Chick embryo skin fibroblasts and vertebral chondroblasts were infected with a temperature-sensitive mutant of Rous sarcoma virus, LA24A, and were grown at permissive (36°C) and nonpermissive (41°C) temperatures. During exponential growth, infected and parallel uninfected cultures were labeled with D-[3H] glucosamine, and newly synthesized glycosaminoglycans were identified by anion exchange chromatography and by selective enzymatic and chemical degradations. Control fibroblasts synthesized low levels of hyaluronic acid (HA), and dermatan sulfate (DS), moderate levels of heparan sulfate (HS), and high levels of chondroitin sulfate (CS). In contrast, control chondroblasts synthesized very low levels of HA and DS, no HS, and very high levels of CS. Following transformation and growth at 36°C, both cell types showed a dramatic increase in HA synthesis and a significant decrease in CS synthesis. In addition, transformed chondroblasts initiated the synthesis of HS and increased their synthesis of DS to levels that matched those of transformed fibroblasts. The CS chains synthesized by control chondroblasts were partially undersulfated, while those synthesized by both normal and transformed fibroblasts were fully sulfated. Upon transformation, chondroblasts grown at 36°C initiated the synthesis of fully sulfated CS chains. Most of the above biosynthetic alterations were completely reversed when infected cells were grown at 41°C, indicating that they were dependent on the transforming gene product of LA24A. Clearly, the profound differences that distinguish normal fibroblasts from normal chondroblasts are lost upon transformation, and these two types of terminally differentiated cells converge toward a common, though not identical, biosynthetic program for glycosaminoglycans.

The role of extracellular matrix and cell surface glycosaminoglycans in the establishment of the malignant phenotype remains unclear. Numerous workers have utilized cell cultures and cell transformation by both DNA and RNA tumor viruses to study the alterations in glycosaminoglycan synthesis and structure which are associated with cell transformation. Early studies on chick embryo cells transformed by RSV demonstrated an increase in HA synthesis (1-4). However, other studies on mammalian cells showed either an increase in HA synthesis in Syrian hamster cells and monkey kidney cells transformed by HSV and SV40 (4) or a decrease in HA synthesis in mouse 3T3 cells and human fibroblasts transformed by SV40 or poloma virus (6).

A similar equivocal picture stems from studies on sulfated glycosaminoglycans, recently reviewed by Kramer (7). For example, hamster and monkey cells transformed by SV40 or HSV were found to produce a significantly higher proportion of HS (5); in contrast, in mouse 3T3 cell cultures transformed by SV40, the synthesis of HS was unaffected (8).

Additional controversy comes from recent studies in which cell growth rate and cell density rather than viral transformation were found to affect the pattern of glycosaminoglycan synthesis (9-11).

It is evident, therefore, that more carefully defined experimental systems are required to examine the role of glycosaminoglycans in the establishment of the malignant phenotype. All the studies mentioned above have utilized either heterogeneous populations of primary cells or established cell lines. In most cases, cells were grown under very different culture conditions. In this study, we have isolated two distinct well characterized types of terminally differentiated cells from chick embryos, vertebral chondroblasts, and skin fibroblasts. The former have been extensively studied and can be grown in vitro as pure populations (12-14). The latter exhibit in vitro both morphological and biochemical characteristics that distinguish them from other types of cells, including other types of fibroblasts (15, 16). Chondroblasts and fibroblasts were grown under identical culture conditions and were infected with the same ts mutant of RSV, LA24A. We have compared the effects of LA24A-induced transformation on the major classes of sulfated and unsulfated glycosaminoglycans, both those associated with the cell layer and those released into the culture medium. This biochemical comparison has allowed us to examine the question of whether, upon transformation, skin fibroblasts and vertebral chondroblasts in vitro express the identical transformed phenotype or whether each cell type expresses its own unique transformed phenotype.

The abbreviations used are: RSV, Rous sarcoma virus; HA, hyaluronic acid; HS, heparan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; ADi-OS, 2-acetamido-2-deoxy-3-O-2-acetamido-2-deoxy-3-O-(α-D-gluco-4-enepyranosyluronic acid)-O-sulfo-D-galactose; ADi-4S, 2-acetamido-2-deoxy-3-O-β-D-gluco-4-enepyranosyluronic acid)-O-sulfo-D-galactose; ADi-4S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; HSV, Herpes simplex virus; CAGs, glycosaminoglycans.
The data in this paper clearly show both qualitative and quantitative differences in the biosynthetic program for sulfated and unsulfated glycosaminoglycans in skin fibroblasts as compared to vertebral chondroblasts. However, upon infection with a ts mutant of RSV and growth at the permissive temperature for transformation, both types of transformed cells express remarkably similar, though not identical, patterns of glycosaminoglycan synthesis. Since the transformed cells reverted to the parental phenotype and synthesized the parental glycosaminoglycans following a shift to nonpermissive temperature, these effects appear to be due to the direct or indirect action of pp60^c/s, the RSV transformation gene product, on the regulatory mechanisms that control glycosaminoglycan synthesis.

HA synthesis increased 10-fold in fibroblasts and 40-fold in chondroblasts following transformation. This observation is in agreement with many previous studies using both RNA and DNA tumor virus-transformed cells of avian and mammalian origin (3-5, 20-22). However, some authors have reported exceptions to this increase in HA (6, 23). Both control fibroblasts and transformed fibroblasts shed about 70% of their newly synthesized HA into the culture medium. Normal chondroblasts synthesized only low levels of HA and shed only 40-50% of it into the medium; however, following cell transformation, the amount shed increased to almost 90%. This increased shedding of HA following transformation of chondroblasts is in agreement with the data reported by Mikuni-Takagaki and Toole (24) using wild type RSV. Following a shift to nonpermissive temperature, both infected fibroblasts and chondroblasts exhibited a large decrease in HA synthesis; however, the residual amount synthesized exceeded control values. The majority of the excess HA synthesized by both infected cell types appeared to be shed into the medium and not retained in the cell-associated extracellular matrix. Thus, increased shedding of HA in the medium in infected chondroblasts is not a reversible phenomenon. We do not have a definitive explanation for this phenomenon. It may be simply caused by the excess synthesis of HA, it may also be due to the abnormal presence of HS in these infected chondroblasts. Conceivably, HS might mask or compete for cell surface binding sites or interfere electrostatically or sterically with HA binding.

Transformation of chondroblasts and fibroblasts by LA24A resulted in a sharp decrease in total CS synthesis, 6.6-fold and 2.8-fold, respectively. Similar decreases in CS synthesis have been reported for SV40-transformed human skin fibroblasts (20) and for RSV-transformed chondroblasts (21). The sharp but incomplete inhibition of CS synthesis in transformed chondroblasts reported here confirms our earlier report on the suppression of the synthesis of the chondroblast-specific Type IV proteoglycan following transformation by ts RSV (13).

The synthesis of Type IV sulfated proteoglycan was replaced by the synthesis of low levels of a smaller CS-rich proteoglycan which migrated with the fibroblast characteristic Type III proteoglycan on sucrose gradients. The alterations in CS structure following transformation of chondroblasts observed in this study support our hypothesis that the Type III and Type IV sulfated proteoglycans are structurally distinct and may represent distinct gene products.

The convergence in phenotype following transformation of chondroblasts and fibroblasts is also indicated by the increase in DS to achieve similar final relative levels of 10-15% (Table III). Interestingly, an increase of DS synthesis has been shown to accompany dedifferentiation of human chondrocytes in culture (41).

Chondroblast CS was found to be undersulfated as revealed by elution at lower ionic strength and by the finding that about 7-13% of its disaccharides released by chondroitinase AC were unsulfated. Since fibroblast CS chains were found to be fully sulfated, the undersulfation of chondroblast CS chains is consistent with the hypothesis that sulfation is an "all or nothing" event and does not occur by a random process (25, 26). Some other hypotheses may be considered. 1) The sulfation machinery in normal chondroblasts is structurally different from that of fibroblasts and is only capable of catalyzing partial sulfation of CS chains. 2) The CS chains in chondroblast proteoglycans are not fully accessible for sulfation. 3) Sulfation is, at least in part, a random event, and its efficiency depends upon the rate and/or amount of CS chains synthesized. Thus, the level of CS synthesis in normal chondroblasts may exceed the capacity of the sulfation enzymes to sulfate all the possible sites. These hypotheses may explain why the reduced CS synthesis and/or different proteoglycan type produced by transformed chondroblasts result in increased CS sulfation. In this connection, it is noteworthy that an inverse relationship has already been demonstrated between the length of CS chains and their rate of synthesis in cultures of Swarm rat chondrosarcoma cells (27, 28).

Our data show that HS constitutes a major class of glycosaminoglycans in normal fibroblasts, but it does not appear to be synthesized by normal chondroblasts. Most of the HS synthesized by fibroblasts was found to be cell layer-associated, in agreement with the current view that HS proteoglycans are plasma membrane components (29). HS present in the culture medium may thus represent a catabolic form. Upon viral transformation of fibroblasts, HS synthesis was reduced over 10-fold. This is at variance with several earlier reports in which other cell systems were used (5, 8, 20, 30, 31). HS was absent in the medium of transformed fibroblasts, indicating that either HS was no longer shed but was metabolized intracellularly or HS was released but rapidly degraded. These effects of viral transformation on both synthesis and metabolism of HS were reversed upon growth of infected fibroblasts at 41 °C.

The absence of HS in normal chondroblasts is consistent with our current knowledge of the glycosaminoglycan composition of the two major cartilage proteoglycans (14, 22-25). In contrast, Kimata et al. (36) have reported that small amounts of HS were synthesized by organ cultured tibias and femurs isolated from chick embryos; however, these authors did not eliminate the possibility that the cellular source of HS synthesis was the perichondrial tissue which cannot be removed completely by microscopic dissection.

The synthesis of HS by transformed chondroblasts at a level comparable to that of transformed fibroblasts was unexpected. These chains eluted at lower ionic strength than fibroblast HS chains, indicating that they were undersulfated. Undersulfation of HS in transformed cells has been previously

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**DISCUSSION**

The absence of HS in normal chondroblasts is consistent with our current knowledge of the glycosaminoglycan composition of the two major cartilage proteoglycans (14, 22-25). In contrast, Kimata et al. (36) have reported that small amounts of HS were synthesized by organ cultured tibias and femurs isolated from chick embryos; however, these authors did not eliminate the possibility that the cellular source of HS synthesis was the perichondrial tissue which cannot be removed completely by microscopic dissection.

The synthesis of HS by transformed chondroblasts at a level comparable to that of transformed fibroblasts was unexpected. These chains eluted at lower ionic strength than fibroblast HS chains, indicating that they were undersulfated. Undersulfation of HS in transformed cells has been previously...
documented (37–39). The biosynthesis of HS requires a battery of enzymes including those involved in macromolecular processing (40). More work will be required to determine whether the newly acquired ability of transformed chondroblasts to synthesize HS depends on the activation of the synthesis of HS core protein(s) or on the activation of the enzymes for the synthesis of HS chains.

The continued synthesis of HS in infected chondroblasts following a shift to nonpermissive temperature was unexpected. While this may be due to the "leakiness" of the LA24A transforming product, the higher synthesis of HS in infected chondroblasts at 41 °C makes this explanation unlikely. Thus, irreversible as well as reversible effects may occur following RSV transformation of chondroblasts.

The abnormal presence of HS in infected chondroblasts at 41 °C may influence their behavior and function, particularly plasma membrane functions. We have suggested above that the presence of HS in these cells may directly or indirectly lower the proportion of HA which is deposited into the cell-associated extracellular matrix. More work on these cells and on normal chondroblasts may elucidate other roles for plasma membrane HS proteoglycans.

In conclusion, most of the profound differences in glycosaminoglycans that distinguish normal fibroblasts from normal chondroblasts are lost following transformation by ts LA24A and growth at permissive temperature. The strikingly similar, though not identical, phenotype that the two types of transformed cells express in culture is thus characterized by very high levels of HA, moderate levels of CS, and low levels of HS and DS. In this respect, the most dramatic effect of LA24A transformation is the induction of the synthesis of HS in transformed chondroblasts to levels that match those of fibroblasts.6 The two cell types used in this study have similar embryological origin in that they both derive from somites. Their convergence to a similar phenotype following transformation by LA24A may be directed by their common embryological origin. It will be most illuminating to determine whether cells with different embryological origin such as the ectodermally derived pigment cells (42) show a similar or a divergent transformed phenotype.

While the transformed fibroblasts and chondroblasts converge to a common phenotype, some significant differences are still reflected in their pattern of glycosaminoglycan synthesis, as documented above. Moreover, the majority of the changes in glycosaminoglycans induced by viral transformation is reversed upon growth at nonpermissive temperature. Therefore, viral transformation does not cancel the original terminal phenotypic program in fibroblasts and chondroblasts, but temporarily blocks its expression. The nonreversible induction of HS synthesis in transformed chondroblasts demonstrates also that the genetic program of terminally differentiated cells can be qualitatively and irreversibly altered by tumor viruses.

We have previously shown that the thymidine analogue 5′-deoxyuridine and the tumor promoter phorbol-12-myristate-13-acetate also alter the biosynthesis of proteoglycans in cultured chondroblasts (43–45). It will be interesting to compare a more detailed analysis of glycosaminoglycan synthesis in chondroblasts treated with these agents with the data presented here. This approach may clarify whether there is one or several ways in which the biosynthetic program for glycosaminoglycans may be altered in terminally differentiated chondroblasts.

REFERENCES


* Experiments are under way to clarify whether the specific activity of the various glycosaminoglycan classes synthesized by the normal versus transformed cells used in this study are similar or dissimilar.

For a discussion of this issue, see Ref. 20.
Glycosaminoglycans in Transformed Fibroblasts and Chondroblasts


Glycosaminoglycans in Transformed Fibroblasts and Chondroblasts

Sodium Pudendal Nerve Tissue. Sodium Pudendal nerve tissue from the rat was minced and digested in 0.25% trypsin for 15 min at 37°C. After digestion, the tissue was washed with phosphate-buffered saline (PBS), and the cells were harvested by filtration through a 100-μm mesh. The cells were then centrifuged at 1,000 g for 10 min, and the supernatant was used for analysis.

Materials and Methods

Materials. - D-Gluconate, calcium chloride, disodium hydrogen phosphate, and sodium bicarbonate were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade. The saline solution used contained (in mM) NaCl 154, KCl 5, CaCl2 2, MgCl2 1, glucose 11, NaHCO3 25, and HEPES 20.

Cell Cultures. - The cells were grown in Falcon tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) in DMEM medium containing 10% fetal bovine serum. The medium was changed every 3 days, and the dishes were subcultured when the cells reached a confluence of 80-90%. The cells were maintained in a humidified incubator at 37°C and 5% CO2.

RESULTS AND DISCUSSION

Synthesis of GAGs. - The synthesis of GAGs in the transformed cells was determined by analysis of the glycosaminoglycan content of the cell cultures. The results are expressed as milligrams of GAG per gram of protein. The results shown in Table 1 indicate that the GAG content of the transformed cells was significantly higher than that of the control cells.

Table 1. GAG synthesis by normal and LPSA-treated cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Temperature</th>
<th>Cytosol</th>
<th>Nucleus</th>
<th>Total GAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control fibroblasts</td>
<td>36°C</td>
<td>1.600</td>
<td>1.600</td>
<td>3.200</td>
</tr>
<tr>
<td>LPSA fibroblasts</td>
<td>36°C</td>
<td>1.600</td>
<td>1.600</td>
<td>3.200</td>
</tr>
<tr>
<td>Control fibroblasts</td>
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<td>5.180</td>
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<tr>
<td>LPSA fibroblasts</td>
<td>41°C</td>
<td>2.590</td>
<td>2.590</td>
<td>5.180</td>
</tr>
</tbody>
</table>

Fig. 1. Arbinic exchange patterns of GAGs synthesized by normal and LPSA-treated cells. The GAGs were separated by gel filtration on Bio-Gel P-60 and were identified as HA, CS, and DS by their elution profiles. The results are expressed as milligrams of GAG per gram of protein.

Table 2. GAG synthesis by normal and LPSA-treated cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Temperature</th>
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<th>Total GAG</th>
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Table 3. GAG synthesis by normal and LPSA-treated cells

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Glycosaminoglycans in Transformed Fibroblasts and Chondroblasts


table ii

percentage of total radioactivity incorporated into gals

<table>
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<tr>
<th>cell type</th>
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<th>cs</th>
<th>4 h wash</th>
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<td>26.5</td>
<td>64.1</td>
</tr>
<tr>
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<td>11.0</td>
<td>5.5</td>
<td>80.9</td>
</tr>
<tr>
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<td>23.0</td>
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<td>28.1</td>
<td>61.6</td>
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<tr>
<td>ov-cot fibroblasts</td>
<td>medium</td>
<td>12.4</td>
<td>24.1</td>
<td>61.6</td>
</tr>
</tbody>
</table>

fig. 3

action exchange elution patterns of hyaluronidase-treated samples.

when transformed fibroblasts were shifted from 36% to 40%., the overall pattern of cell layer and medium gals synthesized by the latter transformed fibroblasts grown in 40% growth medium was compared with that of the cell layer from the 36% growth medium and the difference was not significant.

fig. 4

action exchange elution patterns of cell layer and medium gals synthesized by normal and transformed chondroblasts.

analysis of gals synthesized by normal and transformed chondroblasts -- when aliquots of cell layer and medium gals synthesized by normal and transformed chondroblasts grown in 36% or 40% were analyzed by gel-cellulose action exchange chromatography, all fractions of action exchange chromatographic analysis were pooled, lyophilized, and column fractionated with bacterial hyaluronidase and hyaluronidase. four fractions of action exchange chromatographic analysis were pooled, lyophilized, and column fractionated with bacterial hyaluronidase and hyaluronidase. in the cell layer samples, more than 99% of pool 1 radioactivity was sensitive to hyaluronidase and the remainder eluted in a single peak at fractions 33-36 (fig. 35). the latter peak was resistant to digestion by hyaluronidase but was sensitive to nitrous acid degradation. by these criteria, most of the gals in the cell layer samples were gals which were resistant to bacterial hyaluronidase but sensitive to nitrous acid degradation. the presence of these gals is consistent with a small amount of cell layer-associated hsa revealed by the above analysis.
Glycosaminoglycans in Transformed Fibroblasts and Chondroblasts

Fig. 6. Action exchange elution patterns of hyaluronidase-treated samples. Total cell layer DNA synthesized by A431-transformed chondroblasts grown at 37°C were fractionated by double-action chromatography as shown in Fig. 4. The fraction from the DNA peak (Fig. 6) nontransformed control chondroblasts and chondroblasts treated with DMSO (Fig. 6A). 32P and 3H are represented as indicated in Fig. 5a, b, c, and d, treated with hyaluronidase and re-chromatographed on action exchange columns. A: pool A; B: pools A and B.

As shown in Figs. 5g and 5h, the elution of DNA synthesized by transformed chondroblasts occurred at an overall strength higher than that of normal chondroblasts. DNA of transformed chondroblasts was eluted at a strength higher than 0.68 M NaCl, while the strength of DNA eluted at 0.6 M NaCl. These two lines are more similar to 3H NaCl. In addition, DNA synthesis was determined by the transformed chondroblasts at 0°C and reached around 0.7 M of the total cell layer DNA.

Most of the changes in DNA induced by 3HNaA transformation were reversed when the transformed chondroblasts were shifted to 37°C. The chromatographic behavior of cell layer-A and medium-A (Figs. 5g and 5h) revealed that the proportion of transformed chondroblasts at 37°C reached almost 100% of the total DNA (Table II). Interestingly, the elution position of DNA synthesized by transformed chondroblasts at 4°C was about fraction 70 and thus equal to that of normal chondroblasts DNA.

What bacterial hyaluronidase and chondroblast treatments were performed, they revealed the persistent synthesis of DNA, indicating that DNA synthesis was not altered following the shift from 4°C to 37°C (Table III). The data from the action chromatography (Fig. 6) were used to calculate the absolute amounts of radiolabeled incorporated into DNA (Table III). The transformed chondroblasts at 37°C incorporated about 10-fold more DNA per cell than that incorporated at 4°C and 2.5-fold upon the overall incorporation into DNA and 0.5-fold dropped, respectively, 10-fold and 2.5-fold.

The elution of DNA synthesized at 37°C, the incorporation of radioactive incorporated into DNA increased about 4-6-fold while that into 0.6 M NaCl decreased about 0.5-fold. The level of 0.6 M NaCl elution of untransformed chondroblasts, reached a proportion of about 7.5% (Table II). In the culture medium of transformed chondroblasts, the amount of radioactive incorporated into DNA increased over 100-60 fold while that recovered as DNA decreased about 10-fold. The differential sensitivity of DNA synthesis in transformed chondroblasts toward a common bisacetic acid is shown more clearly in Table I, which shows the remarkable decrease in DNA synthesis in transformed chondroblasts toward a common bisacetic acid.