Studies on Enzymes of Collagen Biosynthesis and the Synthesis of Hydroxyproline in Macrophages and Mast Cells

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The activities of four intracellular enzymes of collagen biosynthesis were assayed in freshly isolated rat peritoneal macrophages and mast cells and compared with the same enzymes in freshly isolated chick-embryo tendon cells. The macrophages were found to contain activities of all four enzymes, those of prolyl and lysyl hydroxylase being 7 and 12% respectively of those in the tendon cells when expressed per cell or 3 and 4% when expressed per unit of soluble cell protein. The corresponding values for hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase activities were about 82 and 68% or 32 and 24% respectively. When the macrophages were incubated in suspension with [^{14}C]proline, they synthesized a small but significant amount of non-diffusible hydroxy[^{14}C]proline. The synthesis per cell was only about 0.1% of that formed by the tendon cells, and its distribution between the cells and the medium also differed from that in the tendon cells. The hydroxy[^{14}C]proline synthesized by the macrophages may be present in the C1q subcomponent of the complement, but its amount was too small to allow any characterization of the protein. All four enzyme activities, and in particular the two hydroxylysyl glycosyltransferase activities, seem to be present in macrophages in a large excess compared with the very low rate of synthesis of hydroxyproline-containing polypeptide chains. The mast cell extract was found to inhibit all four enzyme activities, but even when corrected for this inhibition, prolyl and lysyl hydroxylase activities in the mast cells were less than 0.08% and the two hydroxylysyl glycosyltransferase activities less than 1% of those in the tendon cells. The intracellular enzyme pattern of collagen biosynthesis in the mast cells is thus completely or virtually completely repressed.

The biosynthesis of collagen involves a number of specific post-translational modifications of the initial polypeptide chains, such as hydroxylation of certain prolyl and lysyl residues to 4-hydroxyproline, 3-hydroxyproline and hydroxylysine, and glycosylation of some of the hydroxylysyl residues to galactosylhydroxylysine and glucosylgalactosylhydroxylysine. These intracellular modifications are catalysed by five separate enzymes: prolyl 4-hydroxylase (termed here prolyl hydroxylase), prolyl 3-hydroxylase, lysyl hydroxylase, hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase (for reviews see Kivirikko & Risteli, 1976; Prockop *et al.*, 1976; Kivirikko & Myllylä, 1979). Prolyl hydroxylase activity has been found both in fibroblasts and in a number of non-fibroblastic cell types, including cells of non-mesenchymal origin (see Cardinale & Udenfriend, 1974). Initial reports on several cell types of haematological origin, such as freshly isolated reticulocytes, lymphocytes and macrophages, suggested that these do not possess any prolyl hydroxylase activity either with or without phytohaemagglutinin stimulation (Goldberg & Green, 1969). More recent reports, however, point to the

presence of low but significant prolyl hydroxylase activity in Friend leukaemic cells, thymus-derived and bone marrow-derived lymphocytes (Chen-Kiang *et al.*, 1978), pulmonary alveolar macrophages (Kelleher *et al.*, 1977; R. A. Berg, N. A. Gutzman, S. Gay & D. J. Prockop, personal communication) and platelets (Bates, 1976; Anttinen *et al.*, 1977a). The prolyl hydroxylase activity in the Friend leukaemic cells, lymphocytes (Chen-Kiang *et al.*, 1978) and macrophages (Hance & Crystal, 1975) is not associated with collagen synthesis, and it has also been reported that platelets do not contain any collagen-like molecules (Barber & Jamieson, 1971a). No information is available on the possible presence of other enzymes of collagen biosynthesis in these cells, except that the platelets have also been reported to contain the activities of lysyl hydroxylase (Anttinen *et al.*, 1977a) and the two hydroxylysyl glycosyltransferases (Barber & Jamieson, 1971a,b; Bosmann, 1971; Menashi *et al.*, 1976; Anttinen *et al.*, 1977a).

The present work was undertaken to determine whether macrophages contain any other intracellular enzymes of collagen biosynthesis in addition to

prolyl hydroxylase, and to re-examine the question of whether macrophages synthesize any peptide-bound hydroxyproline, since haemolytic assays indicate that these cells synthesize the C1q subcomponent of complement (see Müller *et al.*, 1978), a protein that is known to contain collagen-like sequences (see Porter & Reid, 1978). An attempt was also made to determine whether any of the enzymes of collagen biosynthesis can be found in the mast cells, as these are of mesenchymal origin (Ginsburg *et al.*, 1978), even though there are no reports on the possible presence of any such enzymes.

Experimental

Materials

L-[U-¹⁴C]Proline (sp. radioactivity 285 Ci/mol), L-[U-¹⁴C]lysine (sp. radioactivity 342 Ci/mol) and uridine diphosphate D-[U-¹⁴C]glucose (sp. radioactivity 283 Ci/mol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.), uridine diphosphate D-[U-¹⁴C]galactose (sp. radioactivity 274 Ci/mol) was from New England Nuclear Corp. (Boston, MA, U.S.A.), and non-radioactive UDP-glucose and UDP-galactose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Denatured [¹⁴C]proline-labelled and [¹⁴C]lysine-labelled procollagen substrates were prepared in freshly isolated chick-embryo tendon cells as described previously (Risteli & Kivirikko, 1976). Gelatinized calf skin collagen for the assay of hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase was prepared as described in detail previously (Myllylä *et al.*, 1975a), and was again heat-denatured immediately before addition to the enzyme incubation mixture (Myllylä *et al.*, 1975b).

Isolation of cell samples for the assays

Macrophages were isolated from rat peritoneal exudate induced by a thioglycollate medium (Orion, Helsinki, Finland) by using the Ficoll-density-gradient-centrifugation method (Zembala & Asherson, 1970). Cells from the first and second interphases (counting from the bottom of the tube) were used. The cell fractions contained 87–97% macrophages, the impurities being lymphocytes and mast cells.

Mast cells were isolated from rat peritoneal and pleural cells by centrifuging through 22.5% (w/v) Metrizamide (Nyegaard and Co. A/S, Oslo, Norway) as described by Yurt & Austen (1977). The resulting preparations contained 96 to 98% mast cells.

Freshly isolated tendon cells were prepared from the tendons of 17 day-old chick embryos by digestion with trypsin and collagenase (Dehm & Prockop, 1971). The cells were filtered through lens paper and washed 5 times with modified Krebs medium contain-

ing 10% (w/v) calf foetus serum (Dehm & Prockop, 1971).

All cell pellets were immediately frozen and stored at -20°C for up to 1 week.

Enzyme assays

On thawing, the cell pellets (5×10^6 – 20×10^6 cells/ml in various experiments) were homogenized with a Teflon/glass homogenizer (1200 rev./min; 50 strokes) in a cold solution containing 0.2 M-NaCl, 0.1 M-glycine, 0.1% (w/v) Triton X-100, 0.01% (w/v) soya-bean trypsin inhibitor and 0.02 M-Tris/HCl buffer, with the pH adjusted to 7.5 at 4°C. The homogenates were centrifuged at 15000g for 30 min at 4°C and the supernatants used for the assays.

Prolyl and lysyl hydroxylase activities were assayed with [¹⁴C]proline-labelled (60000 d.p.m.) or [¹⁴C]lysine-labelled procollagen (non-hydroxylated procollagen) (120000 d.p.m.) substrate (Kivirikko & Prockop, 1972; Tuderman *et al.*, 1975). Hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase activities were assayed as described previously (Myllylä *et al.*, 1975a), omitting the paper electrophoresis (Myllylä *et al.*, 1976), except that 22 µM-UDP-[¹⁴C]galactose (sp. radioactivity 46 Ci/mol) was used for the galactosyltransferase and 34 µM-UDP-[¹⁴C]glucose (sp. radioactivity 35.3 Ci/mol) for the glucosyltransferase activity.

Other assays

Protein content was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Hydroxy[¹⁴C]proline was assayed by the method of Juva & Prockop (1966) after hydrolysis overnight in 6 M-HCl at 120°C.

The specificity of the hydroxylysyl galactosyltransferase, galactosylhydroxylysyl glucosyltransferase and hydroxy[¹⁴C]proline assays was studied by using a Jeol JLC-5AH amino acid analyser connected to a fraction collector (Grant *et al.*, 1972; Myllylä *et al.*, 1975a).

Results

Enzyme activities in macrophages and mast cells

Five separate preparations of macrophages and mast cells were examined, and the enzyme activities were compared with those in freshly isolated chick-embryo tendon cells, which are characterized by a high rate of collagen production, with about two-thirds of the protein synthesized being collagen (Dehm & Prockop, 1971). The assays of all four enzyme activities were carried out under conditions in which the relationship between enzyme concentration and product formation was linear (results not shown).

The activities of prolyl and lysyl hydroxylase, expressed per 10^6 cells and per mg of soluble cell protein, are shown in Table 1. Both of these enzyme activities were found in the macrophages, the activity of prolyl hydroxylase being about 7% of that in freshly isolated tendon cells when expressed per cell, or about 3% when expressed per mg of soluble cell protein, and the corresponding values for lysyl hydroxylase about 12 and 4%. It was not possible to demonstrate these enzyme activities in the mast cells. Additional experiments indicated that the mast-cell extract inhibited both hydroxylase activities when added to the extract of freshly isolated tendon cells, the extract obtained from 5×10^5 mast cells (corresponding to $30 \mu\text{g}$ of soluble mast-cell protein) inhibiting by about 50% the prolyl hydroxylase activity obtained from 2×10^4 tendon cells. Because of this inhibition the results do not exclude the presence of extremely low enzyme activities, but it can be concluded that prolyl hydroxylase activity must be less than 0.07% and lysyl hydroxylase activity less than 0.08% of that in the tendon cells, whether expressed per cell or per mg of soluble cell protein (Table 1).

The two hydroxylysyl glycosyltransferase activities were also present in the macrophages (Table 2). As the assays of these enzymes are not always entirely specific (see Kivirikko & Myllylä, 1979), the findings were verified by using an amino acid analyser connected to a fraction collector, as reported previously (Myllylä *et al.*, 1975a). More than 85% of the products of these reactions were recovered in the positions of the corresponding standards (results not shown). The hydroxylysyl galactosyltransferase activity in the macrophages was about 82% of that in the tendon cells when expressed per cell, or about 32% when expressed per unit of soluble cell protein (Table 2), the corresponding values for galactosylhydroxylysyl glucosyltransferase being about 68 and 24%. No significant hydroxylysyl glycosyltransferase activities could be detected in the mast cells. The mast cell extract inhibited both enzyme activities, the extract obtained from 1×10^5 mast cells (corresponding to $6 \mu\text{g}$ of extracted mast-cell protein) inhibiting by about 50% the galactosylhydroxylysyl glucosyltransferase activity obtained from 2×10^4 tendon cells. Because of this inhibition and owing to the lack of sensitivity and absolute specificity of these enzyme assays (see

Table 1. *Prolyl and lysyl hydroxylase activities in rat peritoneal macrophages and mast cells*

Enzyme activities were assayed as described in the Experimental section. The activities in freshly isolated chick-embryo tendon cells are presented for comparison. The results are expressed as means $\pm$ s.d. for the numbers of samples indicated in parentheses

Cells	Protein (mg/ 10^7 cells)	$10^{-3} \times$ Prolyl hydroxylase activity		$10^{-3} \times$ Lysyl hydroxylase activity	
		(d.p.m./ 10^6 cells)	(d.p.m./mg)	(d.p.m./ 10^6 cells)	(d.p.m./mg)
Freshly isolated tendon cells (3)	0.613 ± 0.045	63.5 ± 6.1	1035 ± 23	24.4 ± 1.9	399 ± 2.1
Macrophages (5)	1.710 ± 0.190	4.66 ± 0.4	28.0 ± 2.4	2.90 ± 0.18	17.1 ± 2.9
Mast cells* (5)	0.594 ± 0.108	<0.04	<0.7	<0.02	<0.3

* The mast-cell extract inhibited both hydroxylase activities, but even when corrected for the inhibition, values exceeding those shown can be excluded.

Table 2. *Hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase activities in rat peritoneal macrophages and mast cells*

Enzyme activities were assayed as described in the Experimental section. The activities of freshly isolated chick-embryo tendon cells are presented for comparison. The results are expressed as means $\pm$ s.d. for the numbers of samples indicated in parentheses

Cells	$10^{-3} \times$ Hydroxylysyl galactosyltransferase activity		$10^{-3} \times$ Galactosylhydroxylysyl glucosyltransferase activity	
	(d.p.m./ 10^6 cells)	(d.p.m./mg)	(d.p.m./ 10^6 cells)	(d.p.m./mg)
Freshly isolated tendon cells (3)	2.55 ± 0.20	41.6 ± 0.14	20.6 ± 3.0	335 ± 24
Macrophages (5)	2.09 ± 0.36	13.1 ± 0.17	14.1 ± 1.5	81.6 ± 6.0
Mast cells* (5)	<0.02	<0.40	<0.20	<3.0

* The mast-cell extract inhibited both hydroxylase activities, but even when corrected for the inhibition, values exceeding those shown can be excluded.

Kivirikko & Myllylä, 1979), values less than 1% of those in the tendon cells cannot be excluded (Table 2).

Proline incorporation into peptide-bound hydroxyproline by macrophages

The possible incorporation of [^{14}C]proline into non-diffusible hydroxy[^{14}C]proline was studied by incubating freshly isolated macrophages (7×10^7 cells) in a suspension with $30 \mu\text{Ci}$ of [^{14}C]proline for 3.5 h, as described previously for tendon cells (Dehm & Prockop, 1971, 1972). Freshly isolated tendon cells (7×10^7) were used as controls. Synthesis by the macrophages of non-diffusible ^{14}C -labelled protein (cells+medium), expressed per cell, was about 26% of that found in the tendon cells (Table 3). In Expt. 1 of Table 3 the formation of non-diffusible hydroxy[^{14}C]proline was assayed both by a chemical procedure (Juva & Prockop, 1966) and by using an amino acid analyser connected to a fraction collector (Grant *et al.*, 1972). The results suggested the formation of a very small but significant amount of non-diffusible hydroxy[^{14}C]proline, but the value obtained in both assays was only about 0.09% of that found in the tendon cells (Table 3, Expt. 1). The distribution of hydroxy[^{14}C]proline between the cells and the medium in the case of the macrophages also differed from that observed with the tendon cells.

As the values for hydroxy[^{14}C]proline as percentages of the total ^{14}C radioactivity in the macrophages were close to the specificity limit of the assay (Juva & Prockop, 1966), and as the peak observed for the fractions from the amino acid analyser was also quite small, Expt. 2 was performed, in which the

radioactivity in the hydroxy[^{14}C]proline was partially purified before the assays. This was achieved by treating the non-diffusible samples from the cells and the medium with highly purified bacterial collagenase and by using the diffusible peptides formed in the subsequent assays. About 1.6% of the non-diffusible radioactivity in the cells and about 2.8% in the medium was found in diffusible peptides after collagenase treatment. Assays by both the chemical procedure (Juva & Prockop, 1966) and by amino acid analyser indicated that about 5.2% of the total radioactivity in these diffusible peptides from the cells and about 3.8% in those from the medium was present as hydroxy[^{14}C]proline, the values obtained by the two procedures agreeing to within 10%. The hydroxy[^{14}C]proline values in the original samples as calculated from these data (Table 3, Expt. 2) are in good agreement with those found in Expt. 1 (Table 3). It is noteworthy that treatment with a very large excess of bacterial collagenase was used in the present study only for partial purification of the samples. As it is not known whether the highly purified enzyme was entirely pure, the values do not indicate that the hydroxy[^{14}C]proline-containing protein possessed only about 5.2 or 3.8% of the total radioactivity as this imino acid.

Discussion

The present results indicate that macrophages contain the four intracellular enzyme activities of collagen biosynthesis studied in the present paper, and that macrophages also synthesize very small but significant amounts of peptide-bound hydroxy-

Table 3. Incorporation of [^{14}C]proline and formation of hydroxy[^{14}C]proline by rat peritoneal macrophages and chick-embryo tendon cells

Freshly isolated rat peritoneal macrophages (7×10^7 cells) and chick-embryo tendon cells (7×10^7 cells) were incubated in suspension with $30 \mu\text{Ci}$ of [^{14}C]proline for 3.5 h in a modified Krebs medium, and the cells and medium were then separated (Dehm & Prockop, 1971, 1972). In Expt. 1 the non-diffusible protein was hydrolysed with 6M-HCl for 18 h at 120°C , and total radioactivity and hydroxy[^{14}C]proline were determined both by the procedure of Juva & Prockop (1966) and by using an amino acid analyser connected to a fraction collector (Grant *et al.*, 1972). In Expt. 2 the total non-diffusible proteins from the cells and the medium were incubated for 2 h at 37°C with $40 \mu\text{g}$ of highly purified bacterial collagenase (type VI from *Clostridium histolyticum*; Sigma Chemical Co.) in 4 ml of 0.12M-NaCl/5 mM- CaCl_2 /2 mM-N-ethylmaleimide/0.05M-Tris/HCl buffer, pH 7.5, and the diffusible peptides were then hydrolysed and assayed for total radioactivity and hydroxy[^{14}C]proline as in Expt. 1. These data were used to calculate the values shown here. In both experiments the hydroxy[^{14}C]proline values obtained with the two procedures agreed to within 10%, the values shown having been obtained by the procedure of Juva & Prockop (1966).

Expt.	Cells	$10^{-4} \times [^{14}\text{C}]\text{Proline radioactivity}$ (d.p.m./ 10^8 cells)			$10^{-3} \times \text{Hydroxy}[^{14}\text{C}]\text{proline radio-}$ activity (d.p.m./ 10^8 cells)		
		Cell	Medium	Total	Cell	Medium	Total
1	Chick-embryo tendon cells	328	506	834	773	1737	2510
	Peritoneal macrophages	187	30	217	1.9	0.4	2.3
2	Peritoneal macrophages	201	21	222	1.7	0.2	1.9

proline. The latter result is consistent with reports indicating the synthesis by macrophages of the C1q subcomponent of complement, a protein containing collagen-like amino acid sequences (Müller-Eberhard, 1975; Müller *et al.*, 1978; Porter & Reid, 1978). As the C1q subcomponent contains not only hydroxyproline, but also hydroxylysine and glucosylgalactosylhydroxylysine (see Müller-Eberhard, 1975; Porter & Reid, 1978), the presence of all four enzymes in the macrophages is not very surprising.

It can be calculated from the data of Müller *et al.* (1978) that cultured macrophages synthesized up to about 1 pmol of haemolytically active C1q subcomponent/10⁹ cells per h, whereas freshly isolated chick-embryo tendon cells would synthesize about 1 nmol of procollagen/10⁹ cells per h under the present conditions (Kao *et al.*, 1977). As the hydroxyproline content of the C1q molecule is about two-thirds of that of the procollagen molecule (see Müller-Eberhard, 1975; Kao *et al.*, 1977), it can be estimated that the synthesis of hydroxyproline by macrophages under the conditions used by Müller *et al.* (1978) should be about 1/1500 of that obtained with freshly isolated tendon cells, provided that the haemolytic assays measure all C1q molecules. The ratio of hydroxy[¹⁴C]proline formation between the macrophages and tendon cells was about 1/1100–1/1300 in the present experiments, which agrees well with the above ratio, but it is noteworthy that the present macrophages were freshly isolated and not cultured, and that it is not known whether the intracellular pools of free proline were similar in the macrophages and tendon cells. It thus seems possible that the hydroxy[¹⁴C]proline synthesized by the macrophages in the present experiment represented the synthesis of subcomponent C1q; but naturally the data do not exclude the possibility that they may also have synthesized very small amounts of procollagen. The amount of hydroxy[¹⁴C]proline synthesized was too small for any characterization of this protein, but its distribution between the cells and medium in the macrophages was clearly different from that found in the tendon cells. The time required for the secretion of the various types of procollagen by different cell types is known to vary markedly (see Grant & Jackson, 1976; Kivirikko & Risteli, 1976; Prockop *et al.*, 1976), and thus the slower secretion by the macrophages may either represent a slower rate of secretion for subcomponent C1q than for type I procollagen or a less efficient secretion potency for macrophages than for tendon cells.

The ratios of the enzyme activities to protein-bound hydroxyproline synthesis were quite high in the macrophages, when compared with those in the freshly isolated tendon cells used as controls. It seems likely, therefore, that all four enzymes, and in particular the two hydroxylysyl glycosyltransferases, are present in a large excess. Previous studies on the effect

of age and hepatic injury on these enzyme activities in tissues (see Risteli & Kivirikko, 1976; Anttinen *et al.*, 1977b) and on changes in the four enzyme activities in cultured 3T6 cells (Risteli *et al.*, 1979) suggest that the two hydroxylysyl glycosyltransferase activities may be regulated less efficiently than the two hydroxylase activities. The present data would be consistent with this suggestion.

Assays of the activities of enzymes involved in collagen biosynthesis, in particular of that of prolyl hydroxylase, have been used to examine actual hepatic collagen synthesis from liver biopsy specimens in patients with liver disease (McGee *et al.*, 1974; for complete references see Kuutti-Savolainen *et al.*, 1979). The presence of these enzymes in macrophages might introduce some difficulties into the interpretation of the results of such assays, although admittedly the hydroxylase activities in the macrophages, expressed per unit of cell protein, were only 3–4% of those in the tendon cells, suggesting that the contribution of the macrophages to the hydroxylase activities in the tissues is probably quite small. Furthermore, the increase in the activities of the two hydroxylases in acute experimental liver injury precedes that in the two hydroxylysyl glycosyltransferases (Risteli & Kivirikko, 1974, 1976), a pattern that is not consistent with that which would be expected if the increased enzyme activities were due to an invasion of macrophages.

As indicated in the introduction section, prolyl hydroxylase activity has been found in a number of different fibroblastic and non-fibroblastic cell types. The majority of the studies have been focused only on this enzyme, but the present results that indicate activities of four intracellular enzymes of collagen biosynthesis in macrophages, and previous reports on the presence of all these enzyme activities in platelets (Anttinen *et al.*, 1977a), suggest that cells that contain prolyl hydroxylase may in general contain all the enzymes of collagen biosynthesis. The absence of enzymes of collagen biosynthesis in the mast cells is unexpected in the light of the occurrence of prolyl hydroxylase in a number of different cell types, since these cells are of mesenchymal origin and typical constituents of loose connective tissue (see Bloom, 1974; Ginsburg *et al.*, 1978). They synthesize glycosaminoglycans, in particular heparin, and a number of enzymes (see Bloom, 1974), including rat skin main neutral proteinase (Seppä, 1978). The present results indicate, however, that mast cells, unlike a number of other cells of various origins, have the intracellular enzyme pattern of collagen biosynthesis completely or virtually completely repressed.

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