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Regulation of collagen post-translational modification in transformed human and chick-embryo cells

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Changes in the regulation of collagen post-translational modification in transformed cells were studied in three established human sarcoma cell lines and in chick-embryo fibroblasts freshly transformed by Rous sarcoma virus. The collagens synthesized by all but one of these and by all the control human and chick-embryo cell lines were almost exclusively of types I and/or III. The relative rate of collagen synthesis and the amounts of prolyl hydroxylase activity and immunoreactive protein were markedly low in all the transformed human cell lines. The other enzymes studied, lysyl hydroxylase, hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase, never showed as large a decrease in activity as did prolyl hydroxylase, suggesting a more efficient regulation of the last enzyme than of the three others. The chick-embryo fibroblasts freshly transformed by Rous sarcoma virus differed from the human sarcoma cells in that prolyl hydroxylase activity was distinctly increased, whereas the decreases in immunoreactive prolyl hydroxylase protein and the three other enzyme activities were very similar to those in the simian-virus-40-transformed human fibroblasts. It seems possible that this increased prolyl hydroxylase activity is only a temporary phenomenon occurring shortly after the transformation, and may be followed by a decrease in activity later. The newly synthesized collagens of all the transformed cells that produced almost exclusively collagen types I and/or III had high extents of lysyl hydroxylation, and there was also an increase in the ratio of glycosylated to non-glycosylated hydroxylysine. The data suggest that one critical factor affecting modification is the rate of collagen synthesis, which affects the ratio of enzyme to substrate in the cell.

The regulation of collagen biosynthesis defines the quantity, type and quality of the protein that is produced (for reviews, see Fessler & Fessler, 1978; Prockop *et al.*, 1979*a*,*b*; Bornstein & Sage, 1980). The term type refers to the genetically distinct collagen polypeptide chains designated as α1(I), α2(I), α1(II), α1(III), α1(IV), α2(IV), αA, αB and αC, which combine to form several different triple-helical molecules. The term quality is used to indicate that even the structure of a single collagen type may vary markedly in terms of the extent to which the α chains have been modified by the post-translational enzymes. The biosynthesis of collagen is characterized by a number of post-translational modifications, the intracellular modifications of the collagen domain of the pro-α chains consisting of the hydroxylation of appropriate prolyl and lysyl residues to 4-hydroxyproline, 3-hydroxyproline and hydroxylysine and the glycosylation of certain hydroxylysyl residues to galactosylhydroxylysine and glucosylgalactosylhydroxylysine (see Kivirikko & Myllylä, 1979, 1980). The changes in collagen quality consist principally of variations in the extent of prolyl 3-hydroxylation, lysyl hydroxylation and hydroxylysyl glycosylation, and in the types of cross-links, whereas the extent of prolyl 4-hydroxylation in a given collagen type varies only within narrow limits (see Kivirikko & Myllylä, 1979, 1980). Relatively little specific information is currently available on the mechanisms regulating collagen quality in various physiological and pathological states.

A number of studies have indicated that the rate of collagen synthesis is markedly decreased in various transformed cells in culture (Green *et al.*, 1966; Levinson *et al.*, 1975; Arbogast *et al.*, 1977; Hata & Peterkofsky, 1977; Kamine & Rubin, 1977), this change being due to decreased amounts of

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procollagen mRNA (Adams *et al.*, 1977; Howard *et al.*, 1978; Rowe *et al.*, 1978; Adams *et al.*, 1979; Sandmeyer & Bornstein, 1979). Transformation may also result in alterations in collagen type (Hata & Peterkofsky, 1977, 1978) and quality, although the data currently available on the latter changes are fragmentary and in part conflicting. The conversion of procollagen into collagen is impaired in at least some types of transformed fibroblasts, possibly because of an absence of procollagen proteinases (Arbogast *et al.*, 1977; Sundarraj & Church, 1978; Vaheri *et al.*, 1978). The transformed cells, unlike the corresponding normal cells, fail to deposit the collagen and fibronectin that they produce (Arbogast *et al.*, 1977; Krieg *et al.*, 1980). Prolyl 4-hydroxylase (termed here prolyl hydroxylase) activity was increased in chick-embryo fibroblasts transformed by Rous sarcoma virus (Levinson *et al.*, 1975), but was unchanged in chemically transformed BHK hamster cells (Smith *et al.*, 1979). Hydroxylysyl glycosyltransferase activities (Bosmann & Eylar, 1968; Bosmann, 1969) are reported to be decreased in virus-transformed fibroblasts, but the assays have been criticized for their lack of specificity (see Kivirikko & Myllylä, 1979). One study indicates a 2-fold increase in the amount of lysyl hydroxylation and hydroxylysyl glycosylation, with an unaltered ratio between non-glycosylated and glycosylated hydroxylysine, in type I and III collagens synthesized by human fibroblasts transformed by simian virus 40 (Sundarraj & Church, 1978). No assays of the corresponding enzyme activities were carried out, and the mechanisms of these changes remained unexplained.

The present work examines changes in the regulation of collagen quality in transformed cells by assaying the activities of four intracellular enzymes of collagen synthesis. The rates of procollagen synthesis and the extents of lysyl hydroxylation and hydroxylysyl glycosylation in the newly synthesized collagen were assayed in the same cell lines, so that it was possible to study the influence of various regulative factors on the extent of modification of the protein.

Experimental

Materials

[^{14}C]Proline (>225 Ci/mol) and [^{14}C]lysine (>270 Ci/mol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.), and UDP-D-[^{14}C]galactose (274 Ci/mol) and UDP-D-[^{14}C]glucose (229 Ci/mol) were from New England Nuclear Corp. (Boston, MA, U.S.A.). Chromatographically purified bacterial collagenase (type VI) from *Clostridium histolyticum* (840 units/mg) and non-radioactive UDP-galactose and UDP-glucose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

[^{14}C]Proline-labelled and [^{14}C]lysine-labelled protocollagen (unhydroxylated 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 activity was prepared as described elsewhere (Myllylä *et al.*, 1975*a*) and was heat-denatured immediately before use (Myllylä *et al.*, 1975*b*).

The cultured human cell lines were adult skin fibroblasts, embryonic skin fibroblasts (Krieg *et al.*, 1979), embryonic lung fibroblasts (WI-38; A.T.C.C. CCL 75, from the American Type Culture Collection), simian-virus-40-transformed WI-38 cells (Va-13/WI-38; A.T.C.C. CCL 75.1), and two lines of rhabdomyosarcoma cells [RD (A.T.C.C. CCL 136) and A-204, obtained from Dr. G. Todaro, National Cancer Institute, Bethesda, MD, U.S.A.]. Cultures of chick-embryo fibroblasts were prepared and transformed with the Rous sarcoma virus and infected with the non-transforming avian leukosis virus RAV-1, as described elsewhere (Arbogast *et al.*, 1977).

Cell cultures and metabolic labelling

For the assay of enzyme activities, the cells were grown to subconfluency at 37°C in a humidified incubator in an atmosphere of air/CO_2 (19:1). Special care was taken to avoid comparing density-inhibited normal cells with over-growing transformed cells. The medium for the human cells was Eagle's minimal essential medium and 10% (v/v) foetal calf serum, supplemented with 50μg of ascorbic acid/ml, 100 units of penicillin/ml and 50μg of streptomycin/ml. The chick cells were grown in Medium 199, 10% (v/v) tryptose phosphate broth and 5% (v/v) calf serum supplemented as above. The cells were harvested by trypsin treatment in Hanks salt solution, and the action of the trypsin was stopped by adding a 3-fold molar excess of soya-bean trypsin inhibitor (Sigma). The cells were isolated by centrifugation at 600**g** for 5 min, washed twice with P_i/NaCl (0.01 M-sodium phosphate/0.14 M-NaCl, pH 7.4), and a sample was counted electronically (Coulter Counter). The remaining cells were stored in the form of a pellet at −70°C for up to 2 weeks.

In experiments involving radioactive labelling of the newly synthesized protein, the cell cultures in 20 cm^2 dishes were washed once with lysine-free medium and labelled for 24 h with 5μCi of [^{14}C]-lysine/ml in a lysine- and serum-free medium containing 0.2% (w/v) bovine serum albumin and antibiotics. Sodium ascorbate (50μg/ml) and β-aminopropionitrile fumarate (50μg/ml) were added to the labelling medium. The labelling was stopped by adding 2 ml of medium containing 3 mg of

unlabelled lysine/ml, proteinase inhibitors were added to final concentrations of 1 mM-EDTA, 0.8 mM-*N*-ethylmaleimide and 0.2 mM-phenylmethanesulphonyl fluoride, and the cells plus medium were exhaustively dialysed against water at 4°C.

Digestion with highly purified collagenase

Samples of the dialysed cells plus medium (above) were heated at 100°C for 10 min to denature any contaminating proteinases (Kao *et al.*, 1979) and then incubated with highly purified bacterial collagenase (35 μg/ml) in the presence of 5 mM-$CaCl_2$, 2 mM-*N*-ethylmaleimide, 0.12 M-NaCl and 50 mM-Tris/HCl buffer, pH adjusted to 7.5 at 4°C. After incubation for 2.5 h at 37°C, the samples were dialysed against 50 ml of water overnight at 4°C, and the diffusible peptides were evaporated to dryness.

Enzyme assays

Thawed cell pellets 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, pH adjusted to 7.5 at 4°C (about 1 ml of solution/10^6 cells). The homogenates were centrifuged at 15000 *g* for 30 min at 4°C, and samples of the supernatants were used for the assays.

Prolyl hydroxylase activity was assayed by measuring the formation of radioactive hydroxyproline in a [^{14}C]proline-labelled protocollagen substrate (see Tuderman *et al.*, 1975*a*), and lysyl hydroxylase activity from the formation of radioactive hydroxylysine in a [^{14}C]lysine-labelled procollagen substrate (Kivirikko & Prockop, 1972). Hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase activities were assayed by determining the radioactive galactosylhydroxylysine and glucosylgalactosylhydroxylysine formed in a gelatinized calf skin collagen substrate (Myllylä *et al.*, 1975*a*, 1976). The UDP-glycoside concentration used in the galactosyltransferase assay was 37 μM-UDP-galactose (33.9 Ci/mol) and in the glucosyltransferase assay 67 μM-UDP-glucose (19.3 Ci/mol).

Immunoreactive prolyl hydroxylase protein was measured with a direct radioimmunoassay based on the displacement of radioactively labelled enzyme from its antibody by non-labelled enzyme and the subsequent precipitation of the enzyme–antibody complex by a cellulose-bound second antibody (Tuderman *et al.*, 1975*b*). Antisera were prepared to pure human (Kuutti *et al.*, 1975) and chick (Tuderman *et al.*, 1975*a*) prolyl hydroxylases, and the corresponding pure enzymes were used as standards in the assay.

Other assays

The peptides prepared by collagenase digestion were assayed either for hydroxy[^{14}C]lysine and [^{14}C]lysine after hydrolysis in 6 M-HCl at 120°C for 16 h or for glucosylgalactosylhydroxy[^{14}C]lysine, galactosylhydroxy[^{14}C]lysine, hydroxy[^{14}C]lysine and [^{14}C]lysine after hydrolysis in 2 M-NaOH at 105°C for 24 h. The hydrolysis, further purification of the products, separation of the products in an amino acid analyser, and assay of the radioactivity were carried out as described previously (Oikarinen *et al.*, 1976*a*).

The extractable cell protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

The statistical significances of the differences between two means were calculated by Student's *t* test.

Results

Enzyme activities of collagen synthesis in human sarcoma cells

The activities of prolyl hydroxylase, lysyl hyroxylase, hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase were assayed in three control and three transformed human cell lines. The control cells were adult skin fibroblasts, embryonic lung fibroblasts (WI-38) and embryonic skin fibroblasts, and the transformed cells were simian-virus-40-transformed WI-38 fibroblasts (Va-13/WI-38) and two lines of rhabdomyosarcoma cells (RD and A-204). The adult and embryonic skin fibroblasts synthesized only collagen types I and III, and the WI-38 and Va-13/WI-38 cells had over 95% of their collagen as these two types, but also synthesized traces of type IV collagen (Alitalo, 1980). The RD cells synthesized mainly type III collagen, but up to 10% of the total collagen consisted of type IV plus chains αA and αB (Krieg *et al.*, 1979). The A-204 cells did not synthesize any detectable quantities of type IV collagen, the main collagenous protein probably being a recently identified (Kumamoto & Fessler, 1980) precursor of type V (K. Alitalo, R. Myllylä & A. Vaheri, unpublished work). Thus the great majority of the collagen synthesized by all but one of the six cell lines was of type I and/or III, which do not differ with respect to the extent of lysyl hydroxylation and hydroxylysyl glycosylation, at least in the tissues of adult animals and human subjects (see Kivirikko & Myllylä, 1979, 1980; Bornstein & Sage, 1980).

The enzyme activities are given per mg of soluble cell protein. The transformed cells had somewhat more extractable protein per cell than did the controls (Table 1), and hence there are some differences in the details of the data when expressed

per 10^6 cells (results not shown), but this does not affect any of the main conclusions.

The three control cell lines had fairly similar enzyme activity patterns, but the WI-38 fibroblasts showed a tendency for slightly higher activities of lysyl hydroxylase and the two hydroxylysyl glycosyltransferases than in the adult and embryonic skin fibroblasts (Tables 1 and 2). The three transformed cell lines had a markedly low prolyl hydroxylase activity, only about 20–35% of that in the controls. In the Va-13/WI-38 cells the other three enzyme activities were likewise low (Tables 1 and 2) compared with the corresponding cell line WI-38, whereas in the two rhabdomyosarcoma cell lines these three enzymes showed a less uniform pattern. The activities in the RD cells were low (lysyl hydroxylase) or similar to those in the control cell lines, whereas those in the A-204 cells that differed from the others with respect to the main collagenous protein synthesized were similar to those in the controls, or even slightly higher.

The three enzyme activities were also expressed in relation to prolyl hydroxylase activity, as it has been found that changes in the latter roughly parallel changes in the rate of collagen synthesis in many situations (see Prockop *et al.*, 1979*a*,*b*; Kivirikko & Myllylä, 1980). Significant increases were found in this ratio in all three transformed cell lines with respect to all three enzyme activities (Tables 1 and 2).

Table 1. *Prolyl hydroxylase and lysyl hydroxylase activities in the human sarcoma cells*

Enzyme activities were assayed as described in the Experimental section and are expressed per mg of extractable cell protein. Lysyl hyroxylase activity is also compared with prolyl hydroxylase activity by calculating the ratio lysyl hydroxylase/prolyl hydroxylase activity. The numbers of samples are indicated in parentheses. The results are given as means ± S.D. The statistical significance was calculated both versus adult skin fibroblasts (first superscript) and versus WI-38 cells (second superscript): $^aP<0.001$; $^bP<0.01$, $^cP<0.05$, ^{n}not significant ($P>0.05$).

Cell line		Protein (μg/10^6 cells)	$10^{-3}\times$ Prolyl hydroxylase activity (d.p.m./mg of protein)	$10^{-3}\times$ Lysyl hydroxylase activity (d.p.m./mg of protein)	Lysyl hydroxylase/ prolyl hydroxylase ratio
Controls					
Adult skin fibroblasts	(8)	201 ± 47	139 ± 24	50.2 ± 16.7	0.36 ± 0.10
WI-38	(7)	185 ± 26^n	127 ± 19^n	58.2 ± 8.5^n	0.45 ± 0.03^n
Embryonic skin fibroblasts	(3)	176 ± 18	145 ± 15	51.8 ± 5.0	0.36 ± 0.01
Sarcoma cells					
Va-13/WI-38	(5)	248 ± 25^{n,b}	48 ± 17^{a,a}	31.2 ± 10.8^{c,a}	0.67 ± 0.13^{a,b}
RD	(6)	214 ± 6^{n,c}	31 ± 3^{a,a}	36.9 ± 4.5^{n,a}	1.19 ± 0.14^{a,a}
A-204	(6)	252 ± 41^{n,b}	40 ± 8^{a,a}	55.5 ± 12.3^{n,n}	1.42 ± 0.39^{a,a}

Table 2. *Hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase activities in the human sarcoma cells*

Enzyme activities were assayed as described in the Experimental section and are expressed per mg of extractable cell protein and in relation to prolyl hydroxylase activity. The numbers of samples are indicated in parentheses. The results are given as means ± S.D. The statistical significance was calculated both versus adult skin fibroblasts (first superscript) and versus WI-38 cells (second superscript): $^aP<0.001$, $^bP<0.01$, $^cP<0.05$, ^{n}not significant ($P>0.05$).

Cell line		$10^{-3}\times$ Galactosyltransferase activity (d.p.m./mg of protein)	Galactosyltransferase/ prolyl hydroxylase ratio	$10^{-3}\times$ Glucosyltransferase activity (d.p.m./mg of protein)	Glucosyltransferase/ prolyl hydroxylase ratio
Controls					
Adult skin fibroblasts	(8)	20.7 ± 2.8	0.15 ± 0.03	59.2 ± 7.4	0.43 ± 0.06
WI-38	(7)	38.4 ± 9.0^a	0.30 ± 0.06^a	67.5 ± 13.6^n	0.53 ± 0.08^c
Embryonic skin fibroblasts	(3)	24.7 ± 2.4	0.17 ± 0.01	59.1 ± 5.8	0.39 ± 0.01
Sarcoma cells					
Va-13/WI-38	(5)	19.5 ± 3.0^{n,b}	0.45 ± 0.16^{a,c}	31.6 ± 4.8^{a,a}	0.73 ± 0.26^{b,n}
RD	(6)	24.0 ± 2.3^{c,b}	0.78 ± 0.10^{a,a}	54.3 ± 6.6^{n,n}	1.74 ± 0.12^{a,a}
A-204	(6)	43.5 ± 10.7^{a,n}	1.09 ± 0.26^{a,a}	71.5 ± 11.6^{c,n}	1.80 ± 0.36^{a,a}

Immunoreactive prolyl hydroxylase protein in the human sarcoma cells

Immunoreactive prolyl hydroxylase protein is found in isolated cells and in intact tissues in two forms: the active enzyme tetramers ($\alpha_2\beta_2$) and an inactive form which corresponds to the smaller enzyme subunit (β-subunit) both in size and by amino acid and peptide-'map' analysis (see Chen-Kiang *et al.*, 1977; Majamaa *et al.*, 1979; Berg *et al.*, 1980; Kao & Chou, 1980; Kivirikko & Myllylä, 1980). The latter is present in most cells in a large excess over the active tetramers, e.g. forming about 80–85% of the total prolyl hydroxylase protein in cultured chick-embryo tendon fibroblasts, which actively synthesize collagen, compared with only 15–20% of the active tetramers (Kao *et al.*, 1975). In many situations associated with a rapid increase in the rate of collagen synthesis, such as the early stages of experimental liver injury (Risteli *et al.*, 1976, 1978), there is a larger increase in the enzyme activity than in the total immunoreactive enzyme protein (see Kivirikko & Myllylä, 1980).

The concentration of total immunoreactive prolyl hydroxylase protein was quite similar in all three control cell lines (Table 3). Distinctly lower amounts of this protein were found in all three transformed cell lines. This decrease was slightly smaller than that in the enzyme activity (active tetramers), and hence there was a tendency for the ratio of enzyme activity per unit of enzyme protein to be slightly lower in the transformed cells.

Extents of lysyl hydroxylation and hydroxylysyl glycosylation in collagens synthesized by human sarcoma cells

The extent of modification of lysyl residues in the newly synthesized procollagen and collagen was studied by incubating the cells with [^{14}C]lysine for 24h as described in the Experimental section, and by assaying the radioactivity of [^{14}C]lysine, hydroxy-[^{14}C]lysine, galactosylhydroxy[^{14}C]lysine and glucosylgalactosylhydroxy[^{14}C]lysine in diffusible peptides produced from the total labelled protein in the cells plus medium by exhaustive digestion with highly purified bacterial collagenase. This procedure was chosen since the intracellular enzyme activities were being assayed in the cells responsible for the synthesis of all the protein, and hence any purification procedure that might involve a selective loss of some hydroxylysine-containing protein fraction, e.g. part of one collagen type, would lead to an erroneous relationship between the enzyme activity and the overall extent of modification of the products formed. In the cells synthesizing almost exclusively collagen types I and/or III, the results are expressed as the percentage hydroxylation of collagen lysine, glycosylation of hydroxylysine and glucosylation of glycosylated hydroxylysine and as residues/35 lysyl plus hydroxylysyl residues, since there are about 35 lysyl plus hydroxylysyl residues per α-chain in these collagen types (see Kivirikko & Myllylä, 1979, 1980; Bornstein & Sage, 1980). The values in the A-204 cells are not shown as residues, since the numbers of lysyl plus hydroxylysyl residues per polypeptide chain differ (see Kivirikko & Myllylä, 1980).

The relative collagen synthesis, expressed in terms of collagenase-sensitive [^{14}C]lysine-labelled protein as a percentage of the total [^{14}C]lysine-labelled protein, was slightly lower in the WI-38 cells than in the adult skin fibroblasts, whereas the extents of the modifications, in particular the extent of total galactosylation (glycosylation of hydroxylysine), were distinctly higher in the WI-38 cells (Table 4). Corresponding differences were found in the enzyme activities (see above).

Table 3. *Immunoreactive prolyl hydroxylase protein in the human sarcoma cells*

Immunoreactive prolyl hydroxylase protein was assayed as described in the Experimental section and is expressed per mg of extractable cell protein. The concentration of the immunoreactive protein was compared with prolyl hydroxylase activity by calculating the ratio of enzyme activity per μg of immunoreactive protein. The numbers of cell samples are indicated in parentheses. The results are given as means ± S.D. The statistical significance was calculated both versus adult skin fibroblasts (first superscript) and versus WI-38 cells (second superscript): $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$, ^{n}not significant ($P>0.05$).

Cell line		Immunoreactive protein (μg/mg of protein)	10^{-3} × Activity (d.p.m./μg of immunoreactive protein)
Control			
Adult skin fibroblasts	(8)	4.51 ± 0.45	31.0 ± 6.5
WI-38	(7)	5.30 ± 0.79^{n}	25.5 ± 4.5^{n}
Embryonic skin fibroblasts	(3)	5.20 ± 0.72	27.0 ± 2.7
Sarcoma cells			
Va-13/WI-38	(6)	$2.03 \pm 0.47^{b,a}$	$22.3 \pm 6.3^{c,n}$
RD	(6)	$1.53 \pm 0.41^{a,a}$	$21.4 \pm 4.2^{b,n}$
A-204	(6)	$1.89 \pm 0.38^{a,a}$	$21.7 \pm 4.3^{c,n}$

Table 4. *Relative collagen synthesis, hydroxylation of lysine and glycosylation of hydroxylysine in collagen synthesized by the human sarcoma cells*

Relative collagen synthesis is expressed in terms of collagenase-sensitive [^{14}C]lysine-labelled protein as a percentage of total [^{14}C]lysine-labelled protein. The degrees of hydroxylation of lysine, glycosylation of hydroxylysine and glucosylation of glycosylated hydroxylysine were calculated from the radioactivities in lysine, hydroxylysine, galactosylhydroxylysine and glucosylgalactosylhydroxylysine in collagenase-sensitive protein. The results are also expressed as residues per 35 lysyl plus hydroxylysyl (Hyl) residues (see the text). Numbers of samples were: collagenase-sensitive [^{14}C]lysine, each cell line, $n = 5$; hydroxylation of collagen lysine, both control cells lines, $n = 4$, all sarcoma cell lines, $n = 3$; other values: WI-38 cells, $n = 3$, other cell lines, $n = 2$. The values are given as means ± S.D., but as means ± absolute deviation in groups with two samples. The statistical significance was calculated both versus adult skin fibroblasts (first superscript) and versus WI-38 cells (second superscript): $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.05$, ^{n}not significant ($P > 0.05$), n.s. = not shown (see the text).

Cell line	Collagenase-sensitive [^{14}C]lysine (%)	Hydroxylation of collagen lysine (%)	Glycosylation of Hyl (%)	Glucosylation of glycosylated Hyl (%)	No. of residues/35 Lys + Hyl residues			
					Total Hyl	Glycosylated Hyl	Gal-Hyl	Glc-Gal-Hyl
Controls								
Adult skin fibroblasts	6.27 ± 0.37	33.1 ± 2.7	25.1 ± 0.2	83.6 ± 1.3	11.6 ± 0.9	2.9 ± 0.0	0.5 ± 0.1	2.5 ± 0.1
WI-38	5.18 ± 0.60^{b}	37.7 ± 1.4^{c}	41.5 ± 1.0^{a}	87.2 ± 1.8^{n}	13.2 ± 0.5^{c}	5.5 ± 0.1^{a}	0.7 ± 0.1^{c}	4.8 ± 0.1^{a}
Sarcoma cells								
Va-13/WI-38	1.03 ± 0.41^{a,a}	50.6 ± 4.5^{a,b}	55.1 ± 4.4^{b,c}	81.0 ± 0.3^{n,b}	17.7 ± 1.5^{a,b}	9.8 ± 0.8^{b,b}	1.9 ± 0.2^{b,b}	7.9 ± 0.6^{b,b}
RD	0.95 ± 0.30^{a,a}	51.8 ± 3.2^{a,a}	61.4 ± 1.9^{a,a}	90.3 ± 2.3^{n,n}	18.1 ± 1.1^{a,a}	11.2 ± 0.3^{a,a}	1.1 ± 0.3^{n,n}	10.1 ± 0.1^{a,a}
A-204	1.00 ± 0.46^{a,a}	53.3 ± 4.9^{a,a}	78.8 ± 1.1^{c,c}	95.0 ± 2.7^{c,c}	n.s.	n.s.	n.s.	n.s.

In all three transformed cell lines the relative collagen synthesis was only about 15–20% of that in the controls (Table 4). Similar low values were found when expressed in terms of collagenase-sensitive [^{14}C]proline-labelled protein as a percentage of the total [^{14}C]proline-labelled protein (results not shown). The extents of lysyl hydroxylation and hydroxylysyl glycosylation were markedly high in the Va-13/WI-38 and RD cell lines, whereas the relative extent (%) of glucosylation of glycosylated hydroxylysine was low in the Va-13/WI-38 cells (compared with the corresponding cell line, WI-38) and unaltered in the RD cells. Owing to the markedly increased percentages of hydroxylation and glycosylation, however, the quantity of glucosylgalactosylhydroxylysyl residues per α-chain was distinctly high in both these transformed cell lines (Table 4). The extents of the modifications in the A-204 cell line were similar to those reported for type V collagen from other sources (see Kivirikko & Myllylä, 1979, 1980), except that the extent of the glucosylation of glycosylated hydroxylysine is very high.

Enzyme activities and prolyl hydroxylase protein in Rous-sarcoma-virus-transformed chick-embryo fibroblasts

To study whether the transformation-associated changes described above all occur relatively rapidly, chick-embryo fibroblasts were transformed with the Rous sarcoma virus. The assays were carried out 120h after the infection and within 24h of subculture, when almost all (>90%) of the cells showed morphological evidence of transformation. Some of the cells were infected with the non-transforming avian leukosis virus to distinguish between changes caused by the RNA-tumour-virus infection and the transformation. No significant changes were found in amount of soluble protein per cell in either the Rous-sarcoma-virus- or avian-leukosis-virus-infected cells (results not shown).

There was no difference in prolyl hydroxylase activity or immunoreactive protein between the control and avian-leukosis-virus-infected cells (Table 5). The Rous-sarcoma-virus-transformed cells had markedly increased prolyl hydroxylase activity, but distinctly decreased immunoreactive enzyme protein (active tetramers plus β-subunit-size protein, see above), and thus the ratio of enzyme activity to total enzyme protein was about 2.8 times that found in the controls. If one assumes that the chick-embryo fibroblasts studied here had about 15% of their total immunoreactive prolyl hydroxylase protein in the form of the active tetramers and 85% as the β-subunit-size protein (Kao *et al.*, 1975), it can be calculated that the concentration of active tetramers in the controls was 0.99 μg/mg of protein and that of the β-subunit-size protein was 5.59 μg/mg. Since the

Table 5. *Prolyl hydroxylase activity and immunoreactive protein in Rous-sarcoma-virus-transformed chick-embryo fibroblasts 120 h after infection*

Prolyl hydroxylase activity and immunoreactive protein were assayed as described in the Experimental section and are expressed per mg of extractable cell protein. The assays were carried out 120 h after infection of the chick-embryo fibroblasts (CEF) with either Rous sarcoma (RS) virus or the non-transforming avian leukosis (AL) virus. The results are also expressed as the ratio of enzyme activity to enzyme protein. The results are given as means ± S.D. for six samples. [a] $P < 0.001$ versus CEF, [n] no significant difference versus CEF ($P > 0.05$).

	Prolyl hydroxylase		
Cell type	10^{-3} × Activity/mg of protein (d.p.m.)	Immunoreactive protein/mg of protein (μg)	10^{-3} × Activity/μg of immunoreactive protein (d.p.m.)
CEF	268 ± 14	6.58 ± 0.66	41.2 ± 5.5
AL-virus-infected CEF	268 ± 20[n]	6.07 ± 0.31[n]	44.2 ± 4.6[n]
RS-virus-infected CEF	529 ± 31[a]	4.49 ± 0.45[a]	116.3 ± 9.6[a]

Table 6. *Lysyl hydroxylase and hydroxylysyl glycosyltransferase activities in Rous-sarcoma-virus-transformed chick-embryo fibroblasts 120 h after infection*

Enzyme activities were assayed as described in the Experimental section and are expressed per mg of extractable cell protein. The assays were carried out 120 h after infection of the chick-embryo fibroblasts (CEF) with Rous sarcoma (RS) virus or the non-transforming avian leukosis (AL) virus. The results are given as means ± S.D. for six samples, [a] $P < 0.001$ versus CEF, [n] no significant difference versus CEF ($P > 0.05$).

Cell type	10^{-3} × Lysyl hydroxylase activity (d.p.m./mg of protein)	10^{-3} × Galactosyltransferase activity (d.p.m./mg of protein)	10^{-3} × Glucosyltransferase activity (d.p.m./mg of protein)
CEF	106 ± 6	15.7 ± 1.7	76.4 ± 5.4
AL-virus-infected CEF	105 ± 4[n]	17.4 ± 1.0[n]	75.2 ± 3.8[n]
RS-virus-infected CEF	63 ± 12[a]	7.5 ± 0.9[a]	31.4 ± 4.2[a]

concentration of the tetramers in the Rous-sarcoma-virus-transformed cells, as measured by enzyme activity, was 1.97 times that in the controls (Table 5), the tetramer concentration in the transformed cells must have been $1.97 \times 0.99\,\mu g/mg = 1.95\,\mu g/mg$. Correspondingly, the concentration of the β-subunit-size protein is $4.49 - 1.95 = 2.54\,\mu g/mg$. This calculation indicates that the concentration of the β-subunit-size protein was decreased to about 45% of that in the controls ($100 \times 2.54\,\mu g/mg \div 5.59\,\mu g/mg = 45$).

The three other enzyme activities were likewise unchanged in the avian-leukosis-virus-infected cells, but significantly decreased in the Rous-sarcoma-virus-transformed cells (Table 6). The decreases in hydroxylsyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase activity were very similar to that calculated above for the free β-subunit-size protein of prolyl hydroxylase, whereas a slightly smaller decrease took place in lysyl hydroxylase activity.

Comparison of enzyme activities between the chick-embryo and human fibroblast controls indicates that the chick-embryo cells had much higher prolyl hydroxylase and lysyl hydroxylase activities (Tables 1, 5 and 6). Galactosyltransferase activity was slightly lower than in the adult human skin fibroblasts and markedly lower than in the WI-38 cells, whereas the glucosyltransferase activity was fairly similar in all these cells (Tables 2, 5 and 6). Corresponding differences were found in the extents of modifications of the collagens synthesized by these cells (compare Table 7, below, with Table 4).

Extents of lysyl hydroxylation and hydroxylysyl glycosylation in collagen synthesized by transformed chick-embryo fibroblasts

The extent of the modification of lysyl residues in the newly synthesized procollagen and collagen was studied by using a 24 h pulse with [^{14}C]lysine (Table 7) as described above for the human cells.

Relative collagen synthesis was not affected by the avian-leukosis-virus infection, but that in the Rous-sarcoma-virus-transformed cells was only about 15% of that in the controls (Table 7). The extent of lysyl hydroxylation was close to the maximal value found for type I collagen even in the controls (see the Discussion section), but a small further increase was seen in the transformed cells. A slight increase was also found in hydroxylysyl glycosylation, the quantity of glycosylated hydroxylysyl residues being 130% of that in the controls (Table 4).

Table 7. *Relative collagen synthesis, hydroxylation of lysine and glycosylation of hydroxylysine in collagen synthesized by the transformed chick-embryo fibroblasts 120 h after infection*

Relative collagen synthesis is expressed in terms of collagenase-sensitive [^{14}C]lysine-labelled protein as a percentage of total [^{14}C]lysine-labelled protein. The assays were carried out 120h after infection of the chick-embryo fibroblasts (CEF) with Rous sarcoma (RS) virus or the non-transforming avian leukosis (AL) virus. The extents of the hydroxylation of lysine, glycosylation of hydroxylysine and glucosylation of the glycosylated hydroxylysine were calculated from the radioactivities in lysine, hydroxylysine, galactosylhydroxylysine and glucosylgalactosylhydroxylysine in the collagenase-sensitive protein. The results are also expressed as residues per 35 lysyl plus hydroxylysyl (Hyl) residues. Number of samples: collagenase-sensitive [^{14}C]lysine, and hydroxylation of collagen lysine, each cell line, $n = 5$; other values in CEF and AL-virus-infected CEF, $n = 3$; other values in RS-virus-infected CEF, $n = 5$. The values are given as means ± S.D. $^{a}P < 0.001$ versus CEF, $^{b}P < 0.01$ versus CEF, $^{c}P < 0.05$ versus CEF, ^{n}no significant difference versus CEF ($P > 0.05$).

Cell line	Collagenase-sensitive [^{14}C]lysine (%)	Hydroxylation of collagen lysine (%)	Glycosylation of Hyl (%)	Glucosylation of glycosylated Hyl (%)	No. of residues/35 Lys + Hyl residues			
					Total Hyl	Glycosylated Hyl	Gal-Hyl	Glc-Gal-Hyl
CEF	7.9 ± 0.4	48.9 ± 0.6	22.6 ± 1.7	82.0 ± 1.9	17.1 ± 0.2	4.6 ± 0.4	0.9 ± 0.1	3.7 ± 0.3
AL-virus-infected CEF	8.3 ± 0.7^{n}	47.9 ± 1.2^{n}	23.3 ± 0.7^{n}	80.5 ± 2.1^{n}	16.8 ± 0.4^{n}	4.6 ± 0.1^{n}	0.9 ± 0.1^{n}	3.7 ± 0.2^{n}
RS-virus-infected CEF	1.2 ± 0.5^{a}	51.2 ± 2.7^{c}	28.0 ± 2.0^{b}	78.7 ± 3.1^{n}	17.9 ± 0.9^{c}	6.0 ± 0.7^{b}	1.3 ± 0.2^{c}	4.7 ± 0.5^{c}

Discussion

The quality of collagen in intact cells may be influenced by a number of factors, such as the rate of pro-α-chain synthesis, the amounts of active enzymes catalysing the modifications, the amounts of cofactors and co-substrates required by the enzymes, and the rate at which the pro-α chains fold into the triple-helical conformation (see Prockop *et al.*, 1979*a*,*b*; Kivirikko & Myllylä, 1979, 1980). Many studies have demonstrated a role of the rate of triple-helix formation on the extents of collagen hydroxylations and hydroxylysyl glycosylations (Prockop *et al.*, 1979*a*,*b*; Kivirikko & Myllylä, 1979, 1980). By contrast, little is known about the dependence of collagen quality on cellular enzyme activities and on the rate of pro-α-chain synthesis. There is no previous report in which changes in the rate of pro-α-chain synthesis, the enzyme activities and the extents of the modifications have been assayed within one study under identical conditions. For these reasons, the present work had two objectives: (*a*) to study changes in the regulation of collagen quality resulting from transformation, and (*b*) to use the transformed cells as a model to study the influence of the rate of pro-α-chain synthesis and the amounts of enzyme activities on collagen quality.

All three transformed human cell lines showed marked decreases in their relative rate of collagen synthesis, their prolyl hydroxylase activity and the amounts of immunoreactive prolyl hydroxylase protein present. A close correlation between the rate of collagen synthesis and prolyl hydroxylase activity has previously been suggested by findings indicating that increases in this enzyme activity generally precede and accompany increases in collagen synthesis (see Prockop *et al.*, 1979*a*,*b*; Kivirikko & Myllylä, 1980). The present findings further emphasize this correlation.

The increase in prolyl hydroxylase activity in the Rous-sarcoma-virus-transformed chick-embryo cells is in contradiction to the notion that changes in prolyl hydroxylase activity generally parallel changes in the rate of collagen synthesis. Interestingly, however, there was a distinct decrease in the β-subunit-size protein. The association of prolyl hydroxylase subunits to active tetramers probably only occurs after the release of the subunits from the ribosomes, and the β-subunit-size protein, either the free subunit or its precursor, is utilized in this process (Majamaa *et al.*, 1979; Berg *et al.*, 1980; Kao & Chou, 1980; Kivirikko & Myllylä, 1980). Many observations suggest that in situations with a rapid increase in the rate of collagen synthesis, such as acute experimental liver injury (Risteli *et al.*, 1976, 1978), there is a rapid increase in the synthesis of the α-subunits, this resulting in an elevated concentration of the tetramers and hence elevated enzyme activity (see Kivirikko & Myllylä, 1980).

One possibility is that acute Rous-sarcoma-virus-induced transformation causes a response similar to that caused by acute tissue injury, and is therefore accompanied by a rapid increase in the synthesis of the α-subunit. This response may well be only a temporary one and may later be followed by a decrease in enzyme activity. The data on the simian-virus-40-transformed human cells, which had been passaged for numerous generations in this transformed state and showed lower prolyl hydroxylase activity, support this possibility.

The other intracellular enzyme activities of collagen synthesis were consistently decreased in the simian-virus-40- and Rous-sarcoma-virus-transformed cells, but showed a less uniform pattern in the two human rhabdomyosarcoma cell lines. The fact that these three enzymes never showed as large a decrease in activity in the human transformed cells as did prolyl hydroxylase suggests a more efficient regulation of prolyl hydroxylase concomitant with the decreased rate of collagen synthesis. Several other studies have likewise demonstrated that the intracellular enzyme activities vary with the rate of collagen biosynthesis and that the changes in the enzymes are not always identical in magnitude (see Kivirikko & Myllylä, 1979, 1980). It is currently not known, however, to what extent and by which mechanisms the synthesis of these enzymes is coupled to the rate of collagen biosynthesis.

The modifications were increased in extent in all transformed cells synthesizing almost exclusively collagen types I and/or III, and, in contrast with data reported previously (Sundarraj & Church, 1978), there was also a distinct change in the ratio of glycosylated to non-glycosylated hydroxylysine. The results thus clearly indicate that collagen types should not be identified on the basis of their carbohydrate composition. Total hydroxylysyl residues per α-chain amounted to about 18 residues in all these transformed cells, a value identical with that seen when hydroxylation is allowed to proceed for a long period of time in the cells by preventing triple-helix formation (Oikarinen *et al.*, 1976*b*, 1977). This value thus probably represents a maximal one for type I and III collagens.

Comparison of the enzyme activities and the extents of the modifications in the control human and chick-embryo fibroblasts indicates a definite dependence of the modifications on the amounts of the corresponding enzymes under conditions of roughly similar rates of collagen synthesis. These findings indicate that the amounts of all the enzyme activities under normal conditions are rate-limiting for the corresponding reactions. This relationship is not seen, however, when the control cells are compared with the transformed cells, for increased modifications of collagen types I and/or III were seen in the transformed cells even when the enzyme activities were decreased. These data thus provide, for the first time, experimental evidence for the suggestion that an additional factor affecting the extent of the modification is the rate of pro-α chain synthesis, which influences the ratio of enzymes to substrate in the cell.

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