EFFECT OF β-XYLOSIDES ON SYNTHESIS OF CARTILAGE-SPECIFIC PROTEOGLYCAN IN CHONDROCYTE CULTURES

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SUMMARY

Monolayer cultures of embryonic chick chondrocytes were incubated with $^{35}SO_4$ in the presence and absence of 1.0 mM p-nitrophenyl-β-D-xylose for 2 days. The relative amounts of chondroitin sulfate proteoglycan and free chondroitin sulfate chains were measured following gel filtration on Sephadex G-200. Synthesis of β-xyloside-initiated polysaccharide chains was accompanied by an apparent decrease in chondroitin sulfate proteoglycan production by the treated cultures. The amount of core protein was determined from equivalent numbers of β-xyloside-treated and untreated cells by a radioimmune assay. Similar amounts of core protein were found in both types of cultures, indicating that decreased synthesis of cartilage-specific core protein is not responsible for the observed decrease in overall chondroitin sulfate proteoglycan production.

Chondrocytes synthesize and secrete chondroitin sulfate-proteoglycan[1] and cartilage-specific collagen, the major macromolecules of extra-cellular matrix. Previous studies have shown that chondrocytes (and other cell types) are also capable of synthesizing free polysaccharide chains initiated with D-xylose or derivatives of β-D-xyloside (1-3). Most likely, the β-xylosides act as initiators of CS chains by joining the intracellular enzyme machinery at the second glycosyltransfer step, thus eliminating the need for core protein and xylosyltransferase. Studies have been initiated to determine the effect of artificial production of high levels of soluble CS chains on the

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[1]The abbreviations used are: CSPG, chondroitin sulfate proteoglycan; CS, chondroitin sulfate chains.

normal synthesis of CSPG and collagen (4-6). Preliminary evidence indicates that a reduction in CSPG synthesis occurs concomitant with the production of free CS chains. Since protein synthesis precedes the addition of carbohydrate chains it was necessary to determine whether β-xyloside treatment caused a decrease in synthesis of core protein. In the studies reported here, a radioimmune assay for cartilage-specific core protein which has recently been developed (7), was used to measure qualitative and quantitative differences in core protein synthesis during production of β-xyloside-induced CS chains by chondrocytes in monolayer.

MATERIALS AND METHODS

Chondrocytes were grown under conditions that have previously been described (8). Briefly, cultures were established from sternal cartilage of 15 day-old chick embryos according to Cahn et al. (9). Cells were plated at initial densities of 2 to 5 x 10^5 cells per 60 mm Falcon tissue culture dishes in Ham's F-12 medium supplemented with 7% horse serum and 10% fetal calf serum. Cells were fed every 2-3 days by a complete change of medium and grown at 37° in a humidified atmosphere of 10% CO_2 and 90% air. Multiple sets of plates were incubated with 5 μCi $H_2{}^{35}SO_4$ (43 Ci/mg) in the presence or absence of 1 mM p-nitrophenyl-β-D-xylose from the 8th to 10th day of culture.

For assay of sulfated glycosaminoglycan, cells and media were collected and extracted for 24 hr at 4° with 5M LiCl. Insoluble material was pelleted at 10,000 x g and the supernatant fluid applied to a column (2.5 x 130 cm) of Sephadex G-200 and eluted with 0.05 M Tris-acetate, pH 5.5, containing 0.25 M KCl and 2.5 M LiCl.

The procedures for preparation of cartilage-specific core protein and antisera to core protein have recently been described (7). Briefly, CSPG from epiphyses of 13 day-old chick embryos or sternae of adult chickens was extracted and purified in the presence of protease inhibitors (10). Following digestion with testicular hyaluronidase (7), the protein portion containing short chains of CS was isolated by gel filtration on Sephadex G-200 (1 x 10 cm) in 0.15 M NaCl. The excluded peak was concentrated and used to elicit antibodies to epiphyseal cartilage core protein in rabbits as previously described (7). A radioactive antigen was also prepared from minced epiphyses by incubating with 25 μCi/ml of [U-^{3}H]-sodium acetate (100μCi/mole) for 6 hr in complete media. CSPG was isolated, treated with hyaluronidase and separated on Sephadex G-200 as described above.

The radioimmune assay system uses the ammonium sulfate precipitation

technique of Farr (11). The method was linear (%precipitation vs. log antibody concentration) between serum dilutions of 1:12 and 1:96, depending on the particular serum used (7). The percent antibody bound radioactivity (%P) was calculated as follows:

$$\% P = 100 - \frac{\text{cpm Ag} - \text{cpm Exp-Ppt}}{\text{cpm Ag} - \text{cpm NRS-Ppt}} \times 100$$

where cpm Ag signifies amount of antigen used, cpm Exp-Ppt is the precipitate in experimental tubes and cpm NRS-Ppt is the precipitate in tubes with only normal rabbit serum. Specific antigens were quantitated by a competition assay with both labeled and unlabeled digested proteoglycan (7). Percent of inhibition was determined using the relationship:

$$\% \text{ inhibition} = \frac{\%P\text{-cont} - \%P\text{-exp}}{\%P\text{-cont}} \times 100$$

where %P-cont is percent of antibody-bound radioactivity in tubes without unlabeled competitor and %P-exp is percent antibody-bound radioactivity in tubes with unlabeled competitor antigen (12).

RESULTS

Synthesis of CSPG by chondrocytes grown in the presence or absence of 1.0 mM p-nitrophenyl-β-D-xyloside for 2 days was assessed by separating and quantitating the $^{35}SO_4$-labeled large molecular weight CSPG and free CS chains by gel filtration on Sephadex G-200. The results (Table I) indicate that synthesis of included material, identified as free CS chains (2) is accompanied by an apparent decrease in production of CSPG (excluded material) in the β-xyloside-treated cultures.

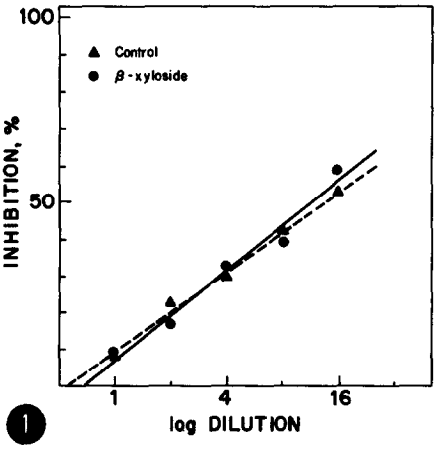
Of the several possibilities that might account for a decreased production of CSPG, the capacity for synthesis of core protein was determined by measuring levels of core protein by a radioimmune assay that has recently been developed (7). Total protein from equivalent numbers of control and β-xyloside-treated cells were prepared by guanidinium chloride extraction and hyaluronidase digestion as described in Methods. Levels of core protein were determined by immunoprecipitation with antisera to core protein in an inhibition assay with labeled core protein of known specific activity. The results of a typical experiment are presented in Fig. 1. Antigens derived from

Table I

Effect of β-Xylosides on Chondroitin Sulfate Synthesis in Chondrocyte Cultures

Experiment	Fraction	$^{35}SO_4$ Incorporation
		cpm x 10^{-6}
Control	CSPG	8.6[a]
	CS	0
β-Xyloside	CSPG	3.2
	CS	20.6

[a] total cpm per 4 plates.



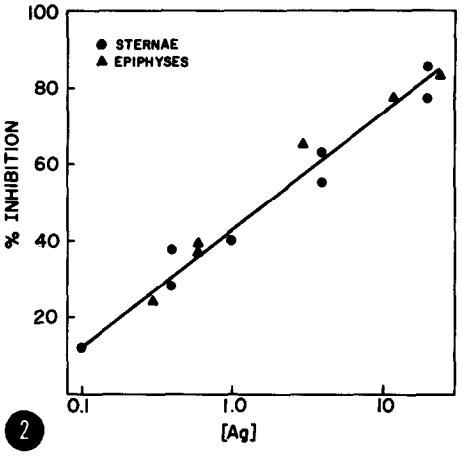


Fig. 1. Inhibition assay using [^{3}H]acetate labeled antigen. Cold antigens compared were obtained from β-xyloside-treated or control chondrocytes. Antigen concentration is arbitrarily expressed as log of dilution of material derived from 5.5 plates of cells per ml where each plate contained approximately 10 x 10^6 cells per plate. The number of cells per plate were comparable for β-xyloside-treated and control cultures.

Fig. 2. Inhibition assay using [^{3}H]acetate labeled antigen and purified, hyaluronidase-digested (7) unlabeled chick sternal or epiphyseal core proteins as competing antigens. Antigen concentration is expressed as micrograms of protein.

β-xyloside-treated and untreated cells gave similar levels of inhibition as indicated by the nearly coincident precipitation curves.

In order to quantitate the amount of core protein produced by both types of cultures, inhibition assays consisting of known amounts of unlabeled chick sternal or epiphyseal cartilage antigen versus labeled core protein antigen were first carried out as described above. As illustrated in Fig. 2, core protein antigen can be measured over the range of 0.1 to 24 μg of protein by this method. Thus, from the percent inhibition obtained with a particular labeled antigen, the concentration of cartilage-specific core protein can be estimated in unknown samples. In the present set of experiments, equivalent amounts of core protein (approximately 1 μg per 1×10^7 cells) were measured in both β-xyloside-treated and untreated cells and represented 1-2% of the total protein in these cultures.

DISCUSSION

It has been possible to develop a radioimmune assay that is reasonably specific for the protein portion of cartilage proteoglycan and to apply this method to measure the synthesis of core protein under conditions in which the levels of CSPG synthesis are reduced. The results indicate that the observed decrease in CSPG is not caused by a reduction in synthesis of core protein since similar amounts of core protein were measured in β-xyloside-treated and untreated cells. Most likely an inhibition of polysaccharide chain addition occurs at a later post-translational step of synthesis. Perhaps the artificial β-xylosides effectively compete with the natural xylosylated-core protein in the first galactosyltransferase reaction.

As previously indicated, production of CSPG is dependent on the prior synthesis of core protein. However, it may be suggested from these data that

synthesis of core protein is not influenced by changes at later steps of CSPG production, since neither increased synthesis of CS chains nor decreased synthesis of CSPG alters the level of core protein production.

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