Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 556-563

SELECTIVE INCREASE IN CYTOKERATIN SYNTHESIS IN CULTURED RAT HEPATOCYTES IN RESPONSE TO HORMONAL STIMULATION

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Received June 2, 1983

SUMMARY: Addition of a combination of insulin, dexamethasone and EGF at seeding time to cultured rat hepatocytes in serum-free medium caused a selective increase in the biosynthesis of particular cytokeratin components. This increase was prominent during the first day in culture. No significant increases were detected in the absence of hormones or in the presence of either hormones added alone or in pairs, except in the case of insulin plus dexamethasone, which yielded an effect close to that obtained with the three factors. Interestingly, the latter condition also maintained a high level of albumin production over a 6-day period in culture.

Cytokeratins constitute a complex family of highly insoluble proteins that belong to the class of intermediate filaments present in keratinizing and non-keratinizing epithelial cells (1-3). Recent biochemical and immunological analyses have revealed that the individual cytokeratins are expressed in different combinations in distinct epithelial cell types (2,3), suggesting that the specific composition of the cytokeratin filaments is important for the performance of differentiated functions (2,4,5). Rat hepatocytes contain 7 cytokeratin components of molecular weights 55K to 41K daltons (6) arranged as filaments interacting with several cytoplasmic structural elements and with desmosomes at the surface membrane (6-10). This supports the view that intermediate filaments may represent a mechanical integrator of the cytoplasm (4,9) and a modulator of cell shape (4,5).

Rat hepatocytes can be cultured as monolayers and their ability to perform cell-type specific functions requires conditions that generate, within a short

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Abbreviations: EGF, epidermal growth factor; Ins, insulin; Dex, dexamethasone; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

0006-291X/83 $1.50

culture period, cell-cell contacts and cellular arrangements equivalent to those observed in vivo (11-14). Using serum-free medium, we recently observed that some hormonal factors induced major changes in the cytoskeleton organization of both cultured neonatal and adult rat hepatocytes (15) and modulated their functional activity (13,14). In this communication we report a selective and prominent increase in the synthesis of two major cytokeratin components in rat hepatocytes during the first day post-seeding, and maintenance of high level of albumin production over a 6-day period in primary culture, in the presence of insulin, dexamethasone and EGF.

MATERIALS AND METHODS

Animals. Hepatocytes were obtained from 2-week neonatal and 3-month old Fisher 344 rats (Charles River Company, Willington, Ma. USA).

Hepatocyte culture. The method for the high yield isolation of hepatocytes from adult and neonatal rats has already been described in detail (13,16). Five ml of hepatocyte suspension at 4.10^5 cells/ml in serum-free and arginine-free William's E medium (17) (Gibco, Burlington, Ont. Canada) were cultured in T-25 flasks (Falcon Labware, Becton/Dickinson, Montréal, Qué. Canada) pre-coated with plasma fibronectin (13,18), at 37°C in a 5% CO_2 atmosphere without supplements, or supplemented with various combinations of insulin (100 ng/ml; Calbiochem, La Jolla, Ca., USA), dexamethasone (1 µM; Sigma, St.Louis, Mo., USA), EGF (100 ng/ml; kindly provided by Dr. P. Walker, CHUL, Sainte-Foy, Qué.Canada); and BSA (0.5%; Sigma). The medium was changed at 3 h post-seeding in order to eliminate unattached hepatocytes.

Analysis of newly synthesized proteins. A) *Cell labeling*. At different times post-seeding, hepatocytes were pulse-labeled for 2 h in methionine-free medium containing 17.5 µCi/ml of [^{35}S]methionine (New England Nuclear, Montréal, Qué. Canada), supplemented with the appropriate hormones. At the end of the incubation period, whole cell proteins were extracted by SDS (19). After heating 3 min at 90°C and precipitation by ethanol at -20°C overnight, the samples were assayed for total protein content (20) and their radioactivity was measured with a scintillation counter. In some experiments, cytoskeleton proteins enriched in cytokeratins were prepared by extraction of the cells with 1% Triton X-100 and 1.5 M KCl before solubilization in 1% SDS, according to the procedure of Franke et al. (6,21). B) *Gel electrophoresis*. The solubilized proteins were fractionated by SDS polyacrylamide gel (10%) electrophoresis according to Laemmli (22). The gels were stained with Coomassie blue and then processed to obtain autoradiograms using XAR5 Kodak film. Exposure time was 5 to 7 days. Protein standards were myosin (200K), β-galactosidase (116K), phosphorylase B (92.5K), BSA (66.2K) and ovalbumin (45K). C) *Immunoblotting*. The newly synthesized proteins fractionated by electrophoresis were further identified by the immunoblot technique (23), using monoclonal antibodies prepared in our laboratory against rat hepatocyte cytokeratins (manuscript in preparation).

Cell number determination. The number of attached hepatocytes was evaluated directly in the flasks using a microscopic procedure previously described (13).

Albumin production. The amount of albumin secreted in the culture medium was measured by solid-phase radioimmunoassay as previously described in detail (13,16).

TABLE I

THE EFFECT OF HORMONES ON ADULT RAT HEPATOCYTES SURVIVAL* IN VITRO

Hormones added	Days in culture		
	1	3	6
Ins,Dex,EGF	111 ± 3	111 ± 11	49 ± 8
Ins,Dex	92 ± 3	85 ± 3	48 ± 5
Dex	86 ± 4	64 ± 6	34 ± 4
Ins	88 ± 5	70 ± 12	15 ± 15
None	72 ± 4.5	44 ± 3	5 ± 2.5

* Expressed as a percent of the number of cells attached at day 0. (average ± S.E.M.). All media contained 0.5% BSA. Values usually correspond to means of duplicate determinations in at least 3 separate experiments.

RESULTS AND DISCUSSION

Table I shows that in presence of Ins, Dex and EGF in primary culture, essentially 100% of the hepatocytes remained attached on day 3 and one-half were still attached on day 6. The removal of EGF led to only a slight decrease in hepatocyte number. In either case, the [^{3}H]TdR labeling index was less than 1% on day 3 and 3% on day 6. Within the first 24 h post-seeding the hepatocytes formed compact monolayers of polygonal cells, indicative of numerous intercellular contacts (10,11,14,15). The various protein species synthesized by adult rat hepatocytes were compared (Fig. 1). In the absence of hormones (lane 1), the predominantly labeled component was a 45K dalton polypeptide. The major effect of the supplements was a selective increase in the labeling of two polypeptides of 55K and 49K daltons; the labeling of the 45K dalton species being slightly increased (lane 2). This increased labeling was evident after 4 h post-seeding and was still detectable at 34 h (data not shown). The 55 and 49K dalton polypeptides co-migrated with the two major Triton-resistant polypeptides of rat hepatocytes (Fig. 2). These proteins were designated by Franke et al. (6) as cytokeratin components A and D. Increased synthesis of the 49 and 55K dalton cytokeratins in response to the addition of Ins, Dex and EGF was also detected in newborn rat hepatocytes (Fig. 4, lanes 5 and 9). The identity of the 55 and 49K dalton components was confirmed by immunoblotting using mono-

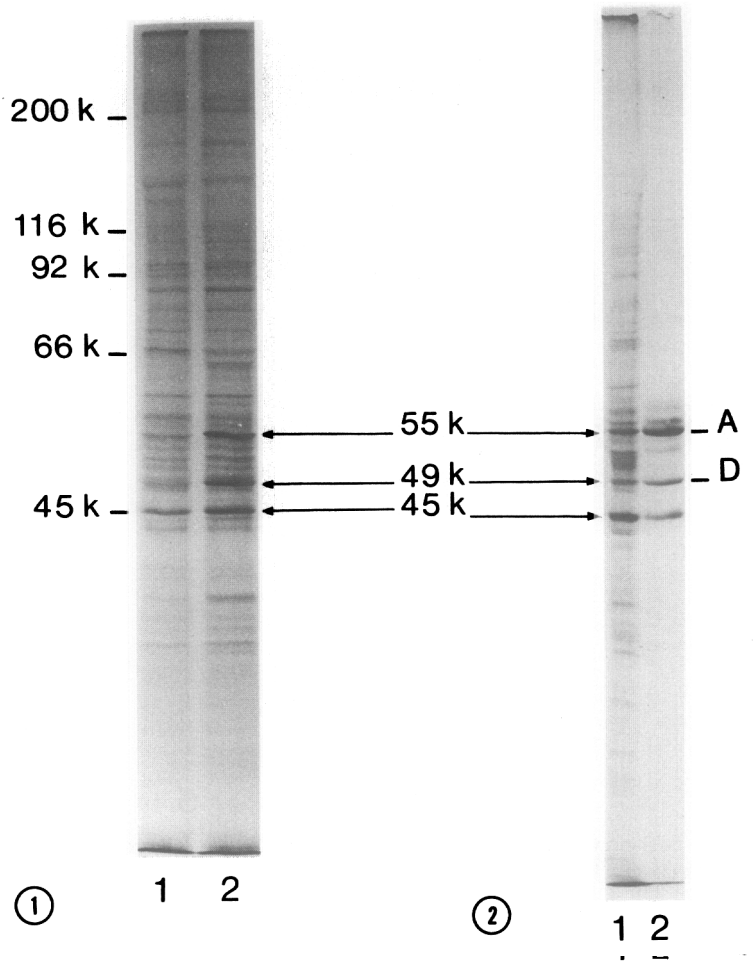

Fig. 1: Autoradiogram of a SDS-polyacrylamide slab gel of proteins extracted from adult rat hepatocytes incubated for 2 h in the presence of [^{35}S]methionine after a 24 h culture in absence (lane 1) or presence (lane 2) of Ins + Dex + EGF. 1.5 x 10^5 cpm were applied to each well. Specific activities: lane 1, 2700 cpm/µg proteins; lane 2, 5800 cpm/µg proteins.

Fig. 2: Autoradiogram of a SDS-polyacrylamide slab gel of proteins extracted from adult rat hepatocytes incubated for 2 h in the presence of [^{35}S]methionine after a 10 h culture in the presence of Ins + Dex + EGF. Lane 1: total cellular extract, 10^5 cpm; lane 2: Triton-resistant fraction, 0.35 x 10^5 cpm.

clonal antibodies prepared in our laboratory against rat hepatocyte cytokeratins A and D (Fig. 3). These two antibodies have been characterized in detail; for example, immunofluorescence microscopy revealed that they decorated distinct fibrillar structures in the cytoplasm of hepatocytes in culture and also in situ (manuscript in preparation). The 45K dalton polypeptide co-migrated with residual actin present in the Triton-resistant material (Fig. 2).

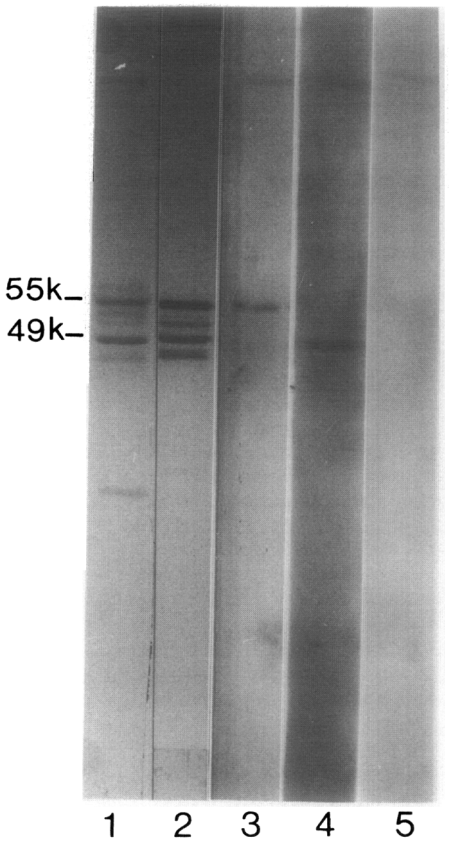

Fig. 3: SDS-polyacrylamide slab gels and nitrocellulose blotting of the Triton-resistant fraction from adult rat hepatocytes cultured for 24 h in the presence of Ins + Dex + EGF. Lane 1: Coomassie blue staining of Triton-resistant proteins; lane 2: autoradiogram of the Triton-resistant fraction from 2 h-pulse labelled cells ([^{35}S]methionine); nitrocellulose strips were incubated with the hybridoma antibodies against cytokeratin A (lane 3) and D (lane 4). Lane 5 shows the non-specific binding with normal mouse serum.

We determined whether the observed changes in cytokeratins occurred in the presence of the three factors added individually or in pairs (Fig. 4). In newborn hepatocyte cultures, there was no increase in cytokeratin synthesis in the presence of one hormone (lanes 2 to 4) or in the presence of EGF and Ins (lane 6) or EGF and Dex (lane 8). Dexamethasone alone (lane 4), or EGF and Dex decreased actin biosynthesis. The action of Ins and Dex (lane 7) however, could mimic to a large extent the effect of the three hormones added together (lane 5). The same results were obtained in adult rat hepatocytes.

The combination of factors that led to this increase in synthesis of particular cytokeratins during the first day in culture also favored the mainte-

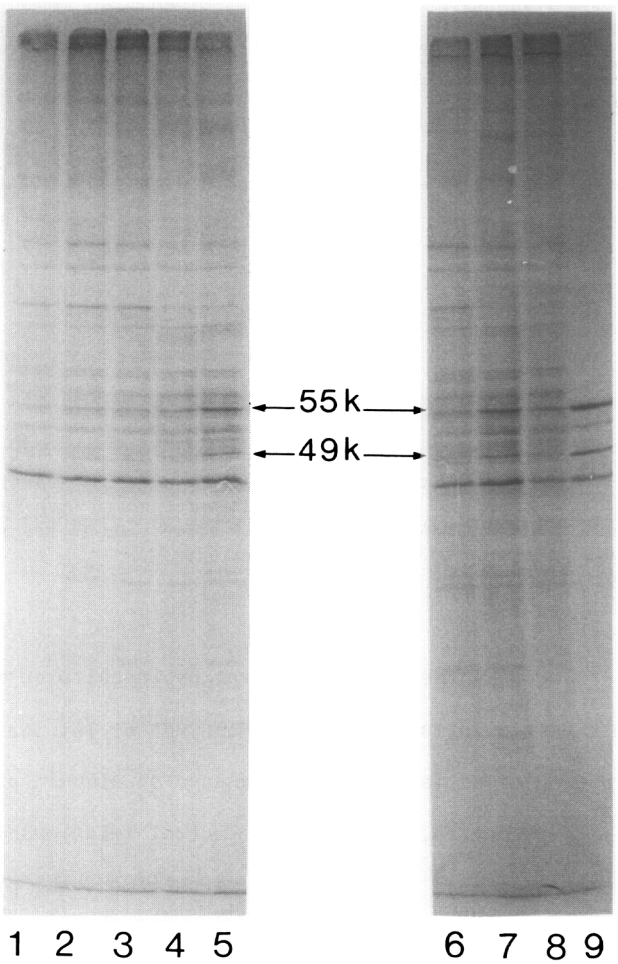

Fig. 4: Autoradiogram of a SDS-polyacrylamide slab gel of proteins extracted from neonatal rat hepatocytes incubated for 2 h in the presence of [^{35}S]methionine after a 24 h culture in the absence (lane 1) or in the presence of EGF (lane 2), Ins (lane 3), Dex (lane 4), Ins + Dex + EGF (lane 5), EGF + Ins (lane 6), Ins + Dex (lane 7), EGF + Dex (lane 8), Ins + Dex + EGF, Triton-resistant fraction (lane 9). 10^5 cpm were applied to each well. Specific activities: (1) 1037 cpm/µg proteins; (2) 1605 cpm/µg; (3) 1495 cpm/µg; (4) 1173 cpm/µg; (5) 2264 cpm/µg; (6) 2535 cpm/µg; (7) 2800 cpm/µg; (8) 1541 cpm/µg.

nance of a typical differentiated function of hepatocytes. Indeed, we observed (table II) that albumin production, an indice of hepatocyte metabolic status, was maintained at a high and constant level over a 6-day period in culture in the presence of Ins, Dex and EGF. In the presence of Ins and Dex, albumin production remained constant for at least a 3-day period but at a lower level. In the presence of a single factor, the albumin secretion decreased markedly after the first day.

TABLE II

THE EFFECT OF HORMONES ON ALBUMIN PRODUCTION* BY ADULT RAT HEPATOCYTES IN VITRO

Hormones added	Days in culture		
	1	3	6
Ins,Dex and EGF	93 ± 5	88 ± 6	92 ± 10
Ins,Dex	55 ± 12	49 ± 11	28 ± 7
Dex	43 ± 19	22 ± 10	15
Ins	50 ± 17	33 ± 1	N.D.
None	56 ± 2	10 ± 1	-

* These values correspond to the percent secreting activity relative to that measured at day 0 (average ± S.E.M.), and expressed in ng/h/10^6 cells. All media contained 0.5% BSA. Values usually correspond to means of duplicate determinations in at least 3 separate experiments.
N.D.: non detectable.

In conclusion, the synthesis of cytokeratins in cultured rat hepatocytes is dependent on the stimulation by Ins, Dex and EGF or Ins and Dex. The same culture conditions favor the maintenance of albumin production, a specific function of hepatocytes. Although the actual relationship between these two hormone-related phenomena remains to be established, the present findings are in line with the current view (2,5,24) suggesting that the expression of cytokeratins could be linked to performance of differentiation-related functions in epithelial cells.

ACKNOWLEDGEMENTS

We thank Drs. William Waithe and Jacques Landry for their critical reading of the manuscript and Ms Chantal Bédard for her secretarial support. This work was supported by the Medical Research Council and the National Cancer Institute of Canada.

REFERENCES

1. Franke, W.W., Schmid, E., Kartenbeck, J., Mayer, D., Hacker, H.J., Bannash, P., Osborn, M., Weber, K., Denk, H., Wanson, J.C., and Drochmans, P. (1979) Biol. Cell. 34, 99-110.
2. Osborn, M., and Weber, K. (1982) Cell 31, 303-306.
3. Moll, R., Franke, W.W., and Schiller, D.L. (1982) Cell 31, 11-24.
4. Lazarides, E. (1980) Nature 283, 249-256.

5. Wu, Y.J., Parker, L.M., Binder, N.E., Beckett, M.A., Sinard, J.H., Griffiths, C.T., and Rheinwald, J.G. (1982) Cell 31, 693-703.

6. Franke, W.W., Denk, H., Kalt, R., and Schmid, E. (1981) Exp. Cell. Res. 131, 299-318.

7. Lazarides, E. (1982) Ann. Rev. Biochem. 51, 219-250.

8. Goodenough, D.A., and Revel, J.P. (1970) J. Cell. Biol. 45, 272-290.

9. French, S.W., Kondo, I., Irie, T., Ihrig, T.J., Benson, N., and Munn, R. (1982) Hepatology 2, 29-38.

10. Guguen, C., Guillouzo, A., Boisnard, M., Le Cam, A., and Bourel, M. (1975) Biol.Gastroentérol. (Paris) 8, 223-231.

11. Laishes, B.A., and Williams, G.M. (1976) In Vitro 12, 521-532.

12. Marceau, N., Noel, M., Deschênes, J. (1982) In Vitro 18, 1-13.

13. Deschênes, J., Valet, J.P., and Marceau, N. (1980) In Vitro 16, 722-730.

14. Guguen-Guillouzo, C., Seignoux, D., Courtois, Y., Brissot, P., Marceau, N., Glaise, D., and Guillouzo, A. (1982) Biol. Cell 46, 11-20.

15. Marceau, N., Goyette, R., and Pelletier, G. Cell.Mol. Biol. (in press).

16. Deschênes, J., Valet, J.P., and Marceau, N. (1981) Cell Biophys. 3, 321-334.

17. Williams, G.M., and Gunn, J.M. (1974) Exp. Cell Res. 89, 139-142.

18. Marceau, N., Goyette, R., Valet, J.P., and Deschênes, J. (1980) Exp. Cell Res. 125, 497-502.

19. Tanguay, R.M., and Nicole, L.M. (1980) Chromosoma 76, 219-235.

20. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

21. Franke, W.W., Mayer, D., Schmid, E., Denk, H., and Borenfreund, E. (1981) Exp. Cell Res. 134, 345-365.

22. Laemmli, U.K. (1970) Nature 227, 680-685.

23. Towbin, H., Stachelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.

24. Schmid, E., Schiller, D.L., Grund, C., Stadler, J., and Franke, W.W. (1983) J. Cell. Biol. 96, 37-50.