PROLYL HYDROXYLASE IN PULMONARY ALVEOLAR MACROPHAGES

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1. Introduction

It has been reported that cell lines which did not orginate from connective tissue cells either synthesize hydroxyproline-containing polypeptides [1–6] or possess prolyl hydroxylase (EC 1.14.11.2) activity [2,3,7]. Since these cell lines had been maintained in culture for some time, the relationship of these observations to the in vivo function(s) of the cells is uncertain [1]. Few instances of either the formation of hydroxyproline-containing polypeptides [8] or the presence of prolyl hydroxylase in homogeneous cell populations obtained directly from the intact animal have been reported [9,10]. Pulmonary lavage in humans [11] and rabbits [12,13] yields a cell population which consists primarily of pulmonary alveolar macrophages (PAM). It has been reported that PAM do not either synthesize collagen [14] or contain prolyl hydroxylase [7]. The present study demonstrated that rabbit and human PAM do contain significant amounts of prolyl hydroxylase activity.

2. Materials and methods

2.1. *Pulmonary alveolar macrophages*

The PAM were obtained from normal male New Zealand white rabbits by pulmonary lavage [12]. The lungs of pentobarbital-killed rabbits were lavaged with calcium- and magnesium-free phosphate buffered saline, pH 7.8 [15]. The cells were tested routinely for viability by dye exclusion (trypan blue) and only batches with 90% or more viable cells were used. Total cell numbers were calculated from haemocytometer counts. Human PAM were obtained from patients who were undergoing the lavage procedure as part of an evaluation of their lung disease.

2.2. *Fibroblast cell lines*

Cultures of WI-38 human fetal lung fibroblasts and CLAC, a fibroblast cell line developed from adult human lung tissue, were maintained by sequential passage of cultures at 3–4 day intervals and at split or passage ratios of 1:2. Sixteen hours prior to harvesting the fibroblasts, fresh culture fluid containing 20 μg/ml sodium ascorbate was added to the cells. The fibroblasts were harvested without using trypsin by scraping into Hank's balanced salt solution (HBSS) with a rubber policeman. Cell counts were performed on parallel fibroblast cultures which were harvested using 0.25% trypsin.

2.3. *Prolyl hydroxylase assay*

Cells were suspended in a solution containing 0.1 mM dithiothreitol, 0.005% (w/v) α-toluene sulfonyl fluoride (added in 0.1 ml 2-propanol/10 ml final volume), 0.01 mM ethylenediamine tetraacetic acid, tetrasodium salt, 50 mM Tris–HCl, pH 7.5, 100 mM NaCl and 0.1% (v/v) Triton X-100. Homogenization was performed at approx. 3°C with a Polytron PT

10-35 fitted with a PT 10-ST generator run at a setting of 10 for 30 s. The homogenate was centrifuged at 15 000 × *g* for 30 min at 3°C and the supernate was used as the source of enzyme.

Prolyl hydroxylase activity was measured by the method of Hutton et al. [16]. Each assay mixture contained 1 mM α-ketoglutarate, 5 mM sodium ascorbate, 1 mM ferrous ammonium sulfate, substrate (80 000 cpm) and enzyme preparation (0.2–1.6 mg protein). The volume of the assay mixture was a adjusted to 3.0 ml with the homogenizing solution lacking Triton X-100. Incubation was carried out for 30 min, aerobically, at 30°C with shaking in a water bath. The reaction was terminated by adding 0.3 ml 50% (w/v) trichloroacetic acid to the incubation mixtures. Enzyme activity was determined by the amount of tritiated water formed in 30 min.

2.4. *Culture of PAM samples*

PAM were washed once with HBSS containing 200 μg streptomycin/ml, 200 units penicillin/ml, 200 μg Kanamycin/ml and 60 μg mycostatin/ml. The cells were suspended in basal medium (Eagle) supplemented with 10% fetal calf serum and one-half the above concentrations of antibiotics. Medium, 4 ml, containing between 2×10^6 and 4×10^6 cells, as plated in 60 mm plastic petri plates (Falcon). Cultures were maintained in a water-saturated atmosphere of 5% CO_2, 95% air at 37°C. To determine the plating efficiency of rabbit lung-derived fibroblasts in the presence of PAM, 200 rabbit lung fibroblasts (cell line RLLF) were added per 60 mm plate to separate cultures of rabbit PAM. Cultures of RLLF alone were plated at a cell density of 200/60 mm plate. The culture medium was changed every 3–4 days. At the end of two weeks of incubation, the culture medium was removed and the cells stained for 18 h with 0.1% 2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride hydrate (Aldrich Chemical Co.) added in growth medium. Following removal of the staining solution and drying of the plates, the number of colonies per plate were counted.

2.5. *Miscellaneous methods*

Protein was measured by the method of Lowry et al. [17]. Amino acid analysis was performed on a Beckman Model 121 amino acid analyzer modified to collect fractions of the eluate. Radioactivity measurements were performed on a Packard liquid scintillation spectrometer employing the scintillation mixture of Prockop and Ebert [18]. Tritium counting efficiency was 30%.

3. Results

The observed prolyl hydroxylase activity of rabbit PAM increased linearly when the enzyme obtained from increasing numbers of cells was assayed. The substrate concentration became rate-limiting when the enzyme present in 5×10^6 to 6×10^6 cells was used in the incubation. Table 1 lists the results of a comparison of enzyme activity present in two human lung fibroblast cell lines with the enzyme activity present in rabbit PAM. The enzyme activity/10^6 cells did not differ among the three types of cells, whereas the enzyme activity per mg protein was higher in the PAM than in the two fibroblast cell lines ($p < 0.001$). Table 2 shows the effect of omitting the known cofactors of prolyl hydroxylase [19] on the enzyme activity in PAM. The enzyme activity exhibited when the assay was performed under an atmosphere of 100% nitrogen was probably due to a failure to obtain complete removal of oxygen from the system.

Amino acid analysis of 5.65 HCl hydrolysates of the substrate after incubation with the enzyme and all cofactors revealed that the amount of radiolabeled hydroxyproline present was equal to the amount of tritiated water which was formed during the incubation. In the absence of α-ketoglutarate no radiolabeled hydroxyproline was detected.

Preparations of pulmonary lavage-obtained cells from normal rabbits contain 90–95% PAM as determined by microscopic criteria, by the ability of cells to adhere to appropriate surfaces and by their ability to phagocytose and kill *Staphylococcus epidermidis* [15]. No cells with the morphology of fibroblasts have been identified by microscopy in the PAM preparations. Few cells with the growth properties of fibroblasts were present in the rabbit lung lavages (table 3). Rabbit PAM did not have any effect on the growth of RLLF. A small number of red blood cells and leukocytes occasionally were present in pulmonary lavage specimens; however, these cells do not contain prolyl hydroxylase activity [7,10].

Prolyl hydroxylase assays were performed on PAM obtained from 7 patients. The amount of enzyme

Table 1
Comparison of prolyl hydroxylase activity in rabbit pulmonary alveolar macrophages and two fibroblast cell lines

Cell type	Enzyme activity[a]			
	n	cpm/10^6 Cells	n	cpm/mg Protein
PAM	16	420 ± 17	13	1630 ± 65
CLAC (passage No. 11)	7	436 ± 28	7	1158 ± 120
WI-38 (passage No. 23)	8	475 ± 39	8	975 ± 109

[a]Mean ± SEM

Enzyme assays were performed as described in the text. The values given for enzyme activity present in PAM represent the results of the indicated number of assays (n) performed on 5 rabbit pulmonary lavage samples (cpm/10^6 cells) and the indicated number of assays (n) performed on 3 rabbit pulmonary lavage samples (cpm/mg protein). The values given for enzyme activity present in cell lines CLAC and WI-38 represent the results of the indicated number of assays (n) performed on 4 replicate cultures studied at the stated passage number

Table 2
Cofactor requirements of prolyl hydroxylase in rabbit pulmonary alveolar macrophages

Incubation conditions	cpm/10^6 Cells[a]	(%) Control activity
Complete system	346	100
Without α-ketoglutarate	0	0
Without ferrous ammonium sulfate	26	8
Without sodium ascorbate	4	1
100% N_2 atmosphere	92	23

[a]Average of two determinations on separate PAM samples from two normal rabbits. Incubation conditions were the same in all studies except for the absence of the designated cofactor

Table 3
Growth of fibroblasts present in cultures of cells from rabbit pulmonary alveolar lavages

Experiment		Fibroblastoid cells/10^6 PAM	Plating efficiency of RLLF cells (%)		Estimated total fibroblastoid[c] cells/10^6 PAM
	n[a]		Alone	With PAM	
1	10	1	n.d.[b]	n.d.	n.d.
2	5	1	11	11	9
3	3	1	11	13	8
4	3	2	5	5	40

[a]n is the total number of cultures plated per lavage sample

[b]Not done

[c]The estimated total number of fibroblastoid cells present in the lavage cell population is calculated on the basis of the plating efficiency of cell line RLLF. For the purpose of this determination, it is assumed that the plating efficiency of fibroblastoid cells present in the lavage cell population is the same as RLLF

activity/10^6 cells was more variable tended to be higher (average, 796 cpm/10^6 cells; range, 399–1103 cpm/10^6 cells) than in rabbit PAM. Prolyl hydroxylase of human PAM had an absolute requirement for α-ketoglutarate.

4. Discussion

The failure of Goldberg and Green to detect significant amounts of prolyl hydroxylase in PAM [7] may have resulted either from not adding an inhibitor of proteolytic enzyme activity to their homogenizing solution or from a failure to obtain optimal solubilization of the enzyme. Proteolytic enzymes can interfere with the measurement of prolyl hydroxylase activity [20]. Prolyl hydroxylase activity in homogenates of PAM made with a Virtis tissue homogenizer but without Triton X-100 was 5–20 times lower than in homogenates made with Triton X-100 (data not shown). Macrophages have been identified as 'avidly phagocytic cells with the cellular enzymes to digest what has been ingested and the cellular apparatus to make more digestive enzymes' [21]. Currently-accepted macrophage functions can be characterized as defensive and/or catabolic in nature [21]. The demonstration of collagenase secretion by PAM [13] is a recent contribution to this concept of macrophage function. Prolyl hydroxylase mediates the hydroxylation of a portion of the proline residues present in the polypeptide precursors of collagen and collagen-related proteins [19,22,23]. This process represents a post-translation step in the synthesis of these proteins [19]; therefore, the presence of prolyl hydroxylase in a cell which has not been subjected to in vitro growth conditions suggests that the cell has a differentiated, anabolic function. Thus, the presence of prolyl hydroxylase in PAM indicates that assigning a purely defensive and catabolic role to these cells may be an incomplete statement of their physiologic function.

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