# INDUCTION OF HYALURONIC ACID SYNTHESIS IN TERATOCARCINOMA STEM CELLS BY RETINOIC ACID

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Received 29 November 1979
Revised version received 16 January 1980

## 1. Introduction

Retinoids are known to reduce the occurrance of epithelial tumors [1,2], to maintain the differentiation of epithelial cells [3–5], to inhibit the growth of certain transformed cells [6–8], and to induce the differentiation of teratocarcinoma stem cells [9,10]. Although the metabolism of retinoic acid has been studied intensively [11], the biochemical reactions underlying these phenomena are not yet understood [12]. They may be mediated by binding proteins [13]. The biochemical alterations induced by retinoids are selective inhibition of chondroitin-sulfate and hyaluronic acid synthesis in chondrocytes [14], stimulation of haematoside synthesis [15] and retention of fibronectin on fibroblasts [15,16] and chondrocytes [17]. Moreover, retinoids are involved in the glycosylation through retinyl–phosphorylmonosaccharides [18]. Thus the action of retinoids in cell differentiation seems to be closely connected to its participation in carbohydrate metabolism. Differentiation of teratocarcinoma stem cells [9,10] is an excellent system to analyze the biochemical changes following retinoic acid administration. Differentiating embryonal carcinoma cells have already been shown to alter the pattern of their cell surface glycopeptides [19].

This report describes the dramatic induction of hyaluronic acid synthesis in teratocarcinoma stem cells stimulated by retinoic acid.

## 2. Materials and methods

### 2.1. *Cell growth and characterization of the glycosaminoglycans*

F9 cells were plated on 4 culture flasks (75 $cm^2$) without collagen coating [9] at $3 \times 10^3$ cells/$cm^2$ in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, supplemented with 2.5 $\mu$Ci/ml [$^3$H]glucosamine and 25 $\mu$Ci/ml [$^{35}$S]sulfate. After 4 days the media were dialyzed against 4 changes of 10 vol. 0.2 M sodium citrate (pH 5.5), 0.5 M NaCl, 5 mM cysteine, 1 mM EDTA and 2 changes of 10 vol. 10 mM Tris–HCl (pH 7.8), 1.5 mM $CaCl_2$, and digested exhaustively for 5 days with pronase. The solutions were applied to a DE-52 ion exchange column (0.8 × 7 cm) which was then eluted with a linear gradient of 0–0.6 M NaCl in 10 mM Tris–HCl buffer (pH 8.4) [20].

After decantation of the media, the cells were washed with cold phosphate-buffered saline and detached from the plastic surface by vigorous shaking. After extraction of glycolipids by chloroform–methanol [21] the aqueous phase was dialyzed against 10 mM Tris–HCl (pH 7.8); 1.5 mM $CaCl_2$ and incubated with pronase. The pronase digest was chromatographed on DE-52 as above.

### 2.2. *Characterization of hyaluronic acid*

The [$^3$H]glucosamine labeled fraction eluted from the DEAE-cellulose column at ~0.3 M NaCl was applied to Sepharose CL 4B and eluted with phosphate-buffered saline in the excluded fraction. This fraction was digested with hyaluronidase from bovine testis [22] and again chromatographed on Sepharose CL 4B. The eluted radioactivity appeared now in the included fractions.

### 2.3. *Hyaluronic acid synthetase activity [23]*

Cells were disrupted by freezing and thawing and suspended at 2.5 mg protein/ml. The lysates (20 $\mu$l) were incubated with 1 $\mu$l 1 M Tris–HCl buffer

(pH 7.2), 1 μl 0.1 M $MgSO_4$, 2 μl 0.01 M UDP–*N*-acetylglucosamine and 2 μl UDP–[$^{14}$C]glucuronic acid (307 Ci/mol) for 60 min at 37°C. The mixture was then applied to Whatman 3MM paper and irrigated with isobutyric acid–1 M $NH_4OH$ (5:3) for 24 h. The radioactivity at the origin was determined.

## 3. Results

F9 stem cells were grown in the absence and presence of $10^{-7}$ M retinoic acid and labeled with *N*-[$^3$H]acetylglucosamine and [$^{35}$S]sulfate. Glucosaminoglycans were isolated from the cells and the media separately, digested with pronase and analyzed by DEAE-cellulose chromatography [20,21] (fig.1,2).

F9 cells produced sulfated glycosaminoglycans corresponding to heparan sulfate and chondroitin sulfate [20], and retinoic acid stimulated their synthesis. In addition, retinoic acid induced the production of an unsulfated glycosaminoglycan which was undetectable in control cells. It was characterized as hyaluronic acid by gel filtration as a large macromolecule being excluded by Sepharose CL 4B and was susceptible to hyaluronidase from bovine testis [22].

Thus retinoic acid induced the synthesis of hyaluronic acid and stimulated the synthesis of heparan sulfate and chondroitin sulfate in teratocarcinoma stem cells.

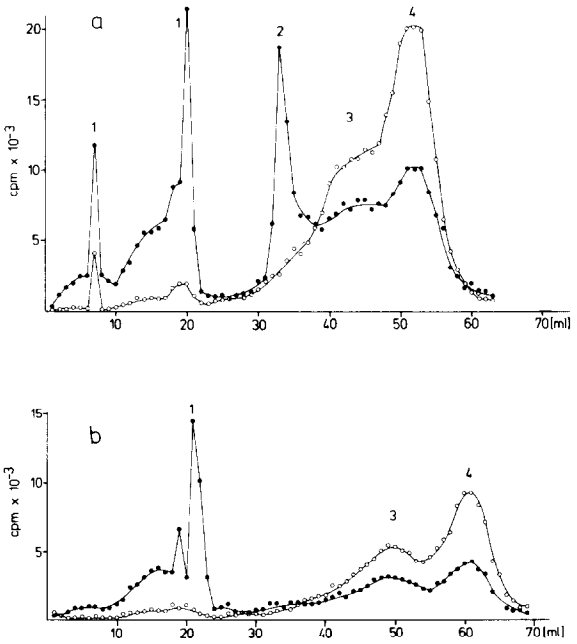


Fig.2. DEAE-cellulose chromatography of glycosaminoglycans of teratocarcinoma stem cells F9. Cells were grown as in fig.1. After decantation of the media, they were washed with cold phosphate-buffered saline and detached from the plastic surface by vigorous shaking. After extraction of glycolipids by chloroform–methanol [30] the aqueous phase was dialyzed against 10 mM Tris–HCl (pH 7.8); 1.5 mM $CaCl_2$ and incubated with pronase. The pronase digest was chromatographed on DE-52 as in fig.1.

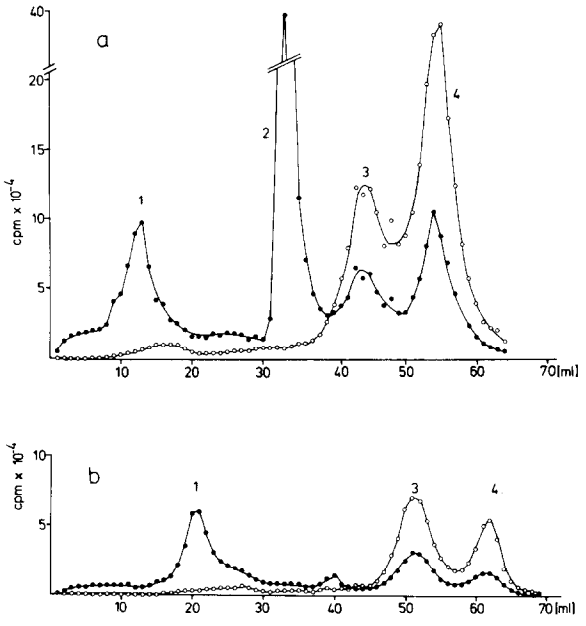


Fig.1. DEAE-cellulose chromatography of glycosaminoglycans from the media of teratocarcinoma stem cells F9 in the presence (a) and absence (b) of $10^{-7}$ M retinoic acid. F9 cells were plated on 4 culture flasks (75 cm$^2$) without collagen coating [9] at 3 × $10^3$ cells/cm$^2$ in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, supplemented with 2.5 μCi/ml [$^3$H]glucosamine and 25 μCi/ml [$^{35}$S]sulfate. After 4 days the media were dialyzed against 4 changes of 10 vol. 0.2 M sodium citrate (pH 5.5), 0.5 M NaCl, 5 mM cysteine, 1 mM EDTA and 2 changes of 10 vol. 10 mM Tris–HCl (pH 7.8), 1.5 mM $CaCl_2$, and digested exhaustively for 5 days with pronase. The solutions were applied to α DE-52 ion exchange column (0.8 × 7 cm) which was then eluted with a linear gradient of 0–0.6 M NaCl in 10 mM Tris–HCl buffer (pH 8.4) [31]. The ordinate represents the total radioactivity incorporated from [$^3$H]-glucosamine (20 Ci/mmol) (•——•) and carrier-free [$^{35}$S]-sulfate (○——○) by 1.5 × $10^8$ cells harvested 4 days after seeding into: (1) glycopeptides; (2) hyaluronic acid; (3) heparan sulfate; (4) chondroitin sulfate.

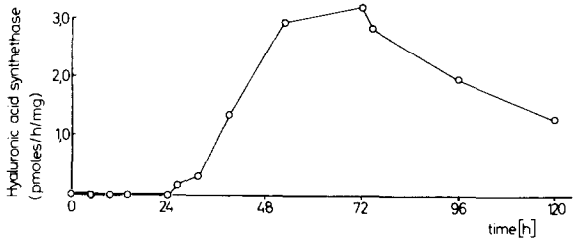


Fig.3. Time course of the induction of hyaluronic acid synthesis after retinoic acid administration at $t = 0$.

The hyaluronic acid synthetase activity was measured after administration of retinoic acid by the incorporation of [$^{14}$C]glucuronic acid as in section 2 (fig.3). The synthetase activity rises after 30 h retinoic acid stimulation and this coincides with the appearance of the new endodermal cell type. When the cell culture reaches confluency at ~72 h, the synthetase activity decreases.

The morphology of the cells was not changed by addition of 20 U/ml testicular hyaluronidase nor by hyaluronic acid to the culture medium.

## 4. Discussion

This study showed that retinoic acid induced the synthesis of hyaluronic acid in teratocarcinoma stem cells simultaneously with the onset of differentiation to endodermal cells. Since the target of retinoic acid action seems to be the cell nucleus [13], the induction of the hyaluronic acid synthesizing genes could be closely associated with differentiation.

The hyaluronic acid synthetase activity has been shown to be dependent on the cell density [24] and the cell cycle [25] in normal fibroblasts. This may explain also the decrease of the hyaluronic acid synthetase in the differentiating teratocarcinoma cells after 72 h, when the cells reach confluency.

Exogenous hyaluronidase or hyaluronic acid did not alter the morphology of the cells and therefore did not seem to influence the differentiation process.

The biosynthesis of hyaluronic acid has been studied in some detail only in *Streptococcus* [26,27], and the mechanism how retinoic acid induces hyaluronic acid synthesis remains to be elucidated. A participation of dolichol intermediates seems to be unlikely [28].

Hyaluronic acid is known to form the structural backbone of aggregated proteoglycans [29] and to mediate anchorage dependence [30,31]. It also plays a central role in differentiation and morphogenesis of embryonic tissue [32,33] and has been implicated in a differentiated function during hybrid formation [34] and in the invasiveness of carcinomas [35]. As hyaluronic acid production accompanied mesenchymal cell migration during chick embryo limb regeneration [36], it seems likely that it is also involved in the spreading and migration of teratocarcinoma stem cells induced to differentiate by retinoic acid [9].

## Acknowledgement

The author thanks Dr S. Strickland for providing the teratocarcinoma stem cell F9.

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