Inhibition of vitamin A action in rat bone cultures by inhibitors of RNA and protein synthesis

A. Kistler[1]

Biological Pharmaceutical Research Department, F. Hoffmann-La Roche & Co. Ltd, CH-4002 Basle (Switzerland), 31 December 1977

Summary. Using explanted humeri of late fetal rats, retinoic acid induced a dose- and time-dependent regression of cartilage. The retinoic acid-induced release of proteoglycan into the medium was blocked by inhibitors of RNA and protein synthesis, suggesting its dependence upon continuous RNA and protein synthesis.

Vitamin A is not only essential for growth and maintenance of specific organ functions such as vision and reproduction, but also plays a key role in normal differentiation of epithelial tissues[2]. Recent studies have shown that vitamin A and its synthetic analogues (retinoids) can prevent the development of epithelial cancer[3-6], but it is not known by which mechanism high doses of retinoids protect against epithelial carcinogenesis or eventually cause general toxic effects. In 1952 Fell and Mellanby[7] have shown that in vitro excess of vitamin A acts directly on the cartilagenous limb rudiments of 6-7-day chick embryos inducing cartilage resorption, which is accompanied by the loss of proteoglycan[8] and an increase in the proteolytic activity of the cartilage rudiment[9]. Using explanted humeri of late fetal rats, we have found that the retinoic acid-induced release of proteoglycan into the medium is blocked by inhibitors of RNA and protein synthesis. From this, we conclude that the catabolic effect of vitamin A is dependent upon continuous RNA and protein synthesis.

Organ cultures were prepared from humeri of late fetal rats and maintained in nutrient mixture F-10 supplemented with 13% fetal calf serum and antibiotics. The time- and dose-dependence of the gross morphological changes induced by retinoic acid in limb bone rudiments is shown in figure 1. Similar changes in response to retinoic acid were found in cultures without fetal calf serum, although cartilage resorption was reduced. Matrix resorption was most conspicuous at the epiphysal ends but lower at the diaphysis of the bone shaft (figure 1). This observation was confirmed by the determination of the macromolecular components DNA, RNA and protein in the 2 parts of the bone rudiment.

Figure 2 shows the changes in length and macromolecular tissue constituents of bone rudiments in response to retinoic acid. Up to 2 days of treatment, no significant changes in length and DNA, RNA and protein were noted, but thereafter a dose-dependent shortening and decrease in the macromolecular constituents were observed. On the other hand, the release of proteoglycan into the medium was already manifest after 2 days of treatment with retinoic acid. Table 1 shows that, after a lag between 7 and 15 h, increasing amounts of proteoglycan were released by retinoic acid. Release of proteoglycan was coincident and quantitatively correlated with the loss of metachromatic staining with toluidin blue, as was also observed in rabbit ear cartilage[8,10]. Vitamin A-alcohol and vitamin A-aldehyde were found to be less potent than retinoic acid, but induced the same changes in the rat bone rudiments (Kistler, unpublished observations).

Humeri, which had been treated for 1-2 days with retinoic acid and subsequently were transferred to control medium, before any morphological changes were discernible, underwent cartilage resorption. This observation and the recent reports on the occurrence of several retinoic acid-binding proteins in mammalian and avian tissues[11-16] which may have some resemblance to steroid-binding proteins, prompted us to investigate whether retinoic acid-induced changes in bone rudiments are dependent upon new protein and RNA synthesis.

Table 1 shows a dose-dependent inhibition by cycloheximide of retinoic acid-induced proteoglycan release from bone rudiments, suggesting its dependence upon de novo synthesis of protein. From table 2 it is evident that 3 inhibitors of RNA synthesis, i.e. actinomycin D, cordycepin, and α-amanitin, which affect transcription at different levels, also depressed the retinoic acid-induced release of proteoglycan. To assess possible toxic effects of these inhibitors, the humeri, after incubation with the drug, were transferred to control medium and further incubated. As shown by table 2 (last column), the inhibition of the retinoic acid-induced release of proteoglycan was relieved in the control medium, except at higher concentrations of actinomycin D or α-amanitin, although no vitamin A acid was present.

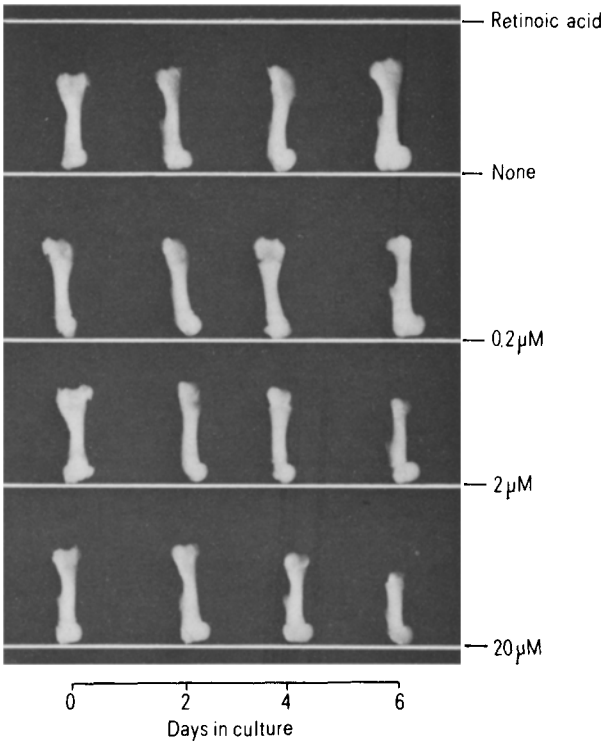

Fig. 1. Time course and dose-dependence of the gross morphological changes induced by retinoic acid in limb bones of late fetal rats. Humeri were prepared from fetal rats (Fü-albino SPF rats, outbread stocks, from the Institute of Biological and Medical Research, Füllinsdorf, Switzerland) at day 22 of gestation in Ringer's salt solution and incubated in nutrient mixture F-10 (Ham, supplemented with 13% fetal calf serum, 60 µg/ml penicillin and 100 µg/ml streptomycin) in cell culture dishes (Falcon, 5.5 cm, final volume 5 ml) in a H_2O-saturated 5% CO_2-air atmosphere at 36 °C. Retinoic acid was dissolved in dimethylsulfoxide (DMSO) before addition to culture; an equivalent amount of DMSO, which never exceeded 1% was added to the control cultures. The medium was changed every 2 days. The distance between the white lines represents 1 cm.

Table 1. Inhibition by cycloheximide of the retinoic acid-induced proteoglycan release into the medium from limb bones of late fetal rats

Cycloheximide (μM)	Retinoic acid (μM)	Proteoglycan release (μg/humerus) 7 h	15 h	24 h	39 h	50 h
None	None	1.9±1.3	4.0±0.4	6.3±1.5	10.2± 0.9	14.4± 0.7
None	20	1.2±1.2	6.8±1.0	21.7±3.3	65.2± 8.4	115.0±12.0
0.05	None	n.d.	n.d.	6.8±2.4	10.4± 2.6	15.2± 1.1
0.5	None	n.d.	n.d.	8.2±1.4	9.4± 3.1	16.8± 2.8
5.0	None	n.d.	n.d.	7.0±2.1	9.8± 1.8	17.4± 1.6
0.05	20	n.d.	n.d.	20.7±2.6	55.8± 5.3	107.2±27.9
0.5	20	n.d.	n.d.	12.2±0.4	33.8±12.6	82.0±29.3
5.0	20	n.d.	n.d.	8.2±1.8	13.0± 1.0	18.0± 2.1

Humeri (2 or 1 per dish, 3.5 cm) were incubated in supplemented culture medium F-10 (final volume 2 ml) at 36 °C. Retinoic acid and cycloheximide (dissolved in ethanol) were added together. At the time indicated aliquots of the medium were collected and the amount of proteoglycan released into the medium determined with Alcian blue[22]. The results are the mean±SD of 4 determinations (n.d. = not determined).

Table 2. Inhibition by actinomycin D, cordycepin, and α-amanitin of the retinoic acid-induced proteoglycan release into the medium from limb bones of late fetal rats

Experiment	Inhibitor (μg/ml)	Retinoic acid (μM)	Proteoglycan release During treatment		After treatment	
			μg/24 h/humerus	%	μg/96 h/humerus	%
A	None	None	11.4±2.6	100	28.8	100
	None	20	24.2±2.2	212	120.0	417
	Actinomycin D					
	0.03	None	11.7±1.2	103	28.8	100
	0.1	None	11.8±1.2	104	20.9	73
	0.03	20	13.8±1.9	121	95.1	330
	0.1	20	8.9±2.5	78	25.7	89
	Cordycepin					
	10	None	8.7±1.4	76	30.1	105
	100	None	9.7±0.8	85	22.7	79
	10	20	10.4±0.9	91	133.4	463
	100	20	8.4±0.7	74	77.0	267
			μg/48 h/humerus	%	μg/48 h/humerus	%
B	None	None	20.5± 2.0	100	16.8	100
	None	20	138.0± 7.1	673	109.6	652
	α-Amanitin					
	3	None	24.3± 1.8	119	30.8	183
	9	None	22.8± 3.7	111	27.6	164
	18	None	18.0± 0.4	88	22.6	135
	3	20	149.7± 9.5	730	106.6	635
	9	20	58.5±14.0	285	80.0	476
	18	20	30.4± 2.5	148	28.0	167

4 humeri per group (1 per dish, 3.5 cm) were incubated in supplemented culture medium F-10 (final volume 2 ml) at 36 °C. In experiment A, actinomycin D was added 2 h before and cordycepin along with retinoic acid and incubated for 1 day. In experiment B, α-amanitin was added 6 h before the addition of retinoic acid and incubated for 2 days. Then 2 humeri were further incubated in control medium for 3 and 2 days in experiment A and B, respectively. The amount of proteoglycan release into the medium was determined in aliquots of the medium with Alcian blue[22]. The results are the mean±SD of 4 determinations (during treatment) or the mean of 2 determinations (after treatment).

This result suggests that continuous synthesis of RNA is needed for retinoic acid-dependent release of cartilage proteoglycan. In addition, it shows that the inducing effect of retinoic acid persists even after its withdrawal. In skin tumor keratoacanthoma, the vitamin A acid-induced mucous metaplasia was also depressed by prior application of actinomycin D[17].

Fell and Dingle[9] have shown that vitamin A caused an increase of proteolytic activity in bone rudiments and suggested that this effect might result from an induced release of lysosomal enzymes. The inhibition of the vitamin A effects on bone rudiments by cycloheximide, and by the inhibitors of RNA synthesis shown in this paper, however, argues against a direct action of vitamin A involving labilization of lysosomal membranes[18]. This conclusion is also supported by our observation that lysosomal stabilizers, like chloroquine and E-aminocaproic acid, had no effect on the retinoic acid-induced cartilage resorption (Kistler, unpublished observation). Since there was a lag between 7 and 15 h until stimulation of the proteoglycan release by retinoic acid could be detected (table 1), it is most likely that enhanced synthesis of hydrolytic enzymes is required for cartilage degradation.

Having shown that the retinoic acid-induced resorption in late fetal bone is dependent upon newly synthesized RNA and protein and that the retinoic acid-binding protein is localized in the nucleus[16], it is tempting to speculate that the vitamin-protein complex is bound to chromatin, like the steroid hormone-receptor complex[19]. It is thought that this nuclear receptor-ligand site stimulates transcription and thus initiates the sequence of biochemical events leading to the overall physiological changes produced by

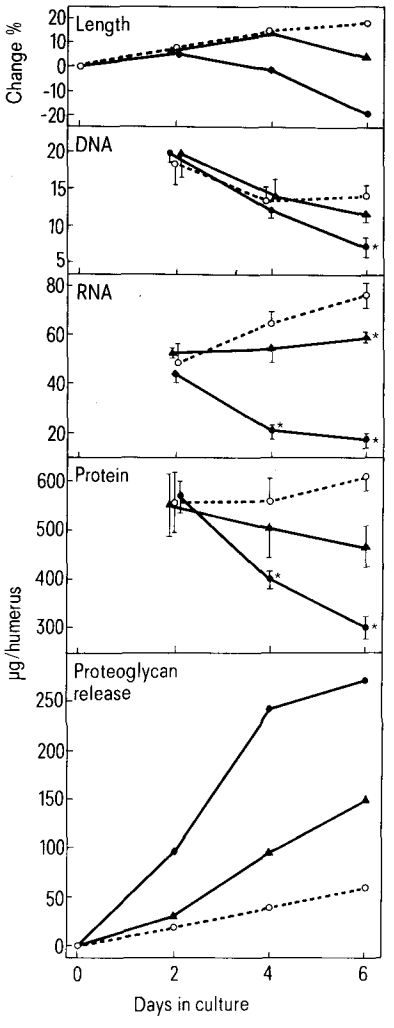

Fig. 2

Fig. 2. Time course of retinoic acid-induced changes in length, DNA, RNA and protein content and in the release of proteoglycan in limb bones of late fetal rats. Humeri (4 per dish) were incubated in supplemented culture medium F-10 (final volume 5 ml) at 36 °C. Before incubation, and then every 2 days, the contour of the magnified bones (× 14.1, using a reversed microscope and a projecting prisma) was drawn and the length determined. At the time indicated, the humeri, after washing in Ringer's salt solution, were extracted in 1 ml of 10% (w/v) trichloroacetic acid and the extract discarded. Nucleic acids were extracted with 10% (w/w) perchloric acid at 70 °C for 30 min and the DNA and RNA determined[23,24]. The remaining tissue was dissolved in 1N NaOH and the protein measured[25]. Aliquots of the culture medium were collected and the amount of proteoglycan released into the medium was measured by the Alcian blue assay[22], using chondroitinsulfate as standard. Since the humeri were distributed randomly, it was assumed that the values at day 0 were similar for all groups. Except for the proteoglycan release, the results are the mean of 4 determinations, ± SD. Treatments were: ○, controls; ▲, 0.6 µM retinoic acid; ●, 20 µM retinoic acid, *$p < 0.01$.

that hormone or vitamin. Since the effects of the hormonal form of vitamin D_3, 1,25-dihydroxycholecalciferol[20,21], are also known to depend upon continous RNA and protein synthesis, it is most likely that cellular effects of certain vitamins, like those of some hormones, are mediated by regulation of protein synthesis, presumably at the transcriptional level. However, further work is needed to ascertain whether the vitamin A effect only represents a quantitative or rather a qualitative change in protein synthesis.

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Effect of infection of mice with Friend leukemia complex viruses on background antibody-forming cell production in vitro[1]

M. Bendinelli, D. Matteucci, A. Toniolo and H. Friedman

Institute of Microbiology, University of Pisa, I-56100 Pisa (Italy) and Department of Microbiology and Immunology, Albert Einstein Medical Center, Philadelphia (Pa. 19141, USA), 16 February 1978

Summary. Friend leukemia complex (FLC) and Rowson-Parr virus (RPV) infections of donor mice depress the production of background antibody-forming cells by splenocytes cultured in the absence of specific antigenic stimulation.

During investigations on the immunodepressive properties of Friend leukemia complex (FLC), it was shown that adult mice infected with viruses belonging to this complex exhibit increased numbers of splenic background antibody-forming cells to various antigens[2,3]. Obviously, this is a paradoxical effect, since in the same mice artifically stimulated antibody responses are suppressed[4]. Here it is shown that after infection of donor mice with FLC viruses the