ABSTRACT

The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) rapidly blocks the terminal differentiation of normal cultured chondroblasts. In TPA, the chondroblasts lose their characteristic polygonal morphology and initiate the synthesis of an atypical type IV sulfated proteoglycan. This atypical proteoglycan has lower molecular size, shorter polysaccharide chains, and different 4S-disaccharide:6S-disaccharide ratio in comparison with type IV sulfated proteoglycan from untreated chondroblasts. When removed from TPA, the cells reacquire their characteristic polygonal morphology and reinitiate the synthesis of their typical cartilage-characteristic type IV sulfated proteoglycan. TPA has no readily detectable effect on the type III sulfated proteoglycan synthesized by fibroblasts.

INTRODUCTION

The cocarcinogen TPA has been shown to affect the differentiation of a variety of normal and transformed cells. For example, TPA inhibits myogenesis at 2 distinct stages by blocking, reversibly, (a) the fusion of mononucleated myoblasts into myotubes (3) and (b) the assembly of the muscle-specific contractile proteins that form the striated myofibrils (23). On the other hand, although TPA blocks the induction of hemoglobin synthesis in many strains of Friend erythroleukemic cells (5, 6, 21), it induces hemoglobin synthesis in Rauscher viral-transformed erythroblasts (16). TPA blocks the differentiation of the gut and skeletal cells in sea urchins (2). The cocarcinogen also delays the formation of adipocytes in populations of 3T3 cells (5), while inhibiting the differentiation of neuroblastoma cells (12).

Chondroblasts reared in TPA promptly (a) adhere to the substrate, (b) assume a fibroblast-like morphology, (c) cease synthesizing their cartilage-characteristic type IV sulfated proteoglycan, (d) cease synthesizing their cartilage-characteristic type II collagen chains, and (e) replicate more frequently (14, 19). Within 24 hr after the TPA is withdrawn, the chondroblasts reacquire their epithelioid polygonal morphology and reinitiate the synthesis of their characteristic type IV sulfated proteoglycan and type II collagen chains.

This report focuses on a detailed analysis of the type of sulfated proteoglycans synthesized by chondroblasts when reared in TPA. As long as the cells are grown in TPA, they synthesize an atypical glycosaminoglycan. When removed from TPA, they reinitiate the synthesis of their cartilage-characteristic glycosaminoglycan.

MATERIALS AND METHODS

Culture media were from Grand Island Biological Co. (Grand Island, N. Y.). Sepharose 2B and 6B were from Pharmacia Fine Chemicals (Piscataway, N. J.). Papain, chondroitinase ABC, and hyaluronic acid were from Sigma Chemical Co. (St. Louis, Mo.), and [35S]sulfate and Aquasol were from New England Nuclear (Boston, Mass.). Pure chondroblast cultures and dermal fibroblast cultures were prepared as described and grown in minimum essential medium containing 10% fetal calf serum (18). When specified, TPA was added at a final concentration of 5 x 10^-8 M. Cells were labeled with [35S]sulfate (10 to 30 μCi/ml) for 6 hr in complete medium. Proteoglycans were extracted from both cell layers and culture medium with 4 M guanidine solution as described previously (18) and were separated on linear sucrose gradients (17). Fractions corresponding to specific types of sulfated proteoglycans were pooled, dialyzed, and lyophilized in the presence of carrier bovine nasal cartilage proteoglycans (kindly provided by Dr. V. Hascall, National Institute of Dental Research, NIH, Bethesda, Md.). Sepharose columns were prepared and eluted with 0.5 M sodium acetate, pH 7.0. When assayed for hyaluronic acid binding, 0.5 mg of lyophilized sample was mixed with 1 to 2% (w/w) of human umbilical hyaluronic acid, kept overnight at 4°, and applied then to Sepharose 2B columns (7). Under these experimental conditions, only the cartilage-characteristic proteoglycans bound hyaluronic acid. We did not determine whether, under different experimental conditions, the sulfated proteoglycans isolated from fibroblasts or other cell types would bind hyaluronic acid, as reported for bovine aorta proteoglycans (15). When assayed for polysaccharide chain length, 0.5 mg of lyophilized sample was digested with 100 μg of papain (twice crystalized) in 0.1 M sodium acetate buffer, pH 7.0–5 mM cysteine for 4 to 5 hr at 65°; the mixture was then applied to a Sepharose 6B column (0.7 x 105 cm). Effluent fractions were assayed for hexuronic acid with an automated carbazole procedure (8) and for radioactivity by counting aliquots in Aquasol. Eighty-five % of total applied radioactivity was recovered from Sepharose columns. Chondroitinase ABC digestion was performed as described (22). DNA content was determined with a fluorometric assay (9).

The inactive analogs of TPA, phorbol diacetate and phorbol, at concentrations of 10^-7 M had no effect on these cultures and so will not be discussed further.

RESULTS

Cell Cultures. The effects of TPA on the morphology of cultured chondroblasts and fibroblasts have been described (14). Briefly, by 48 hr of treatment, 100% of the treated chondroblasts lose their epithelioid arrangement and display...
long fine pseudopodial processes. TPA-treated chondroblasts become multilayered and achieve cell densities that are 6 times higher than those in the control cells. The effects of TPA are totally reversible if the cells are treated with TPA for up to 10 days. Fibroblasts treated with TPA are also induced to form long fine processes but, with this cell type, TPA does not act as a mitogen, nor does it appear to alter the type of sulfated proteoglycan that the fibroblasts synthesize (14).

For the present study, pure populations of "floating" chick embryo chondroblasts were harvested from the medium of primary cultures (18). The chondroblasts were then transferred to secondary cultures. After 24 hr, approximately 30% of the cells reattached to the plastic substrate, while the others remained floating in the medium. The number of floating chondroblasts gradually dwindled and, by Day 8, over 90% had settled on the substrate as polygonal cells. In contrast, when chondroblasts for secondary cultures were plated into medium containing TPA, virtually 100% of the cells attached to the plastic substrate within 15 hr and, by 36 hr, all of them had acquired a fibroblastic morphology. Clearly, TPA greatly enhances the rate at which chondroblasts adhere to the collagen substrate.

\[ \text{[35S]Sulfate Incorporation} \]

TPA rapidly inhibited the incorporation of \([35S]\) sulfate into macromolecules synthesized by cultured secondary chondroblasts. Table 1 shows that, within 48 hr of treatment with TPA, the level of incorporation was only 10% of that of control secondary chondroblasts (mixed floating and substrate-bound populations). It is interesting to note that in control cultures the level of \([35S]\) sulfate incorporation decreased with the age of the culture, whereas in TPA-treated cultures the level showed no further change upon prolonged TPA treatment.

As also shown in Table 1, if treated for 5 days with TPA and then "reversed" by subculturing for 5 days in normal medium, chondroblasts regained a level of \([35S]\) sulfate incorporation which is similar to that of control tertiary chondroblasts or control "old" secondary chondroblasts.

The data reported in Table 1 demonstrate that TPA did not significantly alter the level of \([35S]\) sulfate incorporation into macromolecules synthesized by cultured dermal fibroblasts. The level of incorporation in TPA-treated chondroblasts is strikingly similar to that of control fibroblasts or TPA-treated fibroblasts.

**Chemichophysical Characteristics of the Sulfated Proteoglycans.** When treated with TPA, secondary chondroblasts stop the synthesis of their characteristic type IV sulfated proteoglycan. Instead, these cells synthesize a sulfated proteoglycan which on sucrose gradients appears to be similar to the type III proteoglycan synthesized by cultured fibroblasts (14).

To learn more of the type III proteoglycan induced by treating chondroblasts with TPA, secondary cultures were treated with TPA for 5 days. Part of these cells were labeled with \([35S]\) sulfate, whereas sister cells were subcultured and grown for 5 days in normal medium. Control chondroblasts and dermal fibroblasts were prepared and grown simultaneously.

After labeling with \([35S]\) sulfate, proteoglycans were extracted from the cultured cells and separated on sucrose gradients. After separation on sucrose gradients, the labeled proteoglycans were isolated, mixed with carrier bovine nasal proteoglycans, and then lyophilized. The following lyophilized samples were prepared: (a) type IV sulfated proteoglycan monomers from control chondroblasts; (b) type III-like sulfated proteoglycan monomers from TPA-treated chondroblasts; (c) type IV monomers from "TPA-reversed" chondroblasts; and (d) type III monomers from dermal fibroblasts.

We first tested the ability of these samples to interact with hyaluronic acid. Aliquots were dissolved in 0.5 m sodium acetate buffer, pH 7.0, and then applied to a Sepharose 2B column. Carrier proteoglycans were in vast excess, and their elution profiles were recorded by the uronic acid analysis, whereas experimental proteoglycans were followed by their incorporated \[^{35}S\] radioactivity.

As shown in Chart 1, A, C, and E, in the absence of added hyaluronic acid, all the samples tested were included in the Sepharose 2B column. The average molecular size of all the samples was smaller than that of the carrier proteoglycans (Kav = 0.19). However, when incubated with hyaluronic acid and then run in the column, the type IV sulfated proteoglycans synthesized by both control and TPA-reversed chondroblasts were excluded from the column (Chart 1, B and F), whereas the proteoglycans from TPA-treated chondroblasts (Chart 1D) and from dermal fibroblasts (not shown) remained included in the column. These data demonstrate that only the sulfated proteoglycan monomers synthesized by control and "TPA-reversed" chondroblasts are able to interact with hyaluronic acid under these experimental conditions.

We then determined the size distribution of the chondroitin sulfate chains present in the isolated proteoglycans described above. Samples were digested with papain, and the digests were filtered on Sepharose 6B columns. As shown in Chart 2C, the size of the chondroitin sulfate chains of the type IV macromolecules from control chondroblasts are shorter than those present in bovine nasal carrier (Kav = 0.56 and 0.52, respectively). In contrast, the chain size of the type III-like sulfated proteoglycans synthesized by TPA-treated chondroblasts (Kav = 0.31) is longer than that of control chondroblasts (Chart 2B) and is very similar to the chain size of the fibroblast-type III proteoglycans (Kav = 0.28) (Chart 2A). TPA treatment did not affect the chain size in cultured fibroblasts.

We then determined the relative percentages of 6S- and 4S-disaccharides in the isolated proteoglycans. These macromolecules were digested with chondroitinase ABC, and the digest was chromatographed on Whatman paper. Table 2 shows that the type III-like macromolecules isolated from TPA-treated chondroblasts have a relative ratio of 6S- and 4S-disaccharides.
TPA and Proteoglycan Synthesis

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Chart 1. Sepharose 2B elution profile of the isolated 35S-labeled proteoglycans (—) before and after interaction with hyaluronic acid. Uronic acid assay refers almost exclusively to the carrier proteoglycans (—). V0: excluded column volume; Vt, total column volume. A, control chondroblasts; B, control chondroblasts preincubated with hyaluronic acid; C, TPA-treated chondroblasts; D, TPA-treated chondroblasts preincubated with hyaluronic acid; E, "TPA-reversed" chondroblasts; F, "TPA-reversed" chondroblasts preincubated with hyaluronic acid.

which is different from that found in control chondroblasts and in control or TPA-treated dermal fibroblasts. The table shows also that the TPA effect on chondroblasts is totally reversible and that TPA does not affect significantly the 6S-disaccharide:4S-disaccharide ratio in dermal fibroblasts. In addition, Table 2 shows that over 90% of the isolated macromolecules are digestable by chondroitinase.

DISCUSSION

From the data reported here, we conclude that the main proteoglycan synthesized by TPA-treated chondroblasts exhibits changes in its monomer size, polysaccharide chain length, and hyaluronic acid-binding ability when compared to the type IV sulfated proteoglycan synthesized by control chondroblasts. It is also evident, however, that the type III-like proteoglycan synthesized by TPA-treated chondroblasts exhibits a unique 6S-disaccharide:4S-disaccharide ratio. The latter finding suggests that, as long as chondroblasts are in TPA, they synthesize an atypical polysaccharide chain. It is worth stressing that data to be detailed elsewhere indicate that TPA-treated chondroblasts shift from synthesizing cartilage-characteristic type II collagen chains to atypical fibroblast-like type I collagen chains. The data on the changes in the sulfated proteoglycans and those on the shift in collagen chain types suggest that the TPA-treated chondroblasts approach the fibroblast phenotype.

On the other hand, TPA does not appear to affect proteoglycan synthesis, both quantitatively and qualitatively, in cultured dermal fibroblasts, according to the criteria mentioned above. These differential responses of chondroblasts and fibroblasts to TPA are not understood. However, there is an immense literature suggesting that, under a variety of experimental conditions, fibroblasts can be induced to replicate and yield chondroblasts and, alternatively, that chondroblasts, following considerable replication, yield fibroblast-like cells (1, 11, 13, 20, 24, 25). These experiments with TPA confirm earlier reports that the chondrogenic phenotype can be shifted by exogenous agents following cell replication to approximate that of fibroblast-like cells (10).

It has been suggested that "authentic" fibroblasts isolated

Table 2

<table>
<thead>
<tr>
<th>Cell type</th>
<th>D-6S</th>
<th>D-4S</th>
<th>Undigested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control chondroblasts</td>
<td>63</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>&quot;TPA-reversed&quot; chondroblasts</td>
<td>61</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td>TPA-treated chondroblasts</td>
<td>53</td>
<td>41</td>
<td>6</td>
</tr>
<tr>
<td>Control fibroblasts</td>
<td>81</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>TPA-treated fibroblasts</td>
<td>78</td>
<td>14</td>
<td>7</td>
</tr>
</tbody>
</table>

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from different tissues synthesize different sets of glycosaminoglycans (4). It will be of interest to determine whether TPA can in fact alter the synthetic program of chondroblasts so as to approach the program of some type of authentic in vivo fibroblasts. Lastly, it also will be of interest if the atypical glycosaminoglycans synthesized by chondroblasts in TPA are responsible for the rapid changes that they display in their cell surface and cell morphology (14, 19).

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REFERENCES

12-O-Tetradecanoylphorbol-13-acetate-induced Changes in Sulfated Proteoglycan Synthesis in Cultured Chondroblasts

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