Vol. 111, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1983 Pages 353-359

EFFECT OF THYROTROPIN ON GLYCOSAMINOGLYCANS SYNTHESIZED BY PRIMOCULTURED THYROID CELLS

Annie Giraud and Simone Bouchilloux

Laboratoire de Biochimie Médicale et U 38 INSERM, Faculté de Médecine, 27 Bd J. Moulin, F-13385 Marseille Cédex 5, France

Received December 27, 1982

SUMMARY. The synthesis of glycosaminoglycans (GAGs) was investigated in porcine thyroid cells under the influence or not of thyrotropin. After labelling with [^{3}H] glucosamine and [^{35}S] SO_4^{2-}, enriched GAG-fractions prepared from culture media, cells, and eventually substrate adhering materials, were analyzed by cellulose acetate electrophoresis combined with specific degradations. They comprised heparan sulfate and hyaluronic acid together with an unknown sulfated component labile to endo-β-galactosidase. Whereas global labellings of newly made GAGs were not significantly modified by thyrotropin, we reproducibly observed with the hormone a substantial increase in the proportion of hyaluronic acid [^{3}H] label and, when cells organized into follicles, of the proportion of cell-associated [^{3}H] GAGs. This system thus offers an interesting model to study how the responsiveness to an hormone and the reorganization that follows might implicate specific glycoconjugates.

We showed previously that porcine thyroid cells in primoculture synthesize fibronectin which they mobilize differently depending on their three-dimensional organization : in monolayers the major part of *in vitro* labelled fibronectin was secreted into the culture medium whereas in the presence of thyrotropin (TSH) when cells after a few days became organized in follicle-like structures most of this fibronectin remained within the cell compartment. Immunofluorescence revealed fibronectin at the plasma membrane mainly at the baso-lateral side of follicle-forming cells (1).

There is an increasing evidence that glycosaminoglycans (GAGs), most of them as proteoglycans, interact with fibronectin and contribute somewhat specifically to the adhesive properties of a number of cells (2, 3, 4). They are, together with fibronectin and collagens, present in kidney and possibly other basement membranes (5) and they also constitute major components of extra-

ABBREVIATIONS : TSH, thyrotropin ; GAG, glycosaminoglycan ; HA, hyaluronic acid ; HS, heparan sulfate ; MEM, Eagle's minimum essential medium ; PBM, phosphate-buffered medium : 137 mM NaCl ; 2.7 mM KCl, 8.1 mM $Na_2H\ PO_4$, 1.5 mM $KH_2\ PO_4$, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.02 % NaN_2, 5 mM benzamidine ; SAM, substrate attached material.

0006-291X/83 $1.50

cellular matrices (6). In conjunction with our above mentioned investigation dealing with fibronectin (1) we though interesting to study the synthesis and distribution of specific GAGs by thyroid cells, under stimulation or not.

MATERIALS AND METHODS

Hyaluronic acid, heparin, dermatan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and Proteus vulgaris chondroitinase ABC were from Sigma, DN^{ase}-I and RN^{ase}-A from Worthington, Streptomyces hyalurolyticus hyaluronidase and E. freundii endo-β-galactosidase from Seikagaku Kogyo Co. through Miles. Eagle's minimum essential medium and newborn calf serum were from Gibco, Petri dishes from Corning, cellulose acetate strips (Sepraphore III) from Gelman. Bovine TSH was from Armour.

Cell culture and labelling : Thyroid cells were isolated from porcine thyroid glands by a discontinuous trypsin-EGTA treatment (7). They were suspended at a concentration of 3 x 10^6 cells/ml in Eagle's minimum essential medium (MEM) (8) containing 10 % newborn calf serum, penicillin (200 U/ml) and streptomycin (50 µg/ml) and seeded in tissue culture Petri dishes (4 ml suspension/6 cm-diam. dish). When follicles were to be obtained, TSH (5 mU/ml) was added at the onset of culturing. Incubations took place in a 5 % CO_2 - 95 % air, water-saturated atmosphere at 36° C. Cells seeded at high density do not divide under these conditions.

Na_2 [^{35}S] SO_4 (42 mCi/mmol) and [^{3}H] glucosamine Cl (22 Ci/mmol) both from Amersham were added to the culture medium at a final concentration of 7.8 µCi/ml. Two periods of labelling were studied : the first 24 h and a 48 h period between days 4 and 6 i.e. when TSH cells reach an important degree of folliculisation.

Cell culture compartments after radio labelling : At the end of the incubation, culture media were collected and centrifuged at low speed to clear away cell debris. Cells were washed 3 times in situ with phosphate buffered medium (PBM), then either scrapped with a rubber policeman in the presence of 1 ml PBM/Petri dish and collected by centrifugation at 1100 g (scrapped cells) or alternatively detached from the plastic support by a 15 h incubation at 36° C in a spinner salt solution without Mg^{2+} (8) made 3 mM EGTA and spun as above (EGTA-treated cells). The EGTA-supernatant (EGTA extract) was centrifuged at 10 000 g to clear away cell debris and extensively dialyzed against PBM. Remaining plastic supports were washed with PBM so that no cell remained adherent according to light microscopic controls, before receiving 1 ml of 0.2 % Na dodecyl-sulfate in water. The solubilized material was called substrate attached material (SAM), after Culp et al. (4).

Extraction and analysis of GAGs : Extraction was carried out according to Hronowski and Anastassiades (9) in the presence of carrier cold GAGs. At this stage samples originating from cell compartments were treated by DN^{ase}-I and RN^{ase}-A according to Breen et al.(10). Aliquots corresponding to 40-50 000 dpm (each label) were finally subjected to a sequence of specific degrading enzymes (10) : they were first treated by hyaluronidase, then by chondroitinase ABC, after that by endo-β-galactosidase (11) in order to possibly degrade keratan sulfate and finally by HNO_2 to eliminate heparan sulfate and heparin. Each step was monitored by a monodimensional cellulose acetate electrophoresis corresponding to the first dimension of the procedure described by Hata and Nagai (12). When the radioactivity was too low (SAM samples) GAGs were analyzed without sequential degradation by the two dimensional electrophoresis (12).

Other techniques : After Alcian blue staining, the cellulose acetate sheets were subjected to autoradiography with Kodak no screen film in order to detect ^{35}S. They were then sprayed with the NEN ^{3}H-enhancer and subjected to fluorography at -70° C with Kodak AR X OMAT film in order to also detect ^{3}H. For quantitation of labels the zones corresponding to radioactive products in the

acetate sheet were cut, extracted in 0.750 ml NCS solubilizer (Amersham) and counted with 9 ml of a PPO-POPOP-toluene solution (NEN). A Packard liquid scintillation spectrometer has been preset for ^{35}S, ^{3}H-double label counting and standard curves established. Other samples were counted in Aquasol-2 (NEN).

DNA was determined according to Mc Intire and Sproull (13).

RESULTS

When thyroid cells cultured without or with TSH were labelled with $[^{3}H]$ glucosamine and $[^{35}S]$ SO_4^{2-}, for either 24 h just after seeding or 48 h between days 4 and 6, the total incorporations of radioactivity into their GAGs (sum of individual compartments) were for both isotopes roughly of the same order of magnitude for the 2 periods considered, in the presence or not of the hormone (Table 1).

Fig. 1. documents the distribution of GAG-associated radioactivities between the compartments. Percentages of labels in the scrapped cells (or scrapped cells + SAM) were usually comparable to those in the sum of EGTA-treated cells + EGTA extracts. Radioactivities in the SAM were appreciable only for the cells without TSH i.e. forming a monolayer. Higher percentages of ^{3}H-labels than of ^{35}S-labels were found in the cell layers, probably reflecting the fact that sulfate incorporation into GAG moieties is a late synthetic event preceding their exportation from the cells.

A qualitative inventory of *in vitro* made GAGs was obtained by electrophoretic analysis coupled with specific degradations. By radioautography the presence of heparan sulfate (HS) as a major ^{35}S-sulfated species was revealed in all compartments. Another ^{35}S-labelled component (X) poorly represented in culture media had the property to resist treatments with hyaluronidase, chondroi-

TABLE 1. $[^{3}H]$ and $[^{35}S]$ GAG radioactivities

Cell organization	Day	^{3}H dpm	^{35}S dpm
no TSH	0-1	1356 ± 264	1864 ± 139
TSH	0-1	1814 ± 425	2435 ± 31
no TSH	4-6	1300 ± 124	2065 ± 445
TSH	4-6	1508 ± 808	1815 ± 500

Data expressed as dpm/µg DNA (i.e. 10^5 cells).
Mean of 2 independent expts, each value obtained from 3 or 4 Petri dishes.

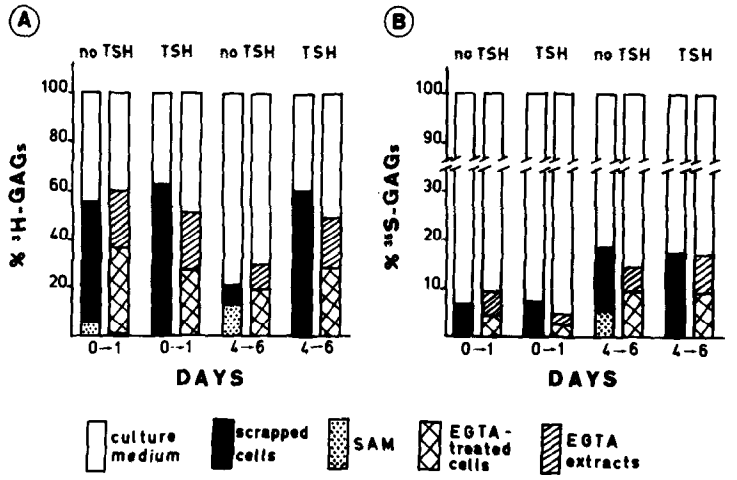

Fig. 1. Per cent distribution of GAG-associated radioactivities (A, [^{3}H] and B, [^{35}S] radioactivity) in the compartments defined in Methods. Two periods of labelling without or with TSH.

tinase ABC and HNO_2 but to disappear with endo-β-galactosidase, thus resembling keratan sulfate. The non sulfated ^{3}H-labelled hyaluronic acid (HA) was revealed by fluorography : it was present in variable proportions depending on culture conditions. In no case were radiolabelled chondroitin sulfate A, B, C nor heparin detectable.

From the radioactivity countings performed with cellulose acetate segments, quantitative estimations were obtained. As shown in Fig. 2 for [^{3}H] glucosamine labellings, a much higher proportion of this label corresponded to HA when the cells were cultured with TSH. This change which was even more pronounced at the first period of labelling also occured at days 4 to 6. It applied to all compartments examined and rather occured at the expense of HS than of the compound X. Also noticeable was the fact that X was in any case rather cellular than medium released. Only the SAM-materials from the cultures without TSH (monolayers) contained enough radioactivity to obtain a compositional estimation : interestingly HS, HA and X were represented all three.

Another significant difference between the two culture conditions concerned the relative distribution of their [^{3}H] GAGs between media and cellular compartments : there was for TSH-cultures at the time of folliculisation a marked increase in association to the cells (noticeable in Fig. 1, days 4-6).

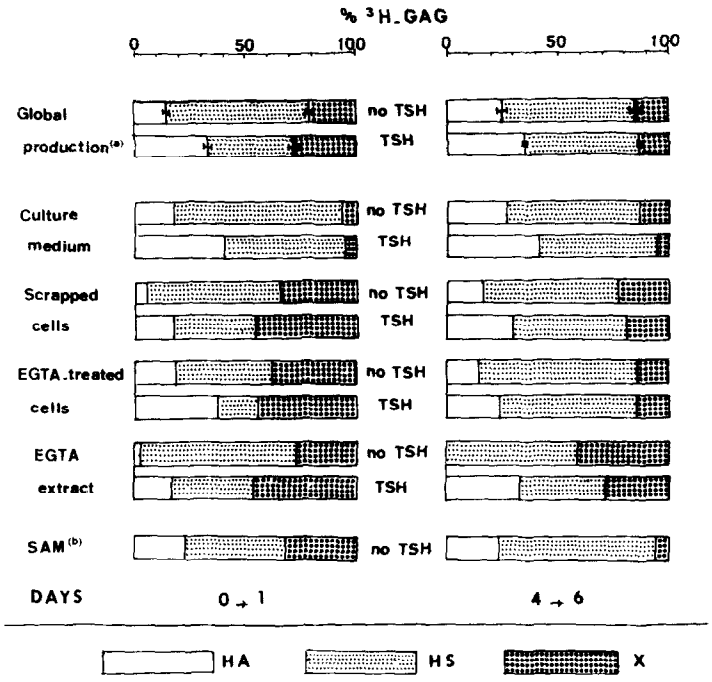

Fig. 2. Per cent distribution of [^{3}H] radioactivity associated with specific GAGs in the different compartments.
Two culture conditions (no TSH, TSH) and 2 periods of labelling. Data from 1 or two ((a) mean values) representative expts. (b) from a bidimensional electrophoresis (see Methods).

As depicted in Fig. 3 this change applied to the 3 components measured as regards their glucosamine labelling. In the same time sulfate labellings at days 4-6 were unmodified by TSH implicating for hormone containing cultures a lower $^{35}S/^{3}H$ ratio in the cell layer.

DISCUSSION AND CONCLUSION

The marked increase in the proportion of HA label observed for TSH-cultures was a salient feature in the present study. This is reminiscent of observations suggesting differential roles for HA and HS in morphogenesis as well as for adhesion of cultured cells (14). Altogether the differences we observed in HA/HS labels depending on culture conditions make sense inasmuch as thyroid cells organizing into follicles are less strongly attached to the plastic support than monolayers and have to move more freely. Even if our primocultures tend to secrete newly made GAGs into culture media and are not able to assemble a fully constituted basement membrane they produce as a major labelled species HS a known constituent of cell surfaces (15, 16) and basement membranes (5).

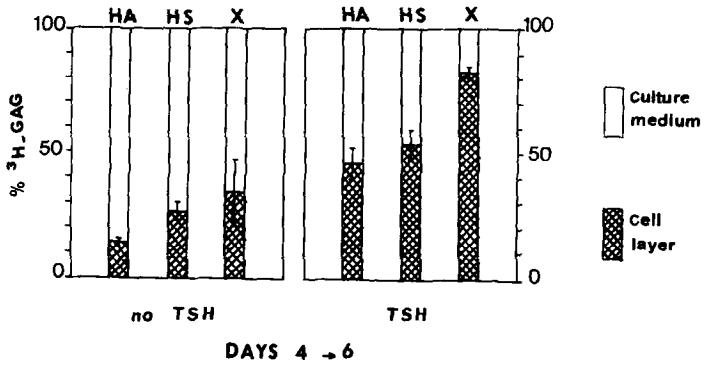

Fig. 3. Comparative location of the [^{3}H] labels related to HA, HS and X between medium and cell layer. Mean of 2 expts.

Further work will be necessary to ascertain the identity of the sulfated species X and one cannot exclude the possibility of a compound bearing a sequence recognizable by an endo-β-galactosidase (11) while not originating from a proteoglycan. When we examined (data not presented here) the GAGs present in the whole porcine thyroid tissue, HS was the main product revealed by Alcian blue, accompanied with chondroitin sulfates and a species similar to X, but with only traces of HA : this underlines how different might be the metabolic states of individual GAG species.

Reminiscent of what we previously found for newly made fibronectin (1), TSH which induces in a few days the reorganization into 3-dimensional follicle-like structures also leads to cell retention of [^{3}H] GAGs (in general). In conclusion, primocultured thyroid cells constitute an hormone-responsive system that might be usefull to explore possible roles of specific GAGs (and proteoglycans) in differentiated tissue organization. Further work is needed to understand to what extent the changes observed here mainly concern synthetic events rather than degradation, and whether or not they include modifications in GAGs (or proteoglycans) receptors.

ACKNOWLEDGEMENTS : We are indebted to Dr J. Mauchamp for gift of thyroid cells, to Miss C. Pelassy, Mrs J. Garcia and J. Lanet for technical assistance.

REFERENCES

1. Giraud, A., Gabrion, J. and Bouchilloux, S. (1981) Exp. Cell. Res. 133, 93-101.
2. Laterra, J., Ansbacher, R. and Culp, L.A. (1980) Proc. Nat. Acad. Sci. (USA) 77, 6662-6666.
3. Perkins, M.E., Tae, H.J. and Hynes, R.O. (1979) Cell 16, 941-952.
4. Culp, L.A., Murray, B.A. and Rollins, B.J. (1979) J. Supramol. Struct. 11, 401-427.
5. Kanwar, Y.S. and Farquhar, M.G. (1979) Proc. Natl. Acad. Sci. (USA) 76, 4493-4497.
6. Hay, E.D. (1981) J. Cell. Biol. 91, 205 s - 223 s.
7. Chambard, M., Gabrion, J. and Mauchamp, J. (1981) J. Cell. Biol. 91, 157-166.
8. Eagle, H. (1959) Science, 130, 432-437.
9. Hronowski, L. and Anastassiades, T.P. (1980) J. Biol. Chem. 255, 9210-9217.
10. Breen, M., Knepper, P.A., Weinstein, H.G., Blacik, L.J., Lewandowski, D.G. and Baltrus, B.M. (1981) Anal. Biochem. 113, 416-422.
11. Fukuda, M.N., Watanabe, K. and Hakomori, S. (1978) J. Biol. Chem. 253, 6814-6819.
12. Hata, R. and Nagai, Y. (1972) Anal. Biochem. 45, 462-468.
13. Mc Intyre, F.C. and Sproull, M.F. (1957) Proc. Soc. Exp. Biol. 95, 458-462.
14. Rollins, B.J. and Culp, L.A. (1979) Biochemistry, 18, 5621-5629.
15. Dietrich, C.P. and Montes de Oca, H. (1978) Biochem. Biophys. Res. Comm. 80, 805-812.
16. Oldberg, A., Kjellen, L. and Höök, M. (1979) J. Biol. Chem. 254, 8505-8510.