EFFECT OF β-D-XYLOSIDES ON COLLAGEN SYNTHESIS IN CULTURED FIBROBLASTS

Nancy B. Schwartz* and Albert Dorfman

Departments of Pediatrics and Biochemistry
Joseph P. Kennedy, Jr. Mental Retardation Research Center
Pritzker School of Medicine
University of Chicago
Chicago, Illinois 60637

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SUMMARY

Cultured normal human skin fibroblasts were incubated with [^{14}C]proline in the presence and absence of 1.0 mM p-nitrophenyl-β-D-xylose. Formation of non-dialyzable hydroxyproline was used as a measure of collagen synthesis. Although total [^{14}C]proline incorporation was similar in the two cultures, [^{14}C]hydroxyproline formation was significantly decreased in the β-xyloside-treated cultures. Increasing the period of incubation increased the radioactivity of the insoluble collagen fraction in untreated fibroblasts, however, in β-xyloside-treated cultures no such increase was observed. In contrast to the decreased production of collagen, growth of cells in the presence of the β-xyloside induced the synthesis of high levels of soluble glycosaminoglycans as measured by $^{35}SO_4$ incorporation into isolated polysaccharide.

Previous studies from this laboratory have shown that β-xylosides stimulate the synthesis of sulfated glycosaminoglycans in several types of non-connective tissue cells, as well as in chondrocytes and fibroblasts (1-3). Most likely the β-xylosides substitute for xylosylated-core protein at the second glycosyltransfer step, thereby acting as initiators of free chondroitin sulfate chains.

Proteoglycans are known to have an intimate relationship with collagen. It has been suggested that chondroitin 4-sulfate is involved in the formation of new collagen and that maturation of fibers may involve removal of this glycosaminoglycan (4). For a preliminary evaluation of the effect of the artificial production of high levels of soluble glycosaminoglycans on the synthesis and/or maturation of collagen, [^{14}C]hydroxyproline formation was compared in normal human fibroblasts grown in the presence and absence of β-xylosides.

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* Established Investigator of the American Heart Association

MATERIALS AND METHODS

Normal human skin fibroblasts, which had undergone 7 transfers, were cultured as described (5) in modified Eagle's medium supplemented with 10% fetal calf serum. Equal numbers of cells (1 x 10^6 cells/plate) were seeded in 100 mM Falcon petri dishes and grown to confluency (approximately 2 x 10^7 cells/plate). Cells were fed 3 times weekly and grown at 37° in a humidified atmosphere of 10% CO_2 and 90% air. On the last day of culture, cells were incubated for 24 or 48 hr at 37° with either 8.3 μCi [^{14}C]proline (260 mCi/mmol), 6.8 μCi [^{14}C]leucine (312 mCi/mmol) or 10 μCi $H_2{}^{35}SO_4$ (43 Ci/mg) in the presence or absence of 1 mM p-nitrophenyl-β-D-xylose and 0.6 mM ascorbate.

For assay of sulfated glycosaminoglycans, cells and media were collected, and the amount of labeled polysaccharide was determined following addition of 2 mg of carrier chondroitin sulfate, papain digestion and cetylpyridinium chloride and ethanol precipitation as described (6).

For estimation of collagen synthesis, the media was removed, cell layer washed 2 times with Hank's BSS, calcium- and magnesium-free and brought into suspension by scraping. Cells were pelleted and extracted with 5 ml of 1 M NaCl in 0.05 M Tris, pH 7.4 for 2 days at 4° (7). The samples were then centrifuged at 10,000 x g for 15 min. The salt soluble, pellet (insoluble fraction) and media plus buffer washes were all dialyzed exhaustively against water. Aliquots plus 20 μmoles each of unlabeled proline and hydroxyproline were hydrolyzed in 6 N HCl at 100° for 20 hr.

Fractionation was carried out according to Lukens (8) using Dowex 50-resin (H^+ form, 0.8 x 22 cm column) by elution with 2.0 N HCl. The elution positions were determined by counting 50 μl aliquots of each fraction (2.5-3.0 ml), to be 30 to 45 ml for hydroxyproline and 60 to 90 ml for proline. The respective fractions were combined and total proline and hydroxyproline content was determined. Radioactivity was measured at 4° in a Packard Tri-Carb liquid scintillation spectrophotometer.

RESULTS

As a measure of collagen synthesis, the amount of non-dialyzable radioactive hydroxyproline synthesized by normal human fibroblasts in the presence and absence of 1 mM p-nitrophenyl-β-D-xylose for 24 hr was determined (Table I-A). Most (60 to 67%) of the total hydroxyproline synthesized in both types of cultures was found in the media. Of the peptide-bound hydroxyproline associated with the cell layer, 64 and 53% was extracted with cold neutral salt solution, and the remainder (36 and 47%) was isolated as insoluble cross-linked collagen fibers in untreated and treated fibroblasts, respectively. However, a significant decrease in hydroxyproline

Table I

[^{14}C]Hydroxyproline Synthesis by Control and β-Xyloside-Treated Fibroblasts in Culture

Fraction	Control (cpm)		β-Xyloside (cpm)	
A. 24 hours				
Cell layer collagen	9,739[a]		5,088	
1 M NaCl extract	6,230	(6.1)[b]	2,690	(5.4)
residue	3,509	(8.8)	2,398	(7.1)
Medium collagen	15,084	(13.6)	10,312	(12.6)
B. 48 hours				
Cell layer collagen	24,296		9,156	
1 M NaCl extract	6,926	(3.3)	5,686	(3.3)
residue	17,370	(9.7)	3,470	(7.9)
Medium collagen	42,566	(9.0)	21,644	(6.8)

[a] Total cpm per plate

[b] Value (hydroxyproline/hydroxyproline + proline) X100

formation in all 3 fractions from the β-xyloside-treated cultures was observed. In all cases the value (hydroxyproline/hydroxyproline + proline) X100 (which represents an estimate of the amount of collagen synthesized in relation to all other proline-containing proteins) decreased, indicating that a reduction in collagen synthesis occurred in the treated cells.

The transfer of radioactive hydroxyproline from a soluble to insoluble collagen fraction was also followed with time (Table I-b). Two additional cultures were incubated as before for 24 hr with [^{14}C]proline in the presence or absence of the β-xyloside. The media was then replaced with fresh media containing [^{14}C]proline, with or without β-xyloside, for an additional 24 hr. In normal cultures there was a relative increase in radioactive hydroxyproline in insoluble collagen with time from 14 to 25%, indicating a transfer of activity from the soluble to insoluble fraction, as expected for the normal

in vitro maturation of collagen. However, in β-xyloside-treated cultures the radioactive hydroxyproline in the insoluble fraction decreased slightly (15 to 11%) between 24 and 48 hr.

Growth of fibroblasts in the presence of 1 mM β-xyloside had no adverse effect on overall protein synthesis, since no difference in [^{14}C]leucine incorporation into TCA precipitable material in the presence or absence of β-xyloside was observed.

As previously reported (1,2) β-xylosides stimulate the synthesis of sulfated glycosaminoglycans by fibroblasts. A 7-fold and 14-fold increase in the chondroitin 4/6-sulfate and dermatan sulfate fractions, respectively, was observed by normal human fibroblasts during a 6 hr incubation with β-xyloside (2). In this study, confluent fibroblasts treated with β-xyloside for 24 hr, were stimulated to produce approximately 100-fold more sulfated glycosaminoglycans (isolated largely from the media) than untreated controls (Table II).

DISCUSSION

It is apparent from these experiments that concurrent with the production of high levels of soluble glycosaminoglycans, there is a decrease in the hydroxyproline formation by normal fibroblasts grown in the presence of β-xylosides. It has previously been shown that most of the peptide-bound hydroxyproline synthesized by human dermal fibroblasts in culture is released into the medium and that the major portion of the medium hydroxyproline is newly synthesized procollagen (9). The present results tend to suggest that β-xyloside-treated fibroblasts have a diminished ability to synthesize procollagen, as indicated by

Table II

Effect of β-Xylosides on Glycosaminoglycan Synthesis by Fibroblasts in Culture

Fraction	Control	β-Xyloside
	($^{35}SO_4$ cpm)	($^{35}SO_4$ cpm)
Cell Layer	7,698	9,238
Medium	8,234	704,792

the 32% and 50% decrease in medium hydroxyproline in 24 and 48 hr cultures, respectively, as well as to process the collagen into a mature insoluble form.

It has been known for many years that aggregation of tropocollagen may be directly dependent upon the presence and amount of glycoprotein and acidic glycosaminoglycans, particularly chondroitin sulfate (10-12). These compounds are also apparently responsible for the production of insoluble collagen fibers (13), and exert some influence on the diameter size of the collagen fiber (4). More specifically, proteolytic digestion of chondroitin sulfate proteoglycan, which reduces the molecular weight from approximately 1-2 x 10^6 to that of polysaccharide chains (22,000), also abolishes the precipitation of collagen in an *in vitro* assay system (Lowther *et al.*, 14). Furthermore, Toole and Linsenmayer (15) showed that insignificant binding of collagen and proteoglycan occurred if the extract of proteoglycan was pre-treated with pronase or mixed with a solution of protein-free chondroitin sulfate in excess, suggesting that the protein component of proteoglycan is essential for binding of collagen. From all of these studies it has become apparent that glycosaminoglycans have an intimate and important structural relationship with collagen. However, this is the first demonstration that the production of one of these components may be influenced by a change in the biosynthesis of the other.

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