁶

The viability of mononuclear cells was estimated by a trypan blue dye exclusion test.¹⁹

The LDH activity in the culture medium was

Fig. 1 Dose dependent inhibition of collagenase production by all-trans-retinoic acid (all-trans-RA) in adherent human mononuclear cell cultures. Peripheral blood mononuclear cells isolated from a patient with haemochromatosis were incubated with all-trans-retinoic acid in the concentrations indicated for 72 hours. Collagenase activity was determined as indicated in 'Materials and methods'. The values are mean (SEM) of four parallel determinations. The statistical significance of the difference, calculated by Student's *t* test, is indicated in the figure (**p* < 0.05; ***p* < 0.01).

Fig. 2 Time dependent inhibition of collagen production by all-trans-retinoic acid. Adherent human mononuclear cells from a patient with haemochromatosis were incubated as in Fig. 1 either without (○), or with 10⁻⁶ M (■) or 10⁻⁵ M (▼) all-trans-retinoic acid. The collagenase activity was determined at the times indicated as described in 'Materials and methods'. The values are mean (SEM) of four parallel determinations.

assayed by a modification of the method of Wroblewski and LaDue²⁰ using a commercial kit (Sigma Chemical Co).

The protein content of cell homogenates after sonication was assayed by a colorimetric method²¹ using commercial reagent (Bio-Rad Laboratories, Richmond, CA).

STATISTICAL ANALYSIS

The results were evaluated for statistical significance by Student's *t* test.

Results

INHIBITION OF COLLAGENASE PRODUCTION BY ALL-TRANS-RETINOIC ACID

The effect of retinoids on collagenase production by adherent human mononuclear cells was first studied by incubating cells isolated from the blood of a patient with haemochromatosis in the presence of varying concentration of all-trans-RA. A dose dependent inhibition of collagenase production was noted in the concentration range 10⁻⁹–5 × 10⁻⁵ mol/l (Fig. 1). The mean inhibition by 10⁻⁵ M all-trans-RA in three independent experiments was 64.7 (16.3)% (mean (SD)) when compared with control cultures incubated with 0.2% ethanol alone. The inhibition by 10⁻⁶ M and 10⁻⁵ M all-trans-RA was also shown to be time dependent (Fig. 2). Similar

inhibition of collagenase production was also observed in monocyte cultures established from two healthy volunteers; these cell cultures required the presence of concanavalin A for collagenase production. In the cultures established from healthy subjects 10^{-5} M all-*trans*-RA inhibited the collagenase production by 60.6 (2.6)% (Table 1, experiment 2) and 58.1 (5.1)% respectively (mean (SD)).

To examine the specificity of all-*trans*-RA on collagenase production several control experiments were performed. Firstly, the total protein synthesis was determined by incorporation of [3 H]leucine in the cell cultures. The results indicated that the incorporation of radioactivity into protein in the control and 10^{-5} M all-*trans*-RA treated cultures was $1.62 (0.05) \times 10^4$ and $1.35 (0.21) \times 10^4$ cpm/ 2×10^6

cells respectively (mean (SD); $p > 0.10$). Also, no difference in the LDH activity released into the medium by cells incubated with or without 10^{-5} M all-*trans*-RA was noted (results not shown). Thus there was no evidence of generalised inhibition of protein synthesis or cell membrane damage.

To examine the possibility that all-*trans*-RA might reduce the collagenase detectable in cell culture medium by directly inhibiting the enzyme activity, medium from control cultures was assayed for collagenase activity directly in the presence of 10^{-5} M all-*trans*-RA. The results indicated that the enzyme activity was not altered in the presence of this retinoid, when compared with control incubation containing the corresponding amount of ethanol.

In further studies the possibility that the retinoid might inhibit the secretion of collagenase from the cells was examined by assaying the intracellular activity. As indicated previously,¹⁶ very little, if any, intracellular enzyme activity was present in either control or all-*trans*-RA treated cells, excluding the possibility that the retinoid might interfere with the secretory process leading to collagenase accumulation in the cellular compartment.

Finally, mixing experiments were performed to examine the possibility that the reduced collagenase activity noted in the retinoid treated cultures might result from induction of an inhibitory molecule rather than from reduced collagenase production. Incubation of control medium with aliquots of media from cultures containing all-*trans*-RA showed that the enzyme activity present in the mixture was the same as the added activities noted in each medium when assayed alone. Furthermore, incubation of control medium with the homogenate of cells from cultures incubated with all-*trans*-RA did not suggest the presence of an inhibitor molecule. Thus these observations suggest that all-*trans*-RA inhibits the collagenase production by adherent mononuclear cells on the level of protein synthesis.

EFFECTS OF OTHER RETINOIDS ON COLLAGENASE PRODUCTION

Several additional retinoids were first tested in monocyte cultures established from the patient with haemochromatosis for their effects on collagenase production. All-*trans*-retinal had a significant effect in the concentrations studied (Table 1, experiment 1). Somewhat surprisingly, 10^{-6} and 10^{-5} M 13-*cis*-RA had no effect on collagenase production, while 62.5% reduction was noted with 10^{-4} M 13-*cis*-RA. This last concentration, however, is unphysiological and may have been toxic to the cells. Ro 10-9359 had no effect in 10^{-6} or 10^{-5} mol/l concentration (Table 1, experiment 1).

Table 1 Effects of various retinoids on collagenase production by adherent human mononuclear cells in culture*

Retinoid tested	Concentration (mol/l)	Collagenase production		p Value‡
		(U/mg cell protein)‡	% Of control	
Experiment 1:				
None (control)	—	6.7 (0.6)	100.0	—
All-trans-RA	10 ⁻⁶	3.9 (0.1)	58.2	<0.01
All-trans-RA	10 ⁻⁵	1.4 (0.1)	20.9	<0.001
13-cis-RA	10 ⁻⁶	6.6 (0.2)	98.5	NS
13-cis-RA	10 ⁻⁵	5.9 (0.5)	88.1	NS
Ro 10-9359	10 ⁻⁶	7.5 (0.5)	111.9	NS
Ro 10-9359	10 ⁻⁵	7.0 (0.5)	104.5	NS
All-trans-retinal	10 ⁻⁶	4.5 (0.1)	67.2	<0.05
All-trans-retinal	10 ⁻⁵	3.7 (0.1)	55.2	<0.01
Experiment 2:				
None (control)	—	3.1 (0.1)	100.0	—
All-trans-RA	10 ⁻⁶	2.6 (0.1)	86.0	<0.05
All-trans-RA	10 ⁻⁵	1.2 (0.1)	39.4	<0.001
13-cis-RA	10 ⁻⁶	2.8 (0.2)	92.2	NS
13-cis-RA	10 ⁻⁵	1.8 (0.1)	57.7	<0.001
Ro 10-9359	10 ⁻⁶	2.8 (0.1)	92.5	NS
Ro 10-9359	10 ⁻⁵	1.8 (0.1)	56.0	<0.001
All-trans-retinal	10 ⁻⁶	2.1 (0.1)	69.1	<0.001
All-trans-retinal	10 ⁻⁵	0.4 (0.1)	14.3	<0.001

*Adherent mononuclear cell cultures were established from peripheral blood of a patient with haemochromatosis (experiment 1) or a healthy volunteer (experiment 2). The cells were incubated for 72 h in serum free medium containing the retinoid in the final concentration indicated. The collagenase activity was then assayed as indicated in 'Materials and methods'. †1 U equals 1 µg collagen degraded per minute; the values are expressed per mg cell protein in the same cultures (mean (SEM) of four parallel determinations).

‡Calculated by Student's *t* test; NS=statistically not significant.

In contrast with the observations made in monocyte cultures from the patient with haemochromatosis, 10^{-5} M 13-*cis*-RA and Ro 10-9359 significantly reduced the collagenase production by normal monocyte cultures (Table 1, experiment 2). The degree of inhibition with these retinoids, however, was still less than that noted with all-*trans*-RA in the same cultures. All-*trans*-retinal had a marked inhibiting effect in both cultures.

Discussion

In the present study we have investigated the effect of retinoids on the production of collagenase by human adherent mononuclear cells in culture. The mononuclear cells from healthy controls synthesise and secrete collagenase only when activated by incubation with concanavalin A.¹⁶ In addition, cells from a patient with haemochromatosis were studied; these cells are active in synthesising collagenase even without concanavalin A activation. The activation of collagenase production by the latter cells may be associated with increased serum iron concentration in the patient with haemochromatosis.²² Since monocyte-macrophage collagenase may contribute to the destruction of connective tissue components in rheumatoid arthritis the inhibition of collagenase production by retinoids such as all-*trans*-RA could potentially result in improvement of rheumatoid arthritis. Similar inhibition of collagenase production by all-*trans*-RA has been previously reported in human skin fibroblast and rheumatoid synovial cell cultures.^{4 23-25}

Previous animal studies examining the effects of retinoids when using collagen induced or adjuvant arthritis as experimental models for human rheumatoid arthritis have been conflicting. Specifically, treatment of adjuvant arthritic rats with 13-*cis*-RA resulted in considerable improvement of the tissue inflammation,³ yet an augmentation of the inflammatory process in the collagen induced arthritis model was observed.⁶ As indicated previously,³ the discrepancy in the effectiveness of 13-*cis*-RA in these two animal models of rheumatoid arthritis could have several explanations, including the retinoid dosage and the type of rats used. An alternative explanation could be offered based on the results of this study, showing that 13-*cis*-RA has a lesser effect on monocyte-macrophage collagenase production. In fact, monocyte cultures established from a patient with haemochromatosis were unresponsive to 10^{-5} M 13-*cis*-RA, while collagenase production by the same cells was readily inhibited by all-*trans*-RA. The reasons for this discrepancy are not apparent, especially since all-*trans*-RA and 13-*cis*-RA share the same cellular binding receptors,

at least in human skin fibroblasts.²⁶ The differences in the postreceptor events between all-*trans*-RA and 13-*cis*-RA and their corresponding receptor complexes may explain the differential effect on the mononuclear cells of a patient with haemochromatosis. Thus if monocyte-macrophage collagenase has a predominant role in the inflammatory-destructive process of the collagen induced arthritis, lack of efficacy of this retinoid could be explained on the basis of predominant macrophage participation.

The exact mechanisms of the inhibition of collagenase production by all-*trans*-RA in mononuclear cell cultures are not clear at this point. Control experiments excluded, however, generalised inhibition of protein synthesis or induction of an inhibitor molecule as an explanation. Furthermore, no evidence of cell toxicity was noted, as monitored by the release of LDH activity into the extracellular milieu or assessed by viability of the cells by trypan blue exclusion. Furthermore, all-*trans*-RA appeared not to interfere with the secretion of collagenase from the cells. Thus it is conceivable that all-*trans*-RA inhibited the collagenase production on the transcriptional or translational level of protein synthesis. Previous studies have shown that the inhibition of collagen production in human skin fibroblast cultures by all-*trans*-RA is accompanied by a similar decrease in the level of type I procollagen messenger ribonucleic acid.²⁴ Thus similar pretranslational mechanisms could be operative in modulating the collagenase production by retinoids in mononuclear cells in culture.

The authors wish to thank Charlene D Aranda for excellent secretarial assistance. This research was supported in part by USPHS, NIH grants AM-28450, GM-28833, and AM-35297, and by a grant from the Arthritis Foundation, Southern California Chapter.

References

- Ohta A, Louie J S, Uitto J. Differential modulation of collagenase production by retinoids in human monocyte cultures [Abstract]. *Clin Res* 1986; **34**: 164A.
- Harris E D Jr. Retinoid therapy for rheumatoid arthritis. *Ann Intern Med* 1984; **100**: 146-7.
- Brinckerhoff C E, Coffey J W, Sullivan A C. Inflammation and collagenase production in rats with adjuvant arthritis reduced with 13-*cis*-retinoic acid. *Science* 1983; **221**: 756-8.
- Brinckerhoff C E, McMillan R M, Dayer J M, Harris E D Jr. Inhibition by retinoic acid of collagenase production in rheumatoid synovial cells. *N Engl J Med* 1980; **303**: 432-6.
- Brinckerhoff C E, Nagase H, Nagel J E, Harris E D Jr. Effects of all-*trans*-retinoic acid (retinoic acid) and 4-hydroxyphenylretinamide on synovial cells and articular cartilage. *J Am Acad Dermatol* 1982; **6**: 591-602.
- Trentham D E, Brinckerhoff C E. Augmentation of collagen arthritis by synthetic analogues of retinoic acid. *J Immunol* 1982; **129**: 2668-72.
- Strauss J S, Windhorst D B, Weinstein G D. Oral retinoids: a workshop. *J Am Acad Dermatol* 1982; **6** (suppl): 573-832.

- 8 Goodman D S. Vitamin A and retinoids: recent advances, introduction, background, and general overview. *Fed Proc* 1979; **38**: 2501-3.
- 9 Nathan C F, Murray H W, Cohn Z A. The macrophage as an effector cell. *N Engl J Med* 1980; **303**: 622-6.
- 10 Davies P, Bonney R J, Humes J L, Kuehl F A. Secretory functions of macrophages participating in inflammatory responses. In: Dingle J T, Gordon S, eds. *Cellular interactions*. Amsterdam: Elsevier/North Holland, 1981: 33-42.
- 11 Wahl L M, Wahl S M, Mergenhagen S E, Martin G R. Collagenase production by endotoxin activated macrophages. *Proc Natl Acad Sci USA* 1974; **71**: 3598-601.
- 12 Werb Z, Gordon S. Secretion of a specific collagenase by stimulated macrophages. *J Exp Med* 1975; **142**: 346-60.
- 13 Horwitz A L, Crystal R G. Collagenase from rabbit pulmonary alveolar macrophages. *Biochem Biophys Res Commun* 1976; **69**: 296-303.
- 14 Mainardi C L, Seyer J M, Kang A H. Type specific collagenolysis: a type I collagen degrading enzyme from macrophages. *Biochem Biophys Res Commun* 1980; **97**: 1108-15.
- 15 Werb Z, Banda M J, Jones P A. Degradation of connective tissue matrices by macrophages. I. Proteolysis of elastin, glycoproteins and collagen by proteinases isolated from macrophages. *J Exp Med* 1980; **152**: 1340-57.
- 16 Louie J S, Weiss J, Ryhnen L, Nies K M, Rantala-Ryhnen S, Uitto J. The production of collagenase by adherent mononuclear cells cultured from human peripheral blood. *Arthritis Rheum* 1984; **27**: 1397-404.
- 17 Boyum A. Separation of leukocytes from blood and bone marrow. *Scand J Clin Lab Invest* 1968; **21** (suppl 97) 77-89.
- 18 Ryhnen L, Rantala-Ryhnen S, Tan E M L, Uitto J. Assay of collagenase activity by a rapid, sensitive and specific method. *Coll Relat Res* 1982; **2**: 117-30.
- 19 Phillips H J. Dye exclusion test for cell viability. In: Kruse P F, Patterson M K Jr, eds. *Tissue culture methods and applications*. New York: Academic Press, 1973: 406-8.
- 20 Wroblewski F, LaDue J S. Lactic dehydrogenase activity in blood. *Proc Soc Exp Biol Med* 1955; **90**: 210-3.
- 21 Bradford M M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-binding dye. *Anal Biochem* 1976; **72**: 248-54.
- 22 Okazaki I, Brinckerhoff C E, Sinclair J F, Sinclair P R, Bonkowski H L, Harris E D Jr. Iron increases collagenase production by rabbit synovial fibroblasts. *J Lab Clin Med* 1981; **97**: 396-402.
- 23 Bauer E A, Seltzer J L, Eisen A Z. Retinoic acid inhibition of collagenase and gelatinase expression in human skin fibroblast cultures. Evidence for a dual mechanism. *J Invest Dermatol* 1983; **81**: 102-69.
- 24 Oikarinen H, Oikarinen A I, Tan E M L, et al. Modulation of procollagen gene expression by retinoids: inhibition of collagen production by retinoic acid accompanied by reduced type I procollagen messenger ribonucleic acid levels in human skin fibroblast cultures. *J Clin Invest* 1985; **75**: 1545-53.
- 25 Abergel R P, Meeker C A, Oikarinen H, Oikarinen A I, Uitto J. Retinoid modulation of connective tissue metabolism in keloid fibroblast cultures. *Arch Dermatol* 1985; **121**: 632-5.
- 26 Oikarinen A I, Oikarinen H, Uitto J. Demonstration of cellular retinoic acid binding protein in cultured human skin fibroblasts. *Br J Dermatol* 1985; **113**: 529-35.