Effects of dideoxyforskolin on proteoglycan synthesis and structure in embryonic chick chondrocyte cultures

Lie-Min Hu, Stephen F. Kemp, Chun-Fu Peng, M. Joycelyn Elders and W. Grady Smith

Departments of Biochemistry and Molecular Biology and Pediatrics, University of Arkansas for Medical Sciences, 4301 W. Markham, Little Rock, AR 72205, USA

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1,9-Dideoxyforskolin inhibits proteoglycan synthesis and xyloside-initiated glycosaminoglycan (GAG) synthesis in chick embryo chondrocytes. Dideoxyforskolin does not affect the length of xyloside-initiated GAG chains secreted into the medium but chains from the dense proteoglycan secreted into the medium appear slightly longer. Incorporation of labeled serine into the dense proteoglycan and subsequent digestion with Pronase revealed a dramatic decrease in percent of total radioactivity associated with GAG chains in the proteoglycan from cultures treated with forskolin or dideoxyforskolin. These observations suggest that these diterpenes have a specific inhibitory effect on chain initiation reactions and thus may be useful tools in the study of proteoglycan synthesis and processing.

Forskolin; Dideoxyforskolin; Cyclic adenosine monophosphate; Chondrocyte; Proteoglycan synthesis

1. INTRODUCTION

We have recently shown that forskolin inhibits proteoglycan synthesis as determined by both sulfate and glucosamine incorporation and results in shorter glycosaminoglycan (GAG) chains when initiation is on an artificial B,D-xyloside acceptor [1,2]. These effects occur in a concentration range well above that required to elicit a maximum response in elevation of cAMP and are not a consequence of impaired cellular energy metabolism [1]. We therefore thought that related labdane diterpenes might prove to be inhibitors of specific steps in proteoglycan synthesis and that a non-cAMP agonistic analog would more clearly separate the direct effects of these compounds from indirect, cAMP-mediated effects. Compounds inhibiting specific steps in the biosynthesis of proteoglycans would facilitate our understanding of proteoglycan synthesis as has recently been accomplished with specific inhibitors of glycoprotein processing [3].

2. MATERIALS AND METHODS

2.1. *Cell culture conditions*

Chondrocyte cultures were established from the sternae of 14-day chick embryos in Ham's F-12 (GIBCO Laboratories, Grand Island, NY) medium and incubated as previously described [1].

2.2. *Incorporation of sulfur-35 sulfate into macromolecules*

Four-day-old cultures were incubated in Ham's F-12 medium with $^{35}SO_4^{-2}$, (amount specified in figure legends), ICN Biomedicals, Irvine, CA) and insulin-like growth factor I (IGF-I), (20 ng/ml, Incell, Milwaukee, WI) and various concentrations of forskolin, (Sigma, St. Louis, MO), or 1,9-dideoxyforskolin (Calbiochem, San Diego, CA), for 8 h as previously described [1,2]. In some cases 4-methylumbelliferyl-B,D-xyloside (0.16 mM) was also present. After incubation the total culture (cells plus medium) was extracted with 2 M guanidine-1% Zwittergent overnight at 4°C in the presence of protease inhibitors [4]. After exhaustive dialysis against 0.1 M NaCl-1 mM Na_2SO_4, the non-dialyzable radioactivity remaining in the dialysis bag was quantitated by liquid scintillation spectroscopy. Experiments using [^{3}H]serine (Du Pont Co., NEN Res. Prod., Boston, MA) did not involve extraction, only medium was used.

Correspondence address: W.G. Smith, Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, 4301 W. Markham, Little Rock, AR 72205, USA

2.3. *Dissociative CsCl density gradient centrifugation*

The medium was dialyzed for 72 h against 4 liters of 0.1 M NaCl-1 mM Na_2SO_4 containing protease inhibitors at one-tenth the usual concentration at 4°C with 4 changes of dialyzing medium, lyophilized and dissolved in dissociative CsCl buffer (4 M guanidine HCl in 0.1 M Na acetate, pH 5.8, density 1.5 g/ml) containing protease inhibitors [5]. This solution was centrifuged for 48 h at 160,000 × *g* at 10°C and then fractionated into 4 parts from the most dense (bottom of the tube), to the least dense. Density of the D_1 fractions was >1.57 g/ml.

2.4. *Isolation of xyloside-initiated GAG chains from the medium*

Following incubation of cultures in the presence of 0.16 mM 4-methylumbelliferyl B,D-xyloside (MUDX, Sigma, St. Louis, MO), $^{35}SO_4^{-2}$ with and without dideoxyforskolin the medium was dialyzed, lyophilized and chromatographed on Sepharose CL-6B as previously described [2].

2.5. *Chromatography on Sepharose CL-2B*

Chromatography on CL-2B was carried out on a 0.9 × 90 cm column equilibrated with 4 M guanidine in 0.1 M Na acetate, pH 5.8.

2.6. *Measurement of protein*

Protein was measured by the dye binding procedure of Bradford [6].

2.7. *Alkaline borohydride elimination and reduction*

Proteoglycans from pooled fractions of CL-2B columns were

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dialyzed to remove guanidine and were treated with 1 M sodium borohydride in 0.05 N NaOH for 48 h at 45°C [7]. Excess borohydride was decomposed with acetic acid and the samples were concentrated by lyophilization prior to chromatography.

2.8. *Measurement of ATP, lactate and cAMP*

These compounds were measured as previously described [1].

2.9. *Pronase digestion*

After dialysis to remove guanidine, material to be digested was lyophilized and dissolved in 1 ml 50 mM Tris buffer, 1.5 mM $CaCl_2$, pH 7.2 and incubated with Pronase (1.5 mg) for 16 h at 37°C [8,9]. Pronase was added to the digest two additional times during the incubation.

3. RESULTS

3.1. *Effect of dideoxyforskolin on cellular metabolism*

Experiments similar to those previously reported [1] were conducted to evaluate the possible inhibitory effects of dideoxyforskolin on chondrocyte metabolism. After 8 h exposure to 100 μM dideoxyforskolin, cellular ATP was 92% of control and not different from control values. Likewise, lactate accumulation in the medium was 91% of control, a slight decrease, but not as great as observed with forskolin [1]. Similarly, the protein content of cultures was identical after 8 h exposure to dideoxyforskolin. Thus, like forskolin, this diterpene is not toxic to embryonic chondrocyte cell cultures. Further, 7 h exposure to this non-cAMP agonistic analog did not increase accumulation of cAMP.

3.2. *Effect of dideoxy forskolin on incorporation of sulfate into total culture macromolecules*

A dose-response relationship for the effect of dideoxyforskolin on incorporation of $^{35}SO_4^{-2}$ into total culture macromolecules is presented in Fig. 1. Inhibition of incorporation is apparent between 1 and 100 μM where inhibition reaches 75%. For comparative purposes, data from an identical experiment with forskolin are included. A direct comparison indicates that 50% inhibition of incorporation is obtained at a concentration of forskolin of about 80 μM whereas with dideoxyforskolin it occurs at about 30 μM. Thus, dideoxyforskolin is about 2.7 times as effective as an inhibitor as is forskolin. The results obtained in this experiment with forskolin agree with those previously published [1].

3.3. *Effect of dideoxyforskolin on the synthesis of sulfated GAG chains initiated on an artificial xyloside acceptor*

When the artificial acceptor 4-methyl umbelliferyl-B,D-xyloside is included in the medium incorporation is stimulated and this incorporation, too, is inhibited by dideoxyforskolin. In an experiment conducted simultaneously with the dideoxyforskolin experiment shown in Fig. 1, the B-xyloside stimulated incorporation of $^{35}SO_4^{-2}$ over two-fold (477,000 cpm/culture vs 1,116,000 cpm/culture) but in the presence of 100 μM

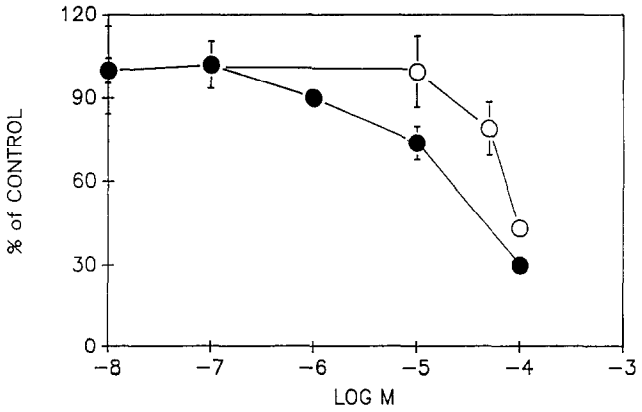

Fig. 1. Effect of forskolin and dideoxyforskolin on proteoglycan synthesis. The cultures were incubated 8 h in the presence of $^{35}SO_4^{-2}$ and the indicated concentrations of forskolin or dideoxyforskolin and total proteoglycans extracted with guanidine-zwittergent. The extracts were dialyzed exhaustively and their content of radioactivity determined. Sixteen μCi per culture (3 ml, 25 cm^2 flasks) were present in the forskolin experiment and 30 μCi per culture were present in the dideoxyforskolin experiment. Control values were 154,000 cpm/culture and 477,000 cpm/culture, respectively. Data are expressed as means ± SEM of 3 replicates. ○, forskolin; •, dideoxyforskolin.

dideoxyforskolin incorporation was reduced to 295,000 cpm. When the extracts from the cultures containing the xyloside were chromatographed on Sepharose CL-6B, a comparison of the radioactivity recovered in the GAG chain region (K_{av} = 0.2–0.8) from the chromatograms for control and treated cultures revealed that the inhibition of incorporation was 73% which agrees with the 75% inhibition noted before the samples were pooled and chromatographed. Additional similar experiments were conducted except that the sulfated, xyloside-initiated GAG chains were recovered from the medium rather than from the medium plus cells. Typical elution profiles are shown in Fig. 2. Extensive control experiments have established that in the absence of a xyloside acceptor, all of the macromolecular radioactivity from sulfate elutes at the void volume of these columns as expected for a large proteoglycan. These sulfated GAG chains chromatograph similarly to chains isolated from the medium of cultures exposed to forskolin except that the peak K_{av} value for chains from treated cultures is not larger than controls. (Forskolin results in chains with a smaller hydrodynamic radius [2].)

3.4. *Effect of dideoxyforskolin on synthesis of dense proteoglycans secreted into the medium*

In order to focus further studies on a more specific class of proteoglycans, we examined the dense proteoglycans secreted into the culture medium in the presence and absence of dideoxyforskolin by subjecting dialyzed, lyophilized culture medium to dissociative CsCl density gradient centrifugation [16]. Data

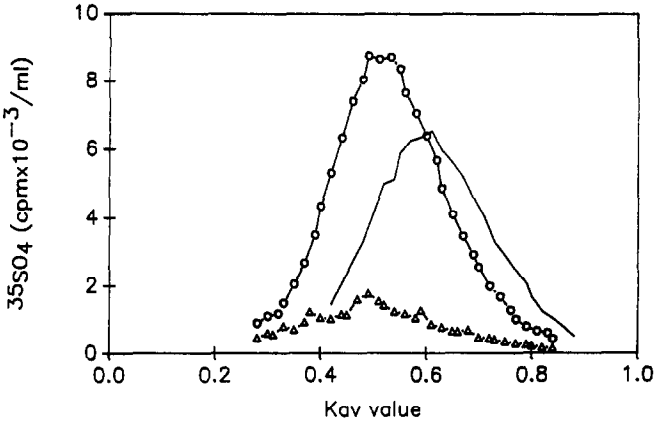

Fig. 2. Effect of dideoxyforskolin on synthesis and size of xyloside-initiated GAG chains. The figure displays the elution profiles for sulfated GAG chains initiated on a B-xyloside and chromatographed on Sepharose CL-6B. In each case, the material chromatographed represents 3 cultures. ○, control; △, dideoxyforskolin-treated (100 μM). The solid line represents data similar to those previously published [2] showing the elution position of chains formed in the presence of forskolin.

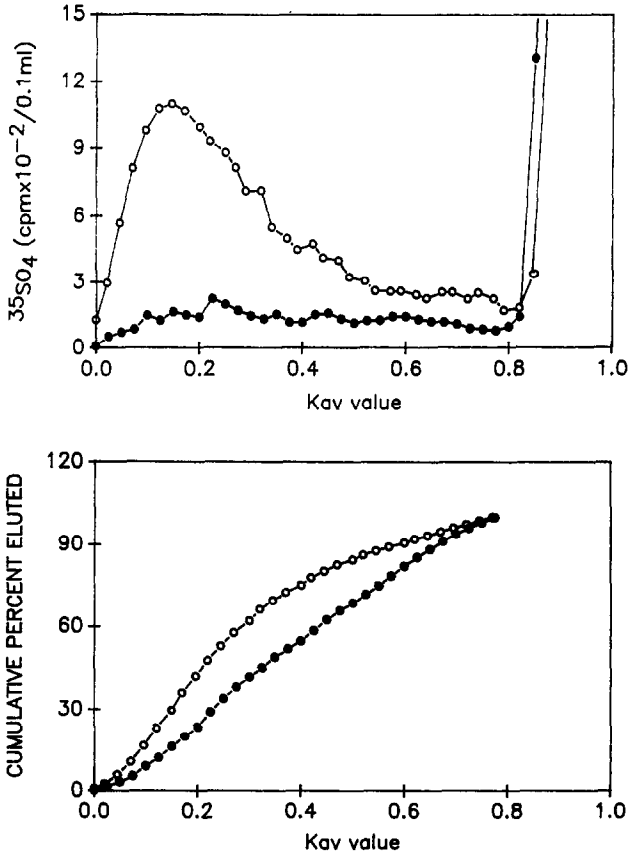

Fig. 3. Effect of dideoxyforskolin on synthesis and size of dense secreted proteoglycan. Cultures were incubated for 8 h in the presence of 100 μM forskolin and 4.3 μCi/ml of radioactive sulfate. The medium was dialyzed, lyophilized and fractionated according to density. The D_1 fraction from dissociative CsCl density gradient centrifugation was chromatographed on Sepharose CL-2B and the radioactivity of each fraction determined. Media from 3 cultures (7 ml, 75 cm^2 flasks) were pooled. ○, control; •, dideoxyforskolin. Data are presented two ways for clarity; in the top panel as conventional elution profiles (cpm/ml vs K_{av}) and in the bottom panel as percent of total radioactivity eluted between K_{av} = 0 and 0.8 vs the K_{av} value.

presented in Fig. 3 demonstrate that incorporation of $^{35}SO_4^{-2}$ into the most dense proteoglycans secreted into the medium (the D_1 fraction of CsCl dissociative density gradient centrifugations) is inhibited by dideoxyforskolin. It is evident from this figure that inhibition is most pronounced for the larger species eluting from the column at $K_{av} < 0.5$. In addition, the hydrodynamic radius of the proteoglycans from cultures treated with dideoxyforskolin tends to be slightly smaller than controls. This difference is most readily apparent in the bottom panel of Fig. 3 in which the cumulative percent of total macromolecular radioactivity eluted between K_{av} = 0 and 0.8 is plotted against the K_{av} value.

3.5. *Effect of dideoxyforskolin on length of proteoglycan GAG chains*

In order to determine the structural basis for the decreased hydrodynamic radius of the proteoglycans, the material chromatographing between K_{av} = 0 and 0.474 on the CL-2B columns was treated with alkaline borohydride to eliminate and reduce GAG chains. These chains were then chromatographed on Sepharose CL-6B to assess their relative hydrodynamic radius. The effect of the dideoxy analog on the length of GAG chains obtained from alkaline borohydride treatment of proteoglycan are shown in Fig. 4. It is obvious that chains are not shorter as a result of this treatment. In fact the treated cultures appear to produce a population of chains that are longer than chains from control cultures.

3.6. *Effect of the diterpenes on number of chains*

A likely explanation for the slightly smaller hydrodynamic radius of the intact proteoglycan in the face of slightly longer GAG chains is that there are fewer chains per core protein molecule in the proteoglycans synthesized in the presence of dideoxyforskolin. To test this hypothesis, we metabolically labeled the secreted proteoglycans with tritiated serine, isolated the D_1 fraction by density gradient centrifugation, chromatographed this on 2B as before and digested the proteoglycans eluting between K_{av} = 0 and 0.59 with Pronase. The digest was subsequently chromatographed on Sepharose CL-6B. GAG chains end-labeled with serine-^3H elute in the same position as the ^{35}S-sulfate labeled GAG chains. Lower molecular weight digestion products elute at or near the total column volume. Therefore, the percentage of tritium that chromatographs as a macromolecule in the GAG chain region is a measure of the relative chain number in comparing treated and control cultures. The results of chromatography of the Pronase digests are shown in

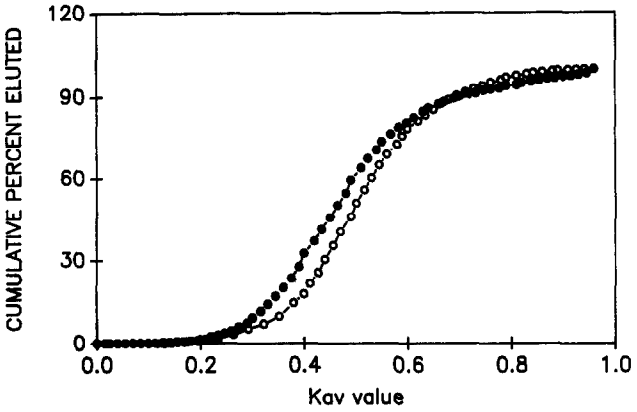

Fig. 4. Effect of dideoxyforskolin on size of GAG chains from dense proteoglycan. The material from the 2B column in Fig. 3 (K_{av} = 0–0.474) was treated with alkaline borohydride and chromatographed on Sepharose CL-6B as described in the text. ○, control; •, dideoxyforskolin.

Fig. 5. In the control, 50% of the radioactivity elutes at a K_{av} value smaller than 0.68, in forskolin-treated cultures the value is 0.94 and for dideoxyforskolin the point of 50% elution is reached at a K_{av} value of 0.98. There is, therefore, a considerable reduction in the fraction of serine attached to GAG chains in the two treated groups, consistent with a reduction in the number of chains per core protein. Of 168,000 cpm chromatographed in the control column, 60% were recovered in the K_{av} = 0.2–0.8 fractions. 71,000 cpm were chromatographed from the forskolin-treated group and 39% were recovered in these fractions. Similarly, from the dideoxyforskolin group, 21% of the 52,000 cpm chromatographed were recovered in the

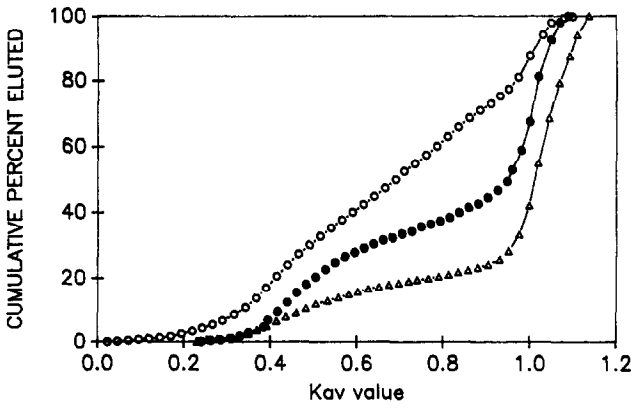

Fig. 5. Effect of forskolin and dideoxyforskolin on number of chains on large dense proteoglycan secreted into the medium. Large, dense proteoglycan was isolated from the medium of cultures metabolically labeled with [^{3}H]serine (11.7 μCi/ml for 8 h) by density gradient centrifugation and Sepharose CL-2B chromatography as described in the text. Proteoglycan between K_{av} = 0 and 0.59 was dialyzed and digested with Pronase. The Pronase digest was chromatographed on Sepharose CL-6B and the tritium measured. The data are expressed as cumulative % eluted, plotted vs K_{av}. Media from 4 cultures (7 ml, 75 cm^2 flasks) cultures were pooled for each group. ○, control; •, 100 μM forskolin; △, 100 μM dideoxyforskolin.

0.2–0.8 K_{av} fractions. These data indicate a reduction in chain number by forskolin of about 33% and by dideoxyforskolin of about 60%.

4. DISCUSSION

The results of this investigation together with other recently published results from our laboratory [1,2] clearly establish an inhibitory effect of the labdane diterpenes, the cAMP agonistic forskolin [10,11] and the non-agonistic, 1,9-dideoxyforskolin [11,12] on proteoglycan synthesis in embryonic chick sternum chondrocyte cultures. Thus proteoglycan synthesis is added to the increasing list of cAMP independent cellular effects of forskolin [13–19]. Both compounds are markedly inhibitory for sulfate incorporation, both result in a reduction in the number of chains per core protein molecule and both inhibit the synthesis of sulfated GAG chains initiated on an artificial B-xyloside.

The structural modifications in proteoglycans revealed by chromatographic analysis are more dramatic in the case of the dideoxy analog. A greater proportion of labeled proteoglycan molecules are present in the smaller molecular weight range with the analog than with forskolin. Also, the GAG chains from the proteoglycans of analog-treated cultures are slightly longer than controls even though synthesis is more severely inhibited. Similarly, dideoxyforskolin does not result in shorter xyloside-initiated GAG chains as does forskolin. These two observations are consistent with a slower overall rate of processing with the analog that somehow allows chains to reach normal or greater lengths as compared to those synthesized by control or forskolin-treated cultures. The idea of competition for available acceptor sites as one determinant of chain length has been previously suggested [20]. The decreased chain number in analog-treated cultures effectively decreases the concentration of acceptor sites for chain synthesis. Presumably a slower rate of chain synthesis could decrease the effect of acceptor concentration on chain length. Our results are therefore compatible with slightly longer chains even though the proteoglycan molecules themselves are slightly smaller as a consequence of the reduced number of chains.

The common denominators in chain initiation on core protein and artificial B-xylosides are the two galactosyl transferases and the first glucuronosyl transferase [21,22]. An inhibition of any of these enzymes, either directly or indirectly as a consequence of some particular insult to the Golgi complex would explain the results herein. The chains that are initiated are processed normally even in the dideoxyforskolin-treated cells. Unprocessed core protein molecules may be degraded or may be involved in a feedback control loop that blocks further synthesis. In any case, they would not be detected by the methods employed in this study and it is

not surprising that inhibition of serine incorporation is roughly similar to inhibition of sulfate incorporation. All our observations are consistent with the hypothesis that these diterpenes inhibit one or more reactions of chain initiation common to both proteoglycan-GAG chains and xyloside-GAG chains. It appears that dideoxyforskolin and similar related compounds may prove to be useful tools for furthering our understanding of proteoglycan synthesis and processing.

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