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INDUCTION OF PROLYL HYDROXYLASE ACTIVITY IN A NONADHERENT POPULATION OF HUMAN LEUKOCYTES

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Summary: A nonadherent population of human monocytes has been shown to express the collagen hydroxylating enzyme prolyl hydroxylase in vitro. Enzyme levels present in freshly isolated nonadherent cells were induced 300% during the first 72 hours of culturing, which could be suppressed by cycloheximide. Maximum induction required both a feeder layer of adherent leukocytes, and 10-15% autologous plasma. Biosynthesis of C1q, a protein which also is hydroxylated by prolyl hydroxylase, by the nonadherent cells was significantly less than the adherent monocytes. Therefore, this collagen biosynthetic marker enzyme was not associated with C1q synthesis, which suggests that the enzyme is present for collagen biosynthesis.

The possibility that cells of hematological origin synthesize collagen has been raised in several previous studies (1,2). Prolyl hydroxylase, the enzyme required for the post-translational formation of hydroxy-L-proline in collagen was present in both stimulated T cells, and cell lines derived from various lymphomas, although intact collagen molecules could not be detected. Prolyl hydroxylase activity has also been demonstrated in both pulmonary alveolar macrophages and peritoneal macrophages (3,4). Myllylä" and Seppä" proposed that the prolyl hydroxylase activity present in macrophages was primarily responsible for biosynthesis of the complement protein C1q, and was not indicative of a collagen biosynthetic role (4).

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The current study was undertaken to obtain biochemical evidence to support our immunofluorescent findings of collagen synthesis by inflammatory cells within healing wounds (5).

Methods

Cell Isolation and Purification

Human peripheral leukocytes were purified from heparinized (100 U/mL) blood of male donors. Buffy coats were obtained by low speed centrifugation, diluted with RPMI-1640 medium, and applied to Histopaque-1077™ cell separation medium. The neutrophils were separated by centrifugation and discarded, with the mononuclear cells washed several times in RPMI-1640. Following initial experiments, cells were further purified by E-rossetting using 2-aminoethylisothiouronium-bromide treated sheep erythrocytes (6). The T-cell depleted monocytes were then plated in RPMI-1640 medium containing 15% autologous human plasma and 25 mM tricine buffer at 5 x 10^6 cells/100 mm dia. dish.

Prolyl Hydroxylase Determinations

Nonadherent mononuclear cells were collected by centrifugation and homogenized two times (15 sec each) in a 50 mM Tris/HCl buffer (pH 7.5) containing 0.1 mM dithiothreitol, 10 mM EDTA, 100 mM NaCl, and 0.1% NP-40 using a glass/glass homogenizer kept on ice. The homogenate enzyme activity was then assayed in a tritium release assay using an under-hydroxylated tritiated collagen substrate (7). The substrate was prepared by incubating 14-day-old embryonic chick calvaria with (3,4-^{3}H)-proline (ICN Pharmaceuticals, Inc., sp. act. 55 Ci/mmole). Enzyme activity was quantitated as cpm tritium released from this substrate during a 20-min incubation at 37°C, and normalized by DNA content which was assayed spectrofluorometrically according to the procedure of Labarca and Paigen (8). Positive controls (3T3 fibroblast homogenates) were run with each analysis, and had to exceed 1000 cpm/ug DNA to accept the results of that analysis.

C1q Determination

C1q biosynthesis was determined in adherent and nonadherent inflammatory cells, depleted of E+ cells, following 48 hr of culturing. Isolated cells were labeled for 24 hr with L-(^{35}S)-methionine (NEN/DuPont, Inc., sp. act. 1103 Ci/mmole, 10.8 mCi/mL). Nonadherent cells were collected by centrifugation (1000 x g for 10 min), washed two times with phosphate-buffered saline, and lysed using a 0.1 M Tris/HCl (pH 7.2) buffer containing 0.15 M NaCl, 0.1% SDS, 1% Triton X-100™, 1% sodium deoxycholate, 100 T.I.U./mL Aprotinin and 0.02% sodium azide (9). The lysate was briefly sonicated, insoluble material removed by centrifugation, and frozen. The adherent leukocyte monolayers were washed twice with phosphate-buffered saline, scraped into lysis buffer, and treated as above. Radiolabeled C1q was immunoprecipitated using Protein A - Sepharose beads as previously described (10), with a rabbit anti-human C1q primary antibody (Calbiochem, Inc.). An additional wash step using 1% bovine serum albumin at 20°C was added to the protocol to further reduce nonspecific binding. Purified human C1q (Calbiochem, Inc.) was radiolabeled with (^{14}C)-formaldehyde (NEN/DuPont, Inc., sp. act. 53.0 mCi/mmole) by the method of Dottavio-Martin and Ravel (11) and used as a standard in the immunoprecipitation procedure.

Results

Prolyl hydroxylase activity was quantitated in the entire nonadherent cell population isolated from human blood. When placed in culture, these

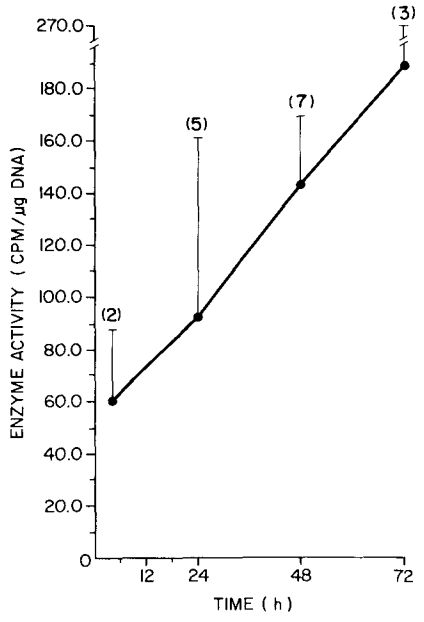

Figure 1. Induction of prolyl hydroxylase activity in nonadherent leukocytes cultured in RPMI-1640 containing 15% plasma. Each value indicates the mean ± S.E.M. from five experiments.

cells demonstrated a substantial increase in enzyme activity during the first 72 hr (Fig. 1). Deletion experiments confirmed that enzyme activity was absolutely dependent on the presence of known prolyl hydroxylase co-factors (Table 1).

Table 1

Co-Factor Requirements for Leukocyte Prolyl Hydroxylase Enzyme Activity

Treatment[a]	N	Enzyme Activity[b]
All Co-Factors	5	180 ± 10[c]
- Ascorbate	6	23 ± 29
- α-ketoglutarate	3	12 ± 10
- Oxygen	4	22 ± 27
- Fe^{++} plus 5 mM a,a'-dipyridyl	4	20 ± 15

a. Each enzyme assay received an identical aliquot of leukocyte homogenate and all co-factors except as indicated. The standard reaction mix contains: 1.0 mM sodium ascorbate, 1.0 mM α-ketoglutarate, 0.2 mM ferrous ammonium sulfate plus catalase, dithiothreitol, and albumin.
b. Enzyme activity is presented as cpm of tritium released.
c. Each value represents the mean ± S.D.

Table 2

Serum Dependence on Prolyl Hydroxylase Activity by Nonadherent Leukocytes[a]

Serum Concentration (%)	N	Enzyme Activity (cpm/ug DNA)
0.0	3	26.6 ± 11.5
5.0	3	20.6 ± 12.4
10.0	4	177 ± 124
15.0	4	120 ± 21.4
20.0	5	65.3 ± 24.9

a. Prolyl hydroxylase activity was quantitated following 48 hr of culturing the cells at the indicated level of serum containing RPMI-1640.

The time-dependent rise in enzyme activity could be partially inhibited by the addition of 1.0 uM cycloheximide 6 hr post-plating. Control cultures at 24 hr had enzyme levels of 75.5 ± 9.4 versus 41.5 ± 1.2 cpm/ug DNA for cycloheximide-treated cultures, thus representing a 45.0% decrease. Prolyl hydroxylase activity on day 2 of culture could also be modulated by varying the medium serum concentration (Table 2). Due to the variability noted for 10% serum, all future experiments were performed at 15% serum, which was considerably more consistent in inducing high levels of enzyme activity.

Prolyl hydroxylase activity in nonadherent monocytes was also dependent on the presence of an adherent monolayer of leukocytes for maximal induction (Fig. 2). If the nonadherent cells were exposed for less than 24 hr to adherent monocytes, enzyme activity was reduced to 30% the level present in cells cultured entirely in the presence of adherent monocytes.

It should be noted that in all experiments prolyl hydroxylase activity could not be demonstrated in the adherent mononuclear cell layer (Table 3). Upon further cell purification using the E-rossetting technique, it was shown that the enzyme activity did not reside within T cells (Table 3). Therefore, subsequent experiments could be performed with a T-cell depleted, nonadherent mononuclear cell preparation.

Biosynthesis of the complement protein Clq which requires prolyl hydroxylase for its synthesis was determined in order to correlate enzyme activity and Clq synthesis in the different cell populations. Biosynthesis was

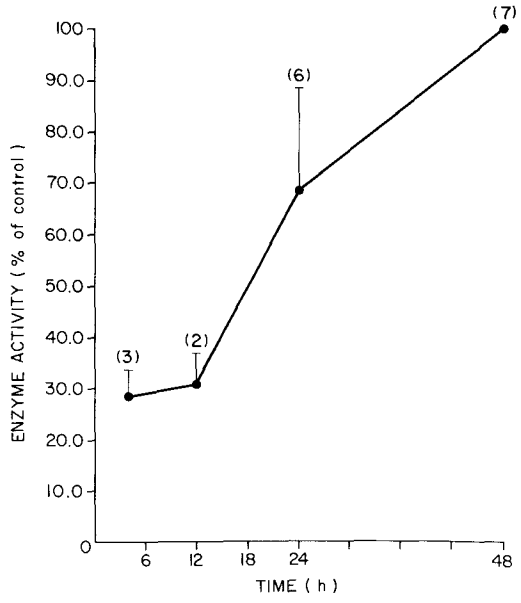

Figure 2. Requirement for the presence of adherent monocytes for the maximal expression of prolyl hydroxylase activity by nonadherent leukocytes. Values are expressed as a percent of the enzyme activity present at 48 hr, if the nonadherent cells were exposed to a monolayer for the entire period. Each value indicates the mean ± S.D. for the number of independent measures given in brackets.

examined by immunoprecipitation of radiolabeled cellular proteins using Protein A - Sepharose beads (Table 4). Significant levels of total protein were being synthesized by both adherent and nonadherent leukocytes. However, five times the relative amount of immunoprecipitable C1q was present in the adherent cells compared to the nonadherent.

Table 3

Distribution of Prolyl Hydroxylase Enzyme Activity
Between Different Cell Subpopulations

Cell Population	N	Enzyme Activity[a]
Experiment 1		
Adherent	6	Non-Detectable
Total Nonadherent	4	186 ± 46
Experiment 2		
Total Nonadherent	3	154 ± 82
E^+ Cells	2	23 ± 0.7
E^- Cells	2	304 ± 174

a. Enzyme activity is expressed in cpm tritium released/ug DNA.
b. Each value represents the mean ± S.D., except for N=2 when it is the mean ± range.

Table 4

Biosynthesis of Clq by Adherent and Nonadherent Leukocytes In Vitro[a]

Cell Population	Total Protein	Immunoprecipitable Clq	Ratio
Nonadherent	512,150 ± 13,676	1343 ± 94	0.262 ± 0.011
Adherent	91,463 ± 16,369	1305 ± 657	1.34 ± 0.47
Standard Clq[c]	17,206 ± 1,285	8155 ± 477	47.5 ± 0.8

a. Cells were incubated with isotope for 24 hr beginning 24 hr following isolation.
b. Each value indicates mean ± range for duplicate analyses (N=2).
c. Purified radiolabeled Clq was immunoprecipitated using the identical reagents to confirm the ability of the procedure to reliably precipitate Clq.

Discussion

This study was performed to ascertain if the collagen biosynthetic enzyme prolyl hydroxylase could be localized within human peripheral leukocytes, and whether this was associated with Clq synthesis. Previous studies demonstrated the enzyme in transformed cells, stimulated T cells, and macrophages (1-4). However, it was concluded that this enzymatic activity was required for Clq synthesis, not collagen biosynthesis (4). Preliminary immunofluorescent analyses of leukocytes within blood clots indicated that collagen was being synthesized by inflammatory cells under certain in vivo conditions (5). Therefore, studies in vitro were initiated to examine the collagen biosynthetic potential of various peripheral human leukocytes using the collagen hydroxylation enzyme prolyl hydroxylase. Since this enzyme is also required for Clq biosynthesis, it became important to analyze the Clq biosynthetic capacity of the cells in relation to prolyl hydroxylase activity.

Prolyl hydroxylase activity, which possessed all of the appropriate cofactor requirements (Table 1), was readily detectable in cultured leukocytes, increasing approximately 300% during 72 hr of culturing (Fig. 1). This activity could be partially blocked by cycloheximide when added 6 hr following culturing. This 6-hr time delay in the experimental protocol was needed to ensure that cells adhered to dishes without inhibition of protein synthesis which could have confounded the analysis. These results suggest that the

nonadherent cells have active enzyme when they are initially isolated, but synthesize more during the culturing period.

The nonadherent cells contained higher levels of enzyme activity in the presence of 10-15% human plasma (Table 2), and when they were cultured in the presence of an adherent monolayer of leukocytes (Fig. 2). This would suggest that the expression of this enzyme activity was dependent on soluble serum factors, and possibly factors secreted by adherent leukocytes. In addition, these results do not support the hypothesis that the enhanced enzyme levels seen after culturing are due to the removal of a serum prolyl hydroxylase inhibitor (12), since the activity was greater with increasing serum. Indeed, the decrease in prolyl hydroxylase activity noted at 20% plasma (Table 2) may result from this inhibitor.

Complement proteins have been shown to be synthesized by human peripheral blood monocytes; however, this study examined synthesis in an adherent cell population (13). Therefore, it was not surprising to detect Clq biosynthesis in the adherent cell monolayer (Table 4). Prolyl hydroxylase activity must be very low in these cells since no enzyme activity has been detected in these cells in our studies. The failure of these experiments to detect prolyl hydroxylase activity in the adherent cells, in contrast to earlier studies with macrophages, probably results from the lower number of cells used to perform the analyses in this study.

These results demonstrate that human peripheral leukocytes contain a subpopulation of non-T-cell, nonadherent cells that contain inducible levels of prolyl hydroxylase. These cells contain detectable, but very low levels of Clq, the only other protein besides collagen that would require this enzyme. Since the adherent cells synthesized five times the amount of Clq produced by nonadherent monocytes, yet possess non-detectable prolyl hydroxylase activity, it is unlikely that the inducible prolyl hydroxylase activity is related to the biosynthesis of the complement protein. These results support the hypothesis that a subpopulation of circulating human leukocytes

participates in collagen deposition under certain environmental conditions *in vivo*.

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