Mononuclear cell supernatants inhibit prolyl hydroxylation

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The effect of phytohemagglutinin(PHA)-activated human peripheral mononuclear cell supernatant (AS) on collagen production by human fibroblasts was examined. The AS inhibited collagen production in a dose- and time-dependent manner. Labeling and pulse chase experiments showed that it did not block collagen secretion, but a greater proportion of molecules synthesized in its presence accumulated within the cells. Amino acid analysis showed that when labeling was done at 24°C prolyl hydroxylation in fibroblasts exposed to the AS was reduced to two-thirds of the cultures treated with control supernatant (CS), however it was not different at 37°C. These results indicate that the AS inhibits collagen hydroxylation, that the un-(under)hydroxylated collagen molecules are degraded at physiological temperature and that suppression of collagen hydroxylation may be a mechanism by which the AS inhibits collagen production.

Collagen synthesis *Lymphokine* *Lymphocyte supernatant* *Prolyl hydroxylation* *Fibroblast*

1. INTRODUCTION

Fibroblasts are the major cell type responsible for synthesis of matrix components. Peripheral blood mononuclear cells activated by a variety of substances such as bacterial endotoxins and phytohemagglutinin (PHA) elaborate mediators that modify the proliferation and synthesis activities of fibroblasts. These substances, which are secreted into the culture medium, have been shown to be stimulatory as well as inhibitory to fibroblast activities [1–8]. A protein which inhibits collagen production by fibroblasts has been partially characterized and it was shown to have an M_r of approx. 55000 [9,10]. The mechanism by which collagen production is inhibited is not known. Here we show that supernatants of mononuclear cells activated by PHA inhibit prolyl hydroxylation; this may be a mechanism by which collagen production is affected.

2. MATERIALS AND METHODS

Human gingival fibroblasts were used for the experiments. PHA-activated human peripheral blood mononuclear cell supernatant (AS) was prepared as in [8]. Briefly, mononuclear cells were isolated by sedimentation in Ficoll hypaque (Litton Bionetics, Kensington, MD), washed twice with Hank's balanced salt solution, and cultured in RPMI-1640 medium at a cell concentration of 2×10^6 cells/ml for 72 h with 15 μg/ml of PHA-P (Difco Laboratories, Detroit, MI). For the control supernatant (CS) the PHA-P was added at the end of incubation. After incubation the media were separated and dialyzed vs RPMI medium prior to use. Fibroblasts were treated with 1:10 dilution of AS or CS for 24 h, except when specified otherwise, and then labeled with 5 μCi/ml of L-[3,4-^{3}H]proline and [2-^{3}H]glycine in RPMI medium containing 0.2% serum in the presence of 50 μg/ml each of ascorbic acid and β-aminopropionitrile and collagen was quantitated by digestion with purified bacterial collagenase (Advance Biofactures, Lynnbrook, NY) [11]. To determine the extent of prolyl hydroxylation, medium and cell proteins were combined, hydrolyzed in 6 M HCl at 108°C for 24 h, radioactive proline and hydroxyproline were separated using a Beckman 120C amino acid analyzer and their radioactivity was measured by liquid scintillation counting [12]. For determination of collagen types,

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procollagens were converted to collagens by limited pepsin digestion, separated on DEAE-cellulose columns at 15°C and on SDS-slab gels, located by fluorography and quantitated by scanning the fluorograms [12].

3. RESULTS AND DISCUSSION

Incubation with AS resulted in inhibition of collagen production, which was dose dependent at dilutions less than 1:50 (fig.1A). (The results for AS and CS were also compared relative to RPMI medium alone, which was not exposed to mononuclear cells. The CS exhibited some inhibition of collagen production relative to the RPMI medium and it was dose dependent. Thus it appears that mononuclear cells elaborate the collagen-inhibitory factor in the absence of PHA and that the elaboration is enhanced by the PHA. The inhibitory factor was not PHA because addition of PHA-P to the fibroblasts had no effect.) However, total incorporation remained similar to the cells treated with CS; this indicated that the production of noncollagenous proteins was not significantly affected. The inhibition of collagen is not reflected in total incorporation probably because of the low proline and glycine content of noncollagenous proteins relative to collagen, however it is noticeable at 1:1 dilution when collagen production is reduced significantly. The inhibition of collagen production could be seen as early as 6 h after exposure to AS and it reached maximum after 24 h (fig.1B). It was not reversible even after 48 h after the removal of AS (not shown).

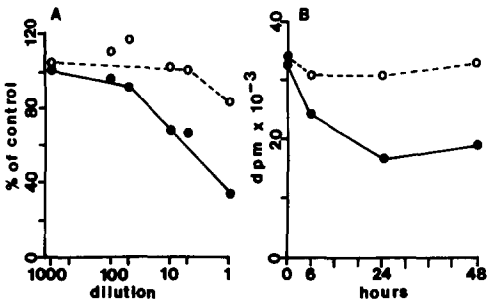

Fig.1. Effect of activated (AS) and control (CS) human mononuclear cell supernatants on protein and collagen production by human fibroblasts. (A) Effects of different dilutions of AS. The results are presented as % relative to the CS for each dilution and they represent the mean of triplicates. (o---o) Total incorporation, (●——●) collagen. (B) Effect of exposure time on collagen production; cells were treated with the supernatants at 1:10 dilution. (o---o) CS, (●——●) AS.

To determine whether the inhibition is directed towards any particular collagen type, the quantities and proportions of types I, III, and V collagens in fibroblasts treated with CS and AS were compared. From table 1 it can be seen that the radioactivity in each collagen type is less in the presence of AS but their proportions remained identical to the CS-treated cultures, indicating that all collagens are affected in a parallel fashion. Our results are in contrast to those of Parrott et al. [5] who observed that activated mononuclear cell supernatants increase fibroblast collagen production, especially type III.

To understand the mechanism by which AS inhibits collagen production the following experiments were done. We compared the membrane transport of [^{3}H]proline and [^{3}H]glycine in fibroblasts treated with either AS or CS, but observed no differences (not shown). Next we examined whether collagen secretion is inhibited so that it accumulates within the cells causing feedback inhibition. Cells were treated for 24 h with the supernatants, then labeled for 6 h and the proportions of collagen in cells and medium were determined. Results showed that a greater proportion of collagen was present in the cells of AS treated fibroblasts relative to the CS (54% vs 37%, table 2, expt 1), indicating that the secretion of collagen synthesized in the presence of the AS may be retarded. To investigate whether the secretion of molecules synthesized under normal conditions is

Table 1

Effect of mononuclear cell supernatants on collagen types made by fibroblasts

Supernatant	cpm × 10^{-3}			Percent		
	I	III	V	I	III	V
Control	302.9	32.1	1.4	90.0	9.5	0.4
Activated	66.0	6.5	0.4	90.5	8.9	0.5

Cells were treated with the supernatants at 1:10 dilutions for 24 h, and then labeled for 24 h. Collagen types were quantitated as described in section 2

affected, fibroblasts were labeled for 6 h in the absence of AS or CS, then the medium was replaced with fresh medium containing AS or CS plus unlabeled proline and glycine. The incubation was continued for 3 h and the distribution of collagen in medium and cells was determined. However, no significant differences were observed (expt 2, table 2).

The above experiments showed that the AS does not totally block collagen secretion, but accumulation of collagen within the cells indicated that molecules made in its presence may be secreted at a slower rate. Because unhydroxylated collagen is secreted at a slower rate [13], we examined whether the AS affects collagen hydroxylation. This was done as follows: two sets of fibroblast cultures were incubated for 24 h with AS or CS. Then one set was labeled at 37°C for 24 h, and the other at 24°C. The prolyl hydroxylation in both cultures was then quantitated. At 24°C un(der)hydroxylated collagen molecules, if made, will remain triple helical and will not be degraded [14,15]; thus the value for prolyl hydroxylation will be less. First we compared the intracellular proteins; however, because a majority of collagen was present in the medium and hydroxyproline counts remaining in the cells at 24°C were very few, media plus cells were combined for analysis. The results showed that when labeling was done at 37°C no differences were present between the AS and CS treated cultures (table 3, expt 1). However, at 24°C the percent prolyl hydroxylation in the presence of AS was reduced to less than two-thirds of the CS treated cultures (expts 2 and 3, table 3).

These results show that hydroxylation of collagen molecules made in the presence of AS is reduced. Steinmann et al. [16] observed that reduction of collagen hydroxylation by one-third by ascorbic acid deficiency causes a four-fold increase in its degradation. Bienkowski et al. [17] showed that prevention of prolyl hydroxylation by *cis*-4-hydroxyproline increases intracellular collagen degradation 2-fold, which was shown to occur in the lysosomes [18]. Therefore, collagen molecules with reduced prolyl hydroxylation and synthesized in the presence of AS are likely to be degraded at 37°C, and if so the remaining molecules will be normally hydroxylated. In addition, the degradation will result in a corresponding decrease in collagen production. Accordingly, the prolyl hydroxylation at 37°C appears normal (table 3), and the reduction in prolyl hydroxylation, which is approximately one-third, and the decrease in collagen production are comparable (cf. table 3 with fig.1). In similar experiments done at 37°C, Jimenez et al. [4] also did not find a reduction in prolyl hydroxylation.

Our observations indicate that suppression of collagen hydroxylation may be a mechanism by which AS inhibits collagen production by fibroblasts. However, how it inhibits prolyl hydroxylation and whether the enzyme prolyl hydroxylase is affected are not known. Whether additional mechanisms such as reduction of collagen mRNA levels operate is also not known.

Table 2

Effect of mononuclear cell supernatants on collagen secretion

No.	Supernatant	Treatment	cpm collagen × 10^{-3} [a]		% collagen in	
			Cells	Medium	Cells	Medium
1[b]	activated	–	10.5 ± 0.4	9.0 ± 0.6	54	46
	control	–	9.8 ± 0.8	16.7 ± 1.5	37	63
2[c]	none	pulse	6.2 ± 0.4	–	100	–
	activated	chase	2.9 ± 0.7	2.0 ± 0.3	59	41
	control	chase	3.1 ± 0.3	1.7 ± 0.3	65	35

[a] Means ± SD of triplicates are presented

[b] Fibroblasts were exposed to AS or CS at 1:10 dilution for 24 h and then labeled

[c] Cells were first labeled for 6 h in the absence of AS or CS, then incubated for 3 h in medium with 1:10 diluted AS or CS

Table 3

Effect of labeling temperature on prolyl hydroxylation in fibroblasts exposed to mononucler supernatants[a]

Expt	Supernatant	Labeling temperature (°C)	cpm × 10^{-3}		% proline hydroxylation[b]
			pro	hyp	
1	activated	37	28.5	5.1	15.2
	control	37	27.9	5.0	15.2
2	activated	24	11.6	1.4	10.7
	control	24	14.2	3.0	17.4
3	activated	24	14.8	2.3	13.5
	control	24	7.9	2.1	21.0

[a] Cells in 75-cm^2 flasks were treated with 1:5 diluted AS or CS for 24 h and then labeled for 24 h. Cell and medium proteins were combined and subjected to amino acid analysis as described in section 3

[b] cpm hyp × 100/(cpm hyp + cpm pro)

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