Effect of dextran on synthesis, secretion and deposition of type III procollagen in cultured human fibroblasts

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When subconfluent cultures of primary human skin fibroblasts are incubated for 20 h in the presence of 5% neutral dextran, the newly synthesized procollagens are shifted from the medium into the pericellular matrix fraction. This is accompanied by an overall decrease by 30–50% in the secretion rates of these proteins, as indicated by the incorporation of tritiated proline into collagenase-sensitive macromolecules and by radioimmunoassays for the propeptide regions of type I and III procollagens. Inhibition of tyrosine sulphation in newly formed type III procollagen by NaClO₃ does not change the distribution of this protein between the medium and matrix fractions either in the presence or in the absence of the polymer. Processing of type III procollagen at its *N*-terminus is incomplete in this situation, irrespective of whether the protein is present mainly in the medium or in the pericellular matrix. The concentrations of the mRNAs for the pro α 1(I)- and pro α 1(III)-chains of procollagens and for actin were monitored for up to 20 h; in the dextran-treated cultures these are not different from the corresponding concentrations in control cultures, indicating that the rapid down-regulation of the synthesis of type I and III procollagens in response to the enhanced pericellular matrix deposition induced by dextran is not due to transcriptional mechanisms.

INTRODUCTION

Fibroblasts maintained in monolayer culture are the system most commonly used for investigating the regulation of collagen production (e.g. Goldberg, 1977; Jimenez *et al.*, 1986; Miskulin *et al.*, 1986; Appling *et al.*, 1989). This situation is highly unphysiological, however, as the cells continuously secrete large amounts of collagenous precursor proteins in soluble form into the culture medium, where their effective concentration yet remains low. In tissues, mesenchymal cells are tightly surrounded by an extracellular matrix containing collagens in an insoluble cross-linked form, and the basal collagen production rate remains low. The mechanisms by which the surroundings of a cell can regulate its collagen synthesis are still to a great extent unknown, but receptors that mediate direct attachment of cells to extracellular collagens have been characterized on the surfaces of several cell types, including connective-tissue cells (Dedhar *et al.*, 1987).

A situation more physiological than monolayer culture can be achieved by culturing the fibroblasts in a three-dimensional collagen gel. Under these conditions the cells attain a different morphology and rapidly slow down their overall collagen and non-collagen protein production (Mauch *et al.*, 1988).

The interstitial collagens, types I to III, are synthesized as larger precursors, procollagens, which contain additional peptide sequences at both ends. The ordered cleavage of these propeptides is a prerequisite for the assembly of the collagen fibres in tissues. Specific proteinases are responsible for the removal of the *N*-terminal and *C*-terminal propeptides, there being one *N*-terminal proteinase for type I and II procollagens and another for type III procollagen and a *C*-terminal proteinase common to all of these procollagen types. In monolayer cell culture the processing of interstitial collagens is slow and incomplete, in contrast with the situation *in vivo*. Type III procollagen, in particular, is often hardly being converted into the respective collagen at all (Goldberg, 1977). In old post-confluent cell cultures the accumulation of a pericellular matrix seems to enhance procollagen processing (Grinnell *et al.*, 1989).

Addition of neutral polymers, such as poly(ethylene glycol) or dextran, has been reported to enhance the deposition of a pericellular matrix and the processing of precursor forms of type I collagen into the collagen proper under cell-culture conditions, with a concomitant decrease in the incorporation of radioactive proline into collagen (Bateman *et al.*, 1986). The present study was undertaken to compare the effects of such a treatment on the synthesis, secretion and subsequent deposition of type III procollagen with those of type I procollagen.

MATERIALS AND METHODS

Materials

The cell-culture reagents were obtained from Kc Biological (Lenexa, KS, U.S.A.) and Gibco Biocult (Paisley, Strathclyde, U.K.). L-[2,3-³H]proline (33.0 Ci/mmol) was from Amersham International (Amersham, Bucks., U.K.), dextran T-40 and Protein A-Sepharose CL-4B were from Pharmacia (Uppsala, Sweden), purified bacterial collagenase (grade CLSPA) was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), β -aminopropionitrile fumarate was from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and nitrocellulose paper (0.45 μ m pore diameter) was from Schleicher und Schüll (Dassel, Germany).

Cell culture, dextran treatment and metabolic labelling

Human dermal fibroblast cultures, established from skin biopsies obtained from several surgical patients with no connective-tissue-affecting diseases, were grown in Dulbecco's modification of Eagle's medium supplemented with newborn-calf serum, ascorbic acid, penicillin and streptomycin (Jukkola *et al.*, 1986). For radioactive labelling of newly synthesized proteins, confluent cultures were incubated overnight in fresh medium without serum but with β -aminopropionitrile fumarate (50 μ g/ml) and L-[2,3-³H]proline (10 μ Ci/ml) and with or without 5% (w/v) dextran T-40.

The cell-layer and medium fractions were analysed separately. After addition of proteinase inhibitors (Jukkola *et al.*, 1986) the medium was exhaustively dialysed at 4 °C against either 0.2 M-

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Table 1. Incorporation of [³H]proline into non-diffusible protein in the medium and into the pericellular matrix by human fibroblast cultures

Confluent cultures were labelled for 20 h with [³H]proline either in the presence or in the absence of 5 % (w/v) dextran T-40. The results represent the means of duplicate determinations of medium samples of several dishes pooled together (*n* = number of dishes). The same fibroblast line was used for all three experiments.

	[³ H]Proline-labelled protein						
	Medium		Matrix		Total		Distribution (% in matrix)
	(d.p.m./ 10 ⁶ cells)	(% of control)	(d.p.m./ 10 ⁶ cells)	(% of control)	(d.p.m./ 10 ⁶ cells)	(% of control)	
Expt. 1 (<i>n</i> = 4)							
Control	53 000	100	6 900	100	60 000	100	12
Dextran	36 000	68	17 000	246	53 000	88	33
Expt. 2 (<i>n</i> = 6)							
Control	122 000	100	26 000	100	148 000	100	17
Dextran	63 000	52	65 000	250	128 000	86	51
Expt. 3 (<i>n</i> = 10)							
Control	113 000	100	41 000	100	154 000	100	27
Dextran	47 000	42	53 000	129	100 000	65	53

NH₄HCO₃ or a solution containing 0.4 M-NaCl, 5 mM-EDTA, 50 mM-Tris/HCl buffer, pH 8.0, 1 % (v/v) Nonidet P-40 and 0.02 % NaN₃ (buffer for immunoprecipitation). Pericellular matrix free of cells was prepared from confluent cultures according to the deoxycholate procedure (Hedman *et al.*, 1979) and scraped into a mixture containing 4 % (w/v) SDS, 2 % (v/v) glycerol, 10 % (v/v) 2-mercaptoethanol and 0.002 % Bromophenol Blue or immunoprecipitation buffer (see above) or 0.2 M-NH₄HCO₃.

Chemical and immunochemical methods

Immunoprecipitation, collagenase digestion, SDS/PAGE and fluorography were carried out as described previously (Jukkola *et al.*, 1986). The radiolabelled bands in the fluorograms were quantified by scanning densitometry. Immunoblotting was carried out as described by Towbin *et al.* (1979). The type III pN-collagen and type III collagen were purified from bovine skin (Timpl *et al.*, 1975).

The radioimmunoassays for the C-terminal propeptide of type I procollagen (Melkko *et al.*, 1990) and for the N-terminal propeptide of human type III procollagen (Risteli *et al.*, 1988) were carried out with the use of sequential saturation to increase the sensitivity. For immunoprecipitation and immunoblotting, the antibodies against the N-terminal propeptide of type III procollagen were purified by affinity chromatography on the respective antigen coupled to Sepharose 4B. Their specificities were verified by the radioimmunoassays.

Assays of mRNA concentrations

The total RNA of the cultured cells was purified (Gough, 1988) and quantified by spectrophotometric absorption at 260 nm. Prehybridization and the specific hybridizations were carried out as described previously (Ala-Kokko *et al.*, 1987). The cDNA probes used were α 12, a 2.4 kb-long human cDNA for pro α 1(I)-chain messenger (Loidl *et al.*, 1984), E6, a 2.4 kb-long human cDNA for pro α 1(III)-chain messenger (Loidl *et al.*, 1984) and pHFG A-1, a 2.1 kb-long cDNA for Γ -actin messenger (Gunning *et al.*, 1983). Plasmid DNA isolation and nick translation were carried out as described by Ala-Kokko *et al.* (1987).

Fig. 1. SDS/PAGE analysis of [³H]proline-labelled macromolecules of the medium of fibroblast cultures after 20 h of continuous labelling

The samples were run under non-reducing conditions (–) or reduced with 2-mercaptoethanol (+). The molecular-mass markers, indicated by arrows, are unreduced type III procollagen (upper 1) and the reduced polypeptide chains pro α 1(III) (lower 1) and pN α 1(III) (2). The samples are medium from control cells (lanes 1 and 5), medium from dextran-treated cells (lanes 2 and 6), medium from control cells digested with bacterial collagenase (lanes 3 and 7) and medium from dextran-treated cells digested with bacterial collagenase (lanes 4 and 8).

RESULTS

Secretion of total protein and collagens in the medium

Dextran T-40 (5 %, w/v), chosen to give a maximal effect on type I procollagen processing (Bateman *et al.*, 1986), in the medium of primary cultures of human fibroblasts decreases the incorporation of radioactive proline into medium macromolecules by 30–60 % during 20 h (Table 1) without affecting cell viability. In controls, 50–60 % of this label is in collagenous proteins, as indicated by sensitivity to digestion with bacterial

Table 2. Immunoprecipitation of [³H]proline-labelled proteins with antibodies against the *N*-terminal propeptide of type III procollagen

Experimental conditions were as indicated in the legend to Table 1. The results show the means of duplicate determinations.

	[³ H]Proline-labelled protein						Distribution (% in matrix)
	Medium		Matrix		Total		
	(d.p.m./ 10 ⁶ cells)	(% of control)	(d.p.m./ 10 ⁶ cells)	(% of control)	(d.p.m./ 10 ⁶ cells)	(% of control)	
Expt. 1							
Control	9500	100	1400	100	10900	100	13
Dextran	3100	32	2300	160	5400	50	43
Expt. 2							
Control	10000	100	1300	100	11300	100	12
Dextran	3300	33	2300	177	5600	50	41

Fig. 2. SDS/PAGE analysis of immunoprecipitates of [³H]proline-labelled medium proteins

Immunoprecipitation with affinity-purified rabbit antibodies to the *N*-terminal propeptide of human type III procollagen was carried out with 1.0 ml of culture medium and 10–40 μ l of antibodies. The samples were run under non-reducing conditions (–) or reduced with 2-mercaptoethanol (+). The samples were medium of control cells (lanes 3 and 5), medium from dextran-treated cells (lanes 4 and 6), standard [³H]proline-labelled type III procollagen (lanes 2 and 7), and ¹²⁵I-labelled *N*-terminal propeptide of human type III procollagen (lanes 1 and 8).

collagenase, whereas in the presence of dextran only about 10 % is sensitive. Thus the polymer selectively inhibits the secretion of procollagens into the medium. Analysis of the [³H]proline-labelled medium proteins by SDS/PAGE agrees with this (Fig. 1): fibronectin and procollagen chains are seen in control cultures, but only fibronectin in the dextran-treated cultures.

The secretion rates of two major collagenous products of fibroblasts were analysed separately with radioimmunoassays for the *C*-terminal propeptide of type I procollagen and for the *N*-terminal propeptide of type III procollagen. Dextran treatment decreases their medium concentrations by 80 % and by 83 % in 20 h respectively. Immunoprecipitation with monospecific antibodies against the *N*-terminal propeptide of type III procollagen also indicates a similar decrease in type III procollagen (Table 2). The sequence recognized by these antibodies is almost completely

Table 3. Secretion of procollagens into the medium as a function of time in cultured human fibroblasts

The concentrations are expressed as those of the *C*-terminal propeptide of type I procollagen (PICP) and the *N*-terminal propeptide of type III procollagen (PIIINP). The PIIINP results show the means and standard deviations, with the number of dishes analysed in parentheses. The PICP values are means of determinations on two dishes for both treated and control at every time point. **P* < 0.05, †*P* < 0.01, ‡*P* < 0.001; the concentration in dextran-treated samples compared with controls at the same time point with Student's *t* test.

Time (h)	PICP (mg/l)		PIIINP (μ g/l)	
	Control	Dextran	Control	Dextran
2	1.9	1.5	6.4 $\pm$ 2.7 (4)	3.8 $\pm$ 1.8 (4)
4	6.4	1.8	17.3 $\pm$ 8.4 (4)	2.2 $\pm$ 0.8* (3)
8	12.2	2.6	48.7 $\pm$ 14.0 (4)	6.7 $\pm$ 1.5† (3)
23	32.0	2.4	116.7 $\pm$ 12.0 (3)	9.0 $\pm$ 6.3‡ (4)

in the form of procollagen or pN-collagen, since there is no free *N*-terminal propeptide in either situation (Fig. 2).

The decrease in the accumulation of type III procollagen in the medium becomes statistically significant after only 4 h of treatment with dextran (Table 3).

Accumulation of collagen precursors in pericellular matrix

The insoluble pericellular matrix fraction of fibroblast cultures normally contains 10–20 % of the total proline radioactivity incorporated into extracellular macromolecules during 20 h, whereas after dextran treatment about half of the label is in this form (Table 1). A similar increase is seen in the amount of type III collagen precursors in the pericellular matrix, as quantified by immunoprecipitation (Table 2). About 80 % of the proline label in the matrix is sensitive to collagenase in both cases, indicating that 80–90 % of the collagenous proteins secreted during dextran treatment become deposited in the matrix. Total collagen secretion is decreased to about 70 % of that of the control cultures (50 %, 70 % and 80 % in Expts. 1, 2 and 3 of Table 1 respectively).

The major [³H]proline-containing constituents of the pericellular matrices of control cultures are collagen α -chains (Fig. 3), with small amounts of pN α - and pro α -chains. The same components are found in larger amounts after dextran treatment, together with some fibronectin.

Fig. 3. Analysis of procollagen-related components in the pericellular matrix

(a) SDS/PAGE of [^3H]proline-labelled macromolecules after 20 h of continuous labelling. All the samples were run after reduction with 2-mercaptoethanol. The samples were pericellular matrix of control cells (lane 1), that of dextran-treated cells (lane 2), matrix of control cells immunoprecipitated with antibodies against the *N*-terminal propeptide of type III procollagen (lane 3), matrix of dextran-treated cells immunoprecipitated with the same antibodies (lane 4) and standard [^3H]proline-labelled type III procollagen (lane 5). Immunoprecipitation with affinity-purified rabbit antibodies to the *N*-terminal propeptide of human type III procollagen was carried out with 1.0 ml of matrix extract and 40 μl of antibodies. The molecular-mass markers, indicated by arrows, were reduced pro α 1(III)- (1) and pN α 1(III)-chains (2) and the α 1(I)- (3) and α 2(I)-chains (4) of type I collagen. (b) SDS/PAGE of non-labelled matrix components. All the samples were reduced with 2-mercaptoethanol. Lanes 1–4 show the protein staining and lanes 5 and 6 immunoblotting of the same gel with antibodies against the *N*-terminal propeptide of human type III procollagen. The samples were bovine type III collagen (lane 1), bovine type III pN-collagen (lane 2), pericellular matrix of control cells (lanes 3 and 5) and that of dextran-treated cells (lanes 4 and 6). Molecular-mass markers were as in (a). In immunoblotting (lanes 5 and 6), the specific dark-brown staining is present only in the bands corresponding to the pro α 1(III)- and pN α 1(III)-chains; the other bands show an unspecific greyish colour.

Fig. 4. Slot-blot hybridization with cDNA probes specific for actin (A) and for the procollagen chains pro α 1(III) (B) and pro α 1(I) (C) of RNA isolated from control fibroblasts (1) and fibroblasts treated with dextran for 8 h (2)

Amounts of 0.2, 0.4 and 0.8 μg of total RNA were bound to nitrocellulose.

The immunoreactivity related to the *N*-terminal propeptide of type III procollagen in control matrices is mostly due to pN α 1(III)-chains, and in a smaller part to pro α 1(III)-chains. In dextran-treated cultures the proportion of the latter is larger and the pN α 1(III)-chain appears as a doublet (Fig. 3a). The *N*-terminal propeptide is not found in free form in the matrix of either type of culture.

In immunoblotting with antibodies against the *N*-terminal propeptide of type III procollagen, larger amounts of pro α 1(III)- and pN α 1(III)-chains are again seen in matrices of the dextran-treated cultures. In protein staining also the amounts of these two chains appear larger (Fig. 3b).

Addition of 10 mM-NaClO₃, which prevents tyrosine *O*-

Fig. 5. Northern-blot hybridization of human procollagen cDNA probes to human fibroblast RNA

Total RNA was isolated, fractionated by 0.8% agarose gel electrophoresis and transferred to nitrocellulose filters, which were hybridized with the cDNA probes for pro α 1(I) collagen (lanes 1 and 2) and for pro α 1(III) collagen (lanes 3 and 4). The samples are control fibroblasts (lanes 1 and 3) and fibroblasts after an 8 h treatment with dextran (lanes 2 and 4).

sulphation in type III procollagen (Jukkola *et al.*, 1990), does not change the distribution of proline-labelled macromolecules between the medium and the matrix. In one experiment 9% and 7.4% of the total radioactivity incorporated into extracellular macromolecules in the absence of dextran were found in the matrix fractions of control and chlorate-treated cultures respectively. The corresponding values for cultures subjected to dextran were 41% and 50% respectively.

Effects on procollagen mRNAs

The mRNAs specific for type I procollagen, type III procollagen and actin were quantified by slot-blot hybridization of total mRNA after various incubation times (Fig. 4). There is little change in any from the beginning of the incubation to 8 h,

and a slow decrease thereafter, with no difference between dextran-treated and control cultures.

In Northern-blot analysis (Fig. 5), similar mRNA species, corresponding to sizes reported previously (Chu *et al.*, 1982; Miskulin *et al.*, 1986), are found in control and dextran-treated cells, with no differences in the relative amounts of the isoforms.

DISCUSSION

Neutral or anionic polymers, such as poly(ethylene glycol), polyvinylpyrrolidone and dextran (Bateman *et al.*, 1986) or dextran sulphate (Bateman & Golub, 1990) respectively, enhance the deposition of type I procollagen into the pericellular matrix in fibroblast cultures and its subsequent processing to type I collagen. Here we show that a similar enhancement of processing does not take place for type III procollagen, the second major collagenous product of fibroblasts, despite the fact that the protein is shifted from the medium to the pericellular-matrix compartment. The processing of type III procollagen, in particular at its *N*-terminus, is slower than that of type I procollagen both *in vivo* and under cell-culture conditions (Goldberg, 1977; Fessler *et al.*, 1981a; Fleischmajer *et al.*, 1985). We investigated fibroblast cultures that had only recently become confluent; it is possible that accumulation of type III procollagen *N*-proteinase only takes place in older cultures and a relatively brief treatment with dextran is not enough to induce secretion of substantial amounts of this enzyme into the matrix.

A down-regulation of collagen synthesis in fibroblasts accompanies the change in the distribution of extracellular procollagens. This effect seems quite specific, compared with other proline-labelled extracellular macromolecules. Generally, when cultured cells are subjected to a chemical treatment, changes in the specific radioactivities of intracellular amino acid pools can cause erroneous results if incorporation of a radioactive precursor substance is the only method used for quantifying a protein produced by the cells. To overcome this possible bias we used two radioimmunoassays to assess type I and III procollagens separately. The results were in good agreement with those obtained with proline-labelling and subsequent collagenase digestion or immunoprecipitation.

The inhibitory effect of dextran on the secretion of type I and III procollagens is discernible in less than 4 h. This is not primarily due to transcriptional control, the cellular concentrations of the corresponding mRNAs remaining unchanged for at least 20 h. Extensive degradation of the mRNAs is not likely either, since the hybridization signals for the procollagen chains in both control and dextran-treated cultures are caused by two mRNA species in similar ratios, with no difference in the proportions of the isoforms between control and dextran-treated cultures. Thus it is likely that the translation efficiency of existing procollagen mRNAs can be rapidly regulated by some yet-unknown mechanism. Other situations with a discrepancy between changes in the numbers of procollagen mRNA transcripts and changes in the synthesis rates of the corresponding proteins are, for example, the different phases of fibroblast cultures (Miskulin *et al.*, 1986) and the rapid enhancement of collagen synthesis induced by ascorbate in cells cultured without this vitamin (Chan *et al.*, 1990).

Fibroblasts grown in a collagen gel that they contract slow down their collagen synthesis (Mauch *et al.*, 1988). The effect of dextran on the synthesis rate may be related to the same basic regulatory mechanism, the critical factor being the effective concentration of collagens in the immediate vicinity of the cell. Fibroblasts grown in a collagen gel also contain much less

mRNA for procollagen chains than do cells grown on a plastic surface. The difference between this finding and ours may be due to the fact that we only studied the effect of short-term treatment on fibroblasts that had just become confluent after being grown in ordinary monolayer culture.

The partially processed pN form of type III procollagen is a commonly found genuine constituent of both fetal and adult tissues (Fleischmajer *et al.*, 1985). Although some type I pN-collagen is also present in fetal tissues, there seems to be a basic difference between the collagen types I and III with respect to their *N*-terminal processing also *in vivo*. The type III pN-collagen molecules are situated on the surface of collagen fibres and probably regulate their further growth (Fleischmajer *et al.*, 1985). The *N*-terminal region of type III procollagen contains sulphated tyrosine residues (Jukkola *et al.*, 1986), whereas type I procollagen does not. Tyrosine *O*-sulphate is also found in type V collagen (Fessler *et al.*, 1986), which is also processed slowly in tissues (Fessler *et al.*, 1981b). Irrespective of the differences in their extracellular processing, the synthesis rates of type I and III procollagens are generally tightly co-ordinated, both in cell culture (Jimenez *et al.*, 1986; Miskulin *et al.*, 1986; Appling *et al.*, 1989; Chan *et al.*, 1990) and *in vivo*, e.g. in healing wounds (Haukipuro *et al.*, 1991). This is also true for their down-regulation by a short-term treatment with dextran.

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