A Pituitary Growth-Promoting Factor for Articular Chondrocytes in Monolayer Culture

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ABSTRACT. A heat-labile anterior pituitary factor exerts a strong mitogenic action on articular chondrocytes in secondary monolayer culture. At the same time secretion of macromolecular radiosulfate into the medium by the stimulated cells is markedly reduced. This factor was found in NIH bovine and ovine TSH as well as LH but not in more purified (Condliffe-Bates, Pierce) preparations of TSH. The response, measured by the DNA content of the cell pellet, was dose-dependent; the lowest effective concentration was 1 μg NIH TSH/ml culture medium. The effect was fairly selective for chondrocytes, rabbit and human, and was displayed to much lesser degree by skin fibroblasts and 4 other cell types studied. FSH had a smaller effect while GH, prolactin, ACTH and a preparation having high EPS activity were relatively ineffective. Of 19 other hormone preparations examined, only crude HCG gave a comparable mitogenic response. Insulin, 0.1 U/ml, had a consistent but small effect. Estradiol-17β and diethylstilbesterol (10 μg/ml) were cytotoxic, as were cyclic AMP and its dibutyryl ester (1.4×10^{-3}M). (*Endocrinology* **90**: 262, 1972)

DURING a study of the effects of hormones on chondrogenic differentiation, we have observed that an anterior pituitary factor has a marked and somewhat selective mitogenic effect on articular chondrocytes in monolayer culture. The factor is associated with thyrotropic and luteinizing hormone fractions but it is not itself thyrotropin. A similar activity is observed in crude human chorionic gonadotropin but not in other pituitary or placental hormones investigated.

Materials and Methods

Cell cultures. The experiments were carried out on secondary cultures of chondrocytes from the shoulder, hip and knee joints of New Zealand albino rabbits two to three months old. The primary cultures were made at different times from 17 animals by methods detailed elsewhere (1,2). The primary cells were grown in Ham's F12 medium supplemented with 10 percent fetal calf serum. When they had become confluent (usually on the 10th day, occasionally the 7th) the chondrocytes were trypsinized. Approximately 2×10^5 cells were inoculated into 25 cm^2 plastic flasks (Falcon Plastics Company) containing 4 ml of a slightly modified Dulbecco-Eagle medium supplemented with 10 percent fetal calf serum and streptomycin-penicillin (10 μg and 10 U/ml respectively). $MgSO_4$ of the medium was replaced by $MgCl_2$, 165 mg/L. All culture media and reagents were obtained from the Grand Island Biological Company (GIBCO), except for some serum supplied by the Baltimore Biological Laboratories. The cultures were gassed with 10 percent CO_2. There were, with a few exceptions, eight flasks in each experimental group.

Under these conditions the chondrocytes do not become "contact inhibited" and continue to proliferate, although at a decreasing rate, for at least 23 days (Table 1). Nevertheless they are not transformed so far as serological tests for group specific leukemia antigens can indicate (Dr. Paul Price, Microbiological Associates), and they continue to secrete sulfated macromolecules.

The cultures were fed with hormone-containing media at the time of the inoculation and again on the 3rd and 5th days. To this last feeding was added $Na_2{}^{35}SO_4$ (New England Nuclear Corporation) at a level of 1.4 μCi/ml. The cells and media were harvested 20 hours later.

Other cell types studied included human articular chondrocytes from a 22 year old man, the cells in their second passage; rabbit skin

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Received July 14, 1971.

TABLE 1. Effect of age of culture on articular chondrocytes

Days in culture	DNA (μg/flask)*	$^{35}SO_4$ (10^4 dpm/μg DNA)*
5	28.9 ± 1.38 (8)	1.43 ± 0.05
12	39.6 ± 1.04 (5)	1.52 ± 0.09
23	43.1 ± 1.86 (5)	1.79 ± 0.13

* Mean ± SE. The figure in parentheses is the number of flasks.

fibroblasts; HeLa; FTRL-4 (diploid fetal rat liver); VERO (polyploid green monkey kidney); F26-10C (Fisher rat embryo).

Hormone preparations. Six preparations of thyrotropin (TSH) with varying specific activity were employed (Table 2): bovine, Ciereszko, Princeton Laboratories, 0.5–0.7 U/mg; bovine, NIH-TSH-B5, 2.21 U/mg; bovine, purified by P. G. Condliffe and R. W. Bates, assaying 20 U/mg; bovine, purified by J. G. Pierce, 25 U/mg; ovine, NIH-TSH-S6, 2.47 U/mg; murine tumor, PC11-113A, prepared by Condliffe and coworkers (3), approximately 1 U/mg. Other anterior pituitary hormones were bovine growth hormone (GH), NIH-GH-B15, 0.88 U/mg; bovine luteinizing hormone (LH), NIH-LH-B7, 1.16 NIH-S1 U/mg; purified bovine LH, LER 1373, 1.6 NIH-S1 U/mg; ovine follicle stimulating hormone (FSH), NIH-FSH-S8, 1.15 U/mg; and ovine prolactin, NIH-P-S9, 30.3 U/mg; the TSH activities of these preparations were .010, .010, .003 and <.001 U/mg respectively. Exophthalmos-producing substance (EPS), which has served as a standard in the laboratory of Dr. Brown M. Dobyns (4), was of ovine origin; ⅛ mg. injected into fish, producing a striking response. It has some unspecified TSH activity. Adrenocorticotropin (ACTH), as the chromatographically purified porcine peptide (150–250 U/mg) was purchased from the Mann Research Laboratories. Non-pituitary peptide

TABLE 2. Effect of pituitary and placental hormones on rabbit articular chondrocytes

Hormone			DNA (μg/flask)		$^{35}SO_4$ (10^4 dpm/μg DNA)	
	Concentration					
Type	μg/ml	U/ml	Control	Treated	Control	Treated
TSH, bovine, Ciereszko	160	.08	38.1 ± 1.38	48.7 ± 1.16†	2.41 ± 0.07	1.16 ± 0.03†
			35.0 ± 0.69	45.4 ± 0.61†	1.67 ± 0.11	0.94 ± 0.07†
	640	.32	38.1 ± 1.38	53.5 ± 0.93†	2.41 ± 0.07	0.46 ± 0.01†
			35.0 ± 0.69	50.5 ± 0.31†	1.67 ± 0.11	0.43 ± 0.02†
TSH, bovine, NIH	35	.08	35.0 ± 0.69	80.8 ± 1.16†	1.67 ± 0.11	0.52 ± 0.02†
	70	.15	35.0 ± 0.69	87.9 ± 1.73†	1.67 ± 0.11	0.53 ± 0.02†
		.15	28.9 ± 1.08	92.1 ± 1.47†	—	—
		.15	36.4 ± 1.68	124.0 ± 1.89†	—	—
TSH, bovine, NIH, heated	70	.15	28.9 ± 1.08	34.5 ± 1.49§	—	—
TSH, bovine, Pierce	6	.15	36.4 ± 1.68	34.5 ± 1.73	—	—
TSH, bovine, Condliffe-Bates	8	.15	36.4 ± 1.68	34.7 ± 0.92	—	—
TSH, ovine, NIH	70	.15	28.9 ± 1.08	96.5 ± 1.34†	—	—
TSH, murine	70	.07	26.9 ± 0.97	33.3 ± 1.71§	1.31 ± 0.05	1.00 ± 0.05§
LH, bovine, NIH	70	.08	36.4 ± 1.68	90.8 ± 1.38†	—	—
LH, bovine, LER 1373	50	.08	33.4 ± 0.59	59.9 ± 0.87†	2.29 ± 0.08	1.01 ± 0.03†
GH, bovine, NIH	10	.0088	27.4 ± 0.85	28.5 ± 0.67	—	—
			31.4 ± 1.43	30.4 ± 1.13	2.23 ± 0.14	1.97 ± 0.11
	100	.088	27.4 ± 0.85	34.2 ± 0.53†	—	—
			31.4 ± 1.43	46.6 ± 2.25†	2.23 ± 0.14	1.97 ± 0.09
ACTH, porcine, peptide	1	.2	31.7 ± 1.29	31.8 ± 1.14	1.90 ± 0.08	1.87 ± 0.09
	10	2	31.7 ± 1.29	27.7 ± 0.85	1.98 ± 0.23	2.01 ± 0.09
Prolactin, ovine, NIH	70	2.12	21.9 ± 0.71	25.3 ± 1.48	1.78 ± 0.07	1.76 ± 0.04
EPS, Dobyns	70	*	21.9 ± 0.71	25.0 ± 1.78	1.78 ± 0.07	1.49 ± 0.17
FSH, ovine, NIH	70	.08	26.9 ± 0.52	40.3 ± 0.90†	2.25 ± 0.07	0.97 ± 0.03†
HCG, Sigma	70	200	27.3 ± 0.97	67.9 ± 1.65†	2.77 ± 0.14	0.70 ± 0.02†
HCG, Organon	13	67	33.4 ± 0.59	31.5 ± 0.68	2.29 ± 0.08	2.35 ± 0.15
	15	200	50.1 ± 0.31	46.7 ± 0.30†	—	—
HPL	70	.7	26.9 ± 0.52	29.9 ± 0.70§	2.25 ± 0.07	1.72 ± 0.08†

* 1/8 mg produces distinct exophthalmos in fish.
† $p < .001$.
§ $p < .01$.

TABLE 3. Effect of TSH on various cell types

Cell type	DNA (μg/flask) Control	TSH*	Δ (%)
Articular chondrocyte, rabbit	28.9 ± 1.38	92.1 ± 1.46	+218
Articular chondrocyte, human	4.0 ± 0.54	11.2 ± 0.38	+181
Skin fibroblast, rabbit	38.3 ± 0.95	51.8 ± 1.90	+ 35
HeLa	37.4 ± 1.72	48.0 ± 1.18	+ 28
VERO	83.2 ± 3.56	94.1 ± 4.15	+ 13
F26-10C	23.6 ± 0.60	25.4 ± 0.30	+ 8
FTRL-4	4.4 ± 1.29	3.1 ± 0.97	†

* Bovine TSH, NIH, 0.15 U/ml.
† These cells, originally maintained in a mixture of F12 and 199 media, did not thrive when passed into the Dulbecco medium used here.

hormones studied (Table 4) included chromatographically purified bovine parathyroid hormone (PTH), 1,000 U/mg (precaution was taken to avoid contact with metal which might inactivate the hormone); crystalline beef zinc insulin, lot T2842, 25.2 U/mg, Lilly; human chorionic gonadotropin (HCG) 2,864 U/mg, Sigma Chemical Company; a highly purified HCG, 13,000 U/mg, Organon batch No. 3; human placental lactogen (HPL), 95% pure, 10 U/mg, Nutritional Biochemicals; relaxin, 2,300 U/mg, Warner-Lambert; porcine calcitonin, Armour, lot K423-079, 88 MRC U/mg peptide. The steroid hormones (Table 5) as well as l-thyroxine, l-norepinephrine, serotonin hydrogen oxalate and dl-isoproterenol were obtained from commercial suppliers. The steroid hormones were made up in a stock solution of propylene glycol.

Other controls employed included biological extracts (Table 6); phytohemagglutinins M and P (DIFCO), pokeweed mitogen and 50% beef embryo extract (GIBCO); and a proprietary extract of bovine cartilage and bone marrow (Rumalon, Lot #04101, Robapharm, Basel, specially prepared without preservative), which has been found by some investigators to increase synthesis of sulfated mucopolysaccharides by cartilage (5). Cyclic 3′–5′ adenosine monophosphate and its dibutyryl ester were purchased from the Sigma Chemical Company, and bovine serum glycoprotein (less than 25% carbohydrate) from Nutritional Biochemicals. The experiment with cyclic AMP was repeated using a second brand of the compound (Calbiochem, A reagent).

Control media were modified by addition of

TABLE 4. Effect of non-steroidal hormones on rabbit articular chondrocytes

Hormone Type	Concentration μg/ml	M	U/ml	DNA (μg/flask) Control	Treated	$^{35}SO_4$ (10^4 dpm/μg DNA) Control	Treated
l-Thyroxine	0.25	3×10^{-7}		37.3 ± 0.91	38.4 ± 0.70	2.45 ± 0.10	2.22 ± 0.15
				37.5 ± 0.86	39.6 ± 0.92	1.47 ± 0.09	1.99 ± 0.08†
	1	1.3×10^{-6}		37.3 ± 0.91	42.4 ± 0.68†	2.45 ± 0.10	2.03 ± 0.06§
				37.5 ± 0.86	39.6 ± 0.80	1.47 ± 0.09	2.12 ± 0.08†
				27.3 ± 1.24	27.0 ± 0.92	1.37 ± 0.08	1.65 ± 0.11
Insulin	0.4	2.5×10^{-8}	.01	27.4 ± 0.85	29.0 ± 0.64	2.56 ± 0.19	2.51 ± 0.09
				38.3 ± 0.77	38.8 ± 0.78	1.89 ± 0.12	1.77 ± 0.14
	4	2.5×10^{-7}	0.1	27.4 ± 0.85	30.4 ± 0.79	2.56 ± 0.19	2.48 ± 0.17
				38.3 ± 0.92*	45.0 ± 1.50*	1.91 ± 0.13*	2.09 ± 0.16*
				22.6 ± 1.16	27.4 ± 1.25	1.77 ± 0.05	2.08 ± 0.11
Parathyroid Hormone	0.5		0.5	27.4 ± 0.85	26.6 ± 0.66	—	—
				25.7 ± 1.07	23.5 ± 0.65	1.35 ± 0.09	1.49 ± 0.08
	2.0		2.0	27.4 ± 0.85	26.3 ± 1.18	—	—
				25.7 ± 1.07	21.6 ± 0.59§	1.35 ± 0.09	1.73 ± 0.11§
Calcitonin	2.3		0.2	22.7 ± 0.84	25.5 ± 1.52	1.52 ± 0.06	1.33 ± 0.07
Relaxin	1		0.4	40.0 ± 1.26	39.9 ± 1.22	2.24 ± 0.10	2.21 ± 0.07
	10		4.4	40.0 ± 1.26	38.9 ± 1.20	2.24 ± 0.10	1.94 ± 0.09
l-Norepinephrine HCl	0.1	4.8×10^{-7}		26.9 ± 0.52	29.1 ± 0.73	2.25 ± 0.07	1.97 ± 0.06†
	1.0	4.8×10^{-6}		26.9 ± 0.52	29.3 ± 1.08	2.25 ± 0.07	2.29 ± 0.07
Serotonin H · oxalate	2.6	10^{-5}		25.7 ± 1.07	26.4 ± 0.92	1.35 ± 0.09	1.13 ± 0.08
dl-Isoproterenol HCl	1	3.8×10^{-6}		33.4 ± 0.59	32.4 ± 0.71	—	—

* 2 specimens only.
† $p < .001$.
§ $p < .01$.

TABLE 5. Effect of steroid hormones on rabbit articular chondrocytes

Hormone			DNA (μg/flask)		$^{35}SO_4$ (10^4 dpm/μg DNA)	
	Concentration					
Type	μg/ml	M	Control	Treated	Control	Treated
Estradiol-17β	1	3.7×10^{-6}	31.0 ± 0.58	29.4 ± 0.94	2.20 ± 0.14	1.80 ± 0.10
			31.5 ± 1.58	28.4 ± 1.09	1.41 ± 0.10	0.94 ± 0.08§
			40.0 ± 1.26	40.7 ± 0.45	2.24 ± 0.10	2.00 ± 0.10
	10	3.7×10^{-5}	31.0 ± 0.58	9.5 ± 0.66†	2.20 ± 0.14	2.60 ± 0.24
			31.5 ± 1.58	7.8 ± 0.31†	1.41 ± 0.10	1.26 ± 0.07
Diethylstilbesterol	10	3.7×10^{-5}	23.6 ± 0.66	3.9 ± 0.73†	1.23 ± 0.08	4.16 ± 0.75§
Testosterone propionate	1	2.9×10^{-6}	31.0 ± 0.58	31.9 ± 0.63	2.20 ± 0.14	2.09 ± 0.14
			31.5 ± 1.58	29.9 ± 0.94	1.41 ± 0.10	1.09 ± 0.08
	10	2.9×10^{-5}	31.0 ± 0.58	32.0 ± 0.76	2.23 ± 0.14	2.36 ± 0.19
			31.5 ± 1.58	27.7 ± 0.94	1.41 ± 0.10	1.04 ± 0.10
Progesterone	1	3.2×10^{-6}	31.0 ± 0.58	32.0 ± 0.81	2.20 ± 0.14	2.11 ± 0.06
			31.5 ± 1.58	26.8 ± 0.64	1.41 ± 0.10	1.08 ± 0.13
	10	3.2×10^{-5}	31.0 ± 0.58	21.9 ± 0.70†	2.20 ± 0.14	2.35 ± 0.10
			30.0 ± 1.09	18.9 ± 0.49†	1.40 ± 0.07	1.89 ± 0.08†
Cortisol	1	2.8×10^{-6}	30.0 ± 1.09	28.5 ± 0.73	1.40 ± 0.07	1.31 ± 0.09
	10	2.8×10^{-5}	30.0 ± 1.09	31.7 ± 1.18	1.40 ± 0.07	0.72 ± 0.04†
			27.9 ± 0.75	25.3 ± 0.68	2.29 ± 0.08	1.76 ± 0.05†
Dexamethasone	10	2.5×10^{-5}	20.3 ± 0.99	17.8 ± 1.03	1.90 ± 0.09	1.68 ± 0.06
Deoxycorticosterone	1	3.0×10^{-6}	30.0 ± 1.09	30.9 ± 0.63	1.40 ± 0.07	1.23 ± 0.07
	10	3.0×10^{-5}	30.0 ± 1.09	29.7 ± 0.82	1.40 ± 0.07	1.23 ± 0.08
Relaxin + Estradiol-17β	1 1		40.0 ± 1.26	40.7 ± 0.65	2.24 ± 0.10	1.92 ± 0.10
Relaxin + Estradiol-17β	10 1		40.0 ± 1.26	39.8 ± 0.74	2.24 ± 0.10	2.02 ± 0.08

† $p < .001$.
§ $p < .01$.

acid, base or propylene glycol in quantities comparable to those used to dissolve the various hormones. These additives usually did not change the values from untreated controls. Adjustments were made for additional quantities of antibiotics in the embryo extracts as well as for its inorganic sulfate content. The latter was found to be 8.8 mg per 100 ml by Dr. William

TABLE 6. Effect of miscellaneous controls on rabbit articular chondrocytes

Factor used				DNA (μg/flask)		$^{35}SO_4$ (10^4 dpm/μg DNA)	
	Concentration						
	% v/v	μg/ml	M	Control	Treated	Control	Treated
Phytohemagglutinin-M	1.5			28.9 ± 1.08	21.9 ± 1.05†	—	—
Pokeweed mitogen	1.0			28.9 ± 1.08	25.0 ± 0.99	—	—
Bovine embryo extract	1			42.1 ± 0.92	55.3 ± 2.53	1.79 ± 0.14	1.56 ± 0.06§
	2			20.0 ± 1.43	43.4 ± 1.88†	—	—
	2			42.1 ± 0.92	59.4 ± 1.67	1.79 ± 0.14	1.59 ± 0.09
Cartilage-bone marrow extract	1			28.5 ± 0.67	28.4 ± 0.98	3.24 ± 0.13	3.09 ± 0.10
				30.5 ± 0.27	30.6 ± 1.22	2.49 ± 0.05	2.18 ± 0.11§
	2			28.5 ± 0.67	27.7 ± 1.05	3.24 ± 0.13	2.69 ± 0.11§
				30.5 ± 0.27	29.9 ± 0.81	2.49 ± 0.05	2.19 ± 0.07§
Bovine serum glycoprotein		70		26.9 ± 0.97	27.7 ± 1.63	1.31 ± 0.05	1.41 ± 0.07
Cyclic AMP		453	1.4×10^{-3}	21.9 ± 0.71	3.2 ± 0.36†	1.96 ± 0.07	2.22 ± 0.41
				50.1 ± 0.31	6.5 ± 0.47†	—	—
Dibutyryl cAMP		515	1.1×10^{-3}	21.9 ± 0.71	6.7 ± 0.61†	1.96 ± 0.07	1.89 ± 0.20
Phenol		3.5	3.7×10^{-5}	29.8 ± 0.50	29.7 ± 0.77	2.76 ± 0.13	2.54 ± 0.10

§ $p < .01$.
† $p < .001$.

C. Alford. The cartilage-bone marrow extract was hypotonic ($\Delta t = -.16$ to $-.18°$ C), and contained 5.6 mg inorganic sulfate/100 ml (data furnished by manufacturer). Accordingly NaCl and Na_2SO_4 were added to make the tonicity and sulfate content of the control and experimental media comparable. All preparations were sterilized by filtering the stock solutions through a Millipore Swinnex filter, pore diameter 0.45 μ. The concentration of the hormones in the control culture media was unknown except in the case of thyroxine; there were less than 14.5 μg/100 ml fetal calf serum (Medical Research Consultants).

Chemical procedures. The quantity of cells in each flask was determined from the DNA content by a slight modification of Burton's method (6). The chondrocytes were detached with trypsin. Gey's solution supplemented with .002% soybean trypsin inhibitor was added and the cells were centrifuged at 2400 rpm for 3 minutes in the cold. The pellet was washed with Gey's solution containing 25% serum, recentrifuged, and stored frozen. They were extracted twice with 1.5 ml of 0.9M $HClO_4$ at 70° C for 15 minutes before the color reaction was developed. The DNA content of rabbit chondrocytes and fibroblasts, counted on sister flasks with an eyepiece micrometer as previously (2), was found to be 9.72 ± 0.33 s.e. μg/10^6 nuclei. This value is higher than that for native rabbit cells obtained by other chemical methods (7,8) but somewhat below certain other cultured diploid mammalian cells measured by Burton's diphenylamine procedure (9,10).

Synthesis of sulfated macromolecules was measured from radioactivity of the culture medium following dialysis for 4 hours against 0.1M $(NH_4)_2SO_4$ and then running tap water overnight. 0.4 ml of the sample was added to 10 ml of scintillation fluid (2% Liquifluor ((Beckman Instruments)) in 1:1 v/v toluene-ethylene glycol monomethyl ether ACS) for counting. Quench and decay corrections were made and the data are reported as dpm.

Results

DNA synthesis. The observation that a pituitary factor has a mitogenic effect on articular chondrocytes was first made on the crude Ciereszko preparation of bovine TSH at a concentration of .08 and .32 U/ml (Table 2). A considerably greater response was obtained when NIH bovine or ovine TSH having a higher specific activity was employed. In concentrations of .15U (70 μg)/ml, the increase of DNA in three different cultures were 150, 218 and 221% respectively. The increase was first apparent microscopically by 72 hours of incubation when the cells became elongated and mitotic forms increased (Fig. 1). The number of cells, counted in a few flasks, paralleled the DNA content. The response was dose-dependent and first detectable at a concentration of 1 μg NIH bovine TSH/ml (Fig. 2). The factor responsible for the increased growth was heat labile. It presumably was not TSH proper because highly purified preparations of Pierce and of Condliffe and Bates elicited no similar response. NIH LH and crude HCG also had a marked mitogenic effect at similar concentrations in terms of weight. The more purified LH retained much of this activity, but highly purified HCG did not. FSH was less effective than TSH or LH while little or no response was obtained with bovine GH, "EPS," prolactin, ACTH, HPL or highly purified HCG.

Although several other cell types exhibited a mild increase of DNA following exposure to NIH TSH, the effect was many times greater on articular chondrocytes, lapine and human (Table 3).

Insulin at a level of 4 μg (0.1 U)/ml had a consistent though mild stimulatory effect on chondrocyte growth, but relaxin, thyroxine, PTH, calcitonin, l-norepinephrine and serotonin did not (Table 4). Among the steroid hormones studied depression of cell growth was sometimes found at high concentrations (10 μg/ml). It was most marked with estradiol-17β and diethylstilbesterol, and to a lesser extent with progesterone and dexamethasone (Table 5). Cyclic AMP and dibutyryl cyclic AMP proved toxic to the cells (Table 6). The several mitogens for lymphocytes were ineffective in this system as was the cartilage-bone marrow extract. Phytohemagglutinin P (0.2% v/v) caused the chondrocytes to adhere tightly to the

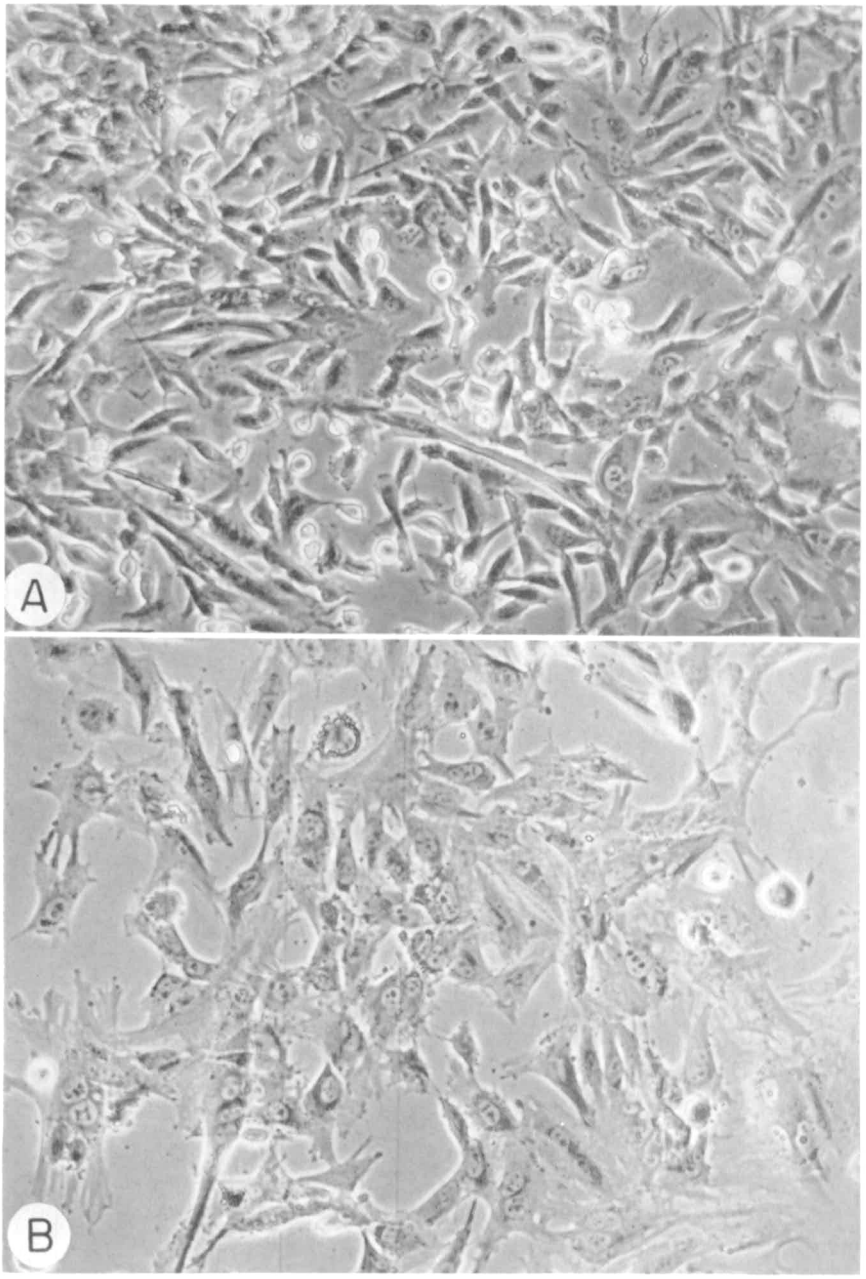

FIG. 1. Rabbit articular chondrocytes, 72 hours incubation. A. Cells grown in medium containing .15 U bovine TSH (NIH) per ml, are more numerous and fusiform than the polyhedral controls (B). Some of the chondrocytes in both groups are binucleate. The refractile round bodies are cells that are dividing and not yet reattached to the surface of the flask. Phase contrast, ×160.

culture flasks; trypsin treatment even overnight at 37°C failed to detach them and the DNA content could not be measured. There was no increase in the cell numbers so far as microscopic scanning could detect. By contrast, bovine embryo extract had an adjuvant effect although considerably below that of the pituitary preparations. Bovine serum glyco-protein, however, did not.

Radiosulfate incorporation. In each instance of mitogenic stimulation, the radioactivity of the dialyzed culture medium (10^4 dpm $^{35}SO_4$/μg DNA) was greatly reduced (Table 2). The reduction was roughly proportional to the increase in the DNA. To determine whether the reduction resulted from a redistribution of extracellular mucopolysaccharide to an adherent cell coat, radioac-

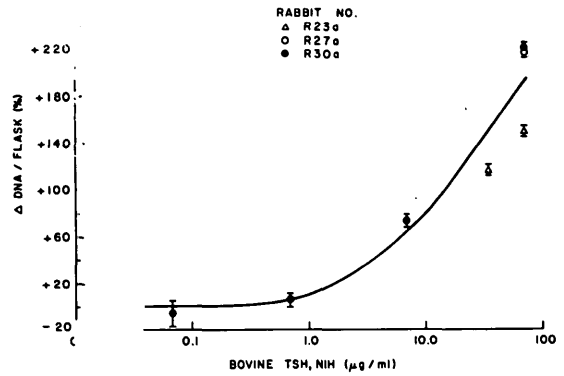

FIG. 2. e response curve of articular chondrocytes t· ·ine TSH (NIH). Data were obtained on succ e cultures from three different rabbits. The bra s on either side of the means enclose ± 2 stan d errors.

tivity was measured also in the trypsin solution used to detach the cells from the flask in one experiment. The counts were low in the trypsin solution as well as in the culture medium.

The steroid preparations used generally had little effect on the amount of sulfated macromolecules in the culture media. Several, including 2.8×10^{-5}M cortisol, reduced it somewhat. Diethylstilbesterol, unlike estradiol-17β, increased the values greatly (Table 5). There was a slight increase in response to PTH, and also to 1 μg/ml thyroxine in two of three experiments (Table 4).

Discussion

Holly and Kiernan (11) made passing mention in another connection that commercial TSH and HCG increased cell division of 3T3 cells. In a personal communication, Dr. Holley has advised us that the concentration of TSH used was .02 U/ml, and that more purified NIH TSH did not have this effect. With this exception, we have found no reports dealing with this phenomenon. The concentration of NIH TSH employed by others (12,13) to effect follicle-like differentiation of thyroid cells in monolayer culture is comparable to that described here. The levels are very high and unphysiological in terms of known pituitary hormone values in the serum (14). Nevertheless we do not know the concentration of the chondrocyte growth factor in the preparations employed and conceivably it may be quite small. Even so, a distinct mitogenic effect was elicited by as little as 1 μg NIH TSH/ml. The evidence is that the active principle is not TSH itself inasmuch as the highly purified preparations, having 9 to 12 times the specific activity of the NIH material, were ineffective in this regard.

The growth-promoting factor is not GH; the bovine GH obtained from the NIH, even in greater concentrations than those used for TSH, LH and HCG, caused only a small increase in the DNA content of the flasks. The GH preparation was contaminated with some TSH activity—at the concentrations used, .001 U/ml of culture medium, equivalent in turn to .45 μg of NIH TSH/ml. Corresponding values for contamination with NIH LH were .0016 U and 1.3 μg respectively. Whatever mitogenic effect the GH had may thus likely be attributable to cocontamination with the factor in the NIH TSH and LH preparations.

Although GH is a powerful stimulator of skeletal and connective tissue (including articular cartilage) growth *in vivo*, there is much though inconsistent evidence that it does not have this action *in vitro* (15–22). The so-called sulfation factor which appears in the serum of hypophysectomized animals following administration of GH has variously been speculated to be a core component of GH or a new compound elaborated as a result of a more remote action of GH on the liver (17,23). The *in vitro* use of primary isolated, though not cultured, chondrocytes has recently been proposed for assaying sulfation factor (24). The present pituitary mitogen is not sulfation factor insofar as it had a marked suppressive rather than stimulatory effect on the net production of sulfated mucopolysaccharide. HPL, which has certain immunologic and physiological similarities to GH, has much less effect than GH in stimulating thymidine incorporation into hypophysectomized rat cartilage *in vitro*

(25,26). It failed to increase proliferation of chondrocytes in monolayer culture.

The pituitary and chorionic (27) hormones in the preparations displaying mitogenic activity are glycoproteins, closely related to each other chemically, immunologically and physiologically. TSH and LH share a similar peptide subunit (28). There are in pituitary extracts quantities of presently unidentified glycoprotein (29). It is tempting to speculate whether the present growth factor is part of this glycoprotein and conceivably a hormone. Glycoprotein growth promoters for other *in vitro* cell systems have been described (30).

An alternative possibility is that the mitogenic factor in the hormonal preparations is a contaminating enzyme. There is evidence that certain other tissue growth factors are esteropeptidases (31). A spectrum of peptidases has been identified in anterior pituitary extracts (32). Most of these are in the fraction rich in FSH, and plasmin is found in GH preparations. Proteolytic activity has apparently not been reported in the glycoprotein preparations studied here. If the chondrocyte growth factor is indeed a contaminant enzyme, it must have a specificity different from the others indicated. The fact that crude urinary HCG was effective may militate against this hypothesis because large contaminant enzymes are unlikely to pass the glomerular barrier.

Of the other hormones employed, only insulin at the higher concentrations (0.1 U/ml) had a distinct though small growth promoting action. Insulin is known to support growth in some though not all other cell culture systems. This function is more obvious in serum-free media which provide no other source of insulin than those, which like the present one, do contain serum (33–35).

Corticosteroid hormones frequently reduce growth and mucopolysaccharide synthesis in other connective tissue cell systems although the effects differ greatly depending on the dosage employed and whether or not serum is present in the medium. Even at concentrations in excess of 2×10^{-5}M, they had little effect in reducing proliferation of the chondrocytes here. In striking contrast to the corticosteroid response, was the marked inhibitory effect of estradiol-17β, diethylstilbesterol, and a somewhat less marked one of progesterone. The sensitivity of the chondrocytes to these steroids differs markedly from that of certain other cell types (36) and is thus not a non-specific cytotoxicity. Because the estrogens are phenolic compounds and this might be the source of their cytotoxicity, phenol was added to control cultures at a comparable concentration (3.7×10^{-5}M); it did not inhibit cell division.

Several other hormones studied have well documented physiological effects on cartilage or other skeletal tissues *in vivo*. Relaxin has apparently not been studied in tissue cultures previously; it had no effect on the chondrocytes. A commercial preparation of PTH has been reported to change the aggregation and stainable sulfated product of embryonic mouse chondrocytes in monolayer culture (37). The purified hormone employed here at a comparable dosage in terms of units had no detectable effect on cell proliferation but did increase the quantity of sulfated macromolecules in the culture medium significantly. Serotonin, which has been reported to stimulate growth of cultured fibroblasts (38), was ineffective at a concentration of 10^{-5}M on articular chondrocytes.

Phytohemagglutinin M and pokeweed mitogen had no growth-promoting effect on the chondrocytes, nor did bovine serum glycoprotein. Cyclic AMP and dibutyryl cyclic AMP in the high concentrations often required to simulate peptide hormone responses had a pronounced cytotoxic effect. Similar findings have been noted by others studying HeLa and L cells (39). This response to cyclic AMP was observed with two different brands of the compound. It may well be due to phosphodiesterase activity of the serum in the medium since Johnson and coworkers (40) have reported

adenosine monophosphate to be cytotoxic. These same investigators found that dibutyryl cyclic AMP, although less susceptible to such hydrolysis, often contains toxic quantities of butyric acid. Dr. Johnson has tested the effect of the dibutyryl compound from which the butyric acid was extracted on our chondrocytes and found a much smaller (20 percent) reduction in the number of cells. These data indicate that the mitogenic response of articular chondrocytes to the glycoprotein hormone preparations is not mediated by the cyclic AMP mechanism operating when the latter act on their specific endocrine targets.

The increase in the number of chondrocytes was consistently associated with a marked reduction in the macromolecular radiosulfate incorporation. On the assumption that there was no other change in the sulfate pool, this indicates a reduction in the net quantity of sulfated mucopolysaccharide they synthesized and secreted into the culture medium. The reduction in the radioactivity of the latter was not caused by a diversion of the radiosulfate to a mucopolysaccharide cell coat. The pituitary factor thus has a mitogenic but not a differentiation-favoring effect on the chondrocytes. This may be another example of the usual dissociation between the ability of cells to divide and to synthesize specialized cell products. There are some exceptions to this principle in other connective tissue cells (41), and its validity in the case of chondrocytes in cell culture is debated (42,43). Crowding of cells in vitro sometimes favors differentiation of cells but Griffiths (35) suggests that it may also reduce the efficiency of transport of nutrients across cell membranes. Crowding of articular chondrocytes during spontaneous unaccelerated growth is not associated with a reduction of sulfated mucopolysaccharide secretion (Table 1). The reduction during the hormonally stimulated mitogenesis probably is related, for this reason, to the increased rate of growth rather than the crowded state proper.

There is little information on the effect of these hormones on articular cartilage *in vivo*. Thyrotropin injected into young puppies is said to stimulate growth of the epiphyseal cartilage of the femoral head (44). It also has been reported to increase radiosulfate incorporation by cartilage as well as other soft tissues of guinea pigs (45). There are a number of presently unexplained clinical disorders of joints in which endocrine factors seem to play a role. These include Heberden's nodes and degenerative joint disease (46) and rheumatic complaints associated with hypothyroidism (47). In one paper, menopausal osteoarthritis was associated with elevated levels of LH, TSH and FSH in the serum (48). These data are difficult to evaluate because the control groups were not matched for age and interval following menopause. Furthermore there were increased titers of GH and other pituitary hormones as well. Urinary excretion of gonadotropins was no greater in elderly women who had Heberden's nodes than in those who did not (49).

The biological and clinical implications of the present observations must await further characterization of the chondrocyte growth factor. We are carrying out studies to this end.

Addendum

We have recently tested and found moderate chondrocyte growth-promoting activity in ovine LH (NIAMD).

Acknowledgments

We are indebted to Dr. Robert W. Bates for invaluable advice throughout this study; and to the following for generous contributions of hormones: the Hormone Distribution Program of the National Institute of Arthritis and Metabolic Diseases for the preparations indicated in the text; Dr. Gerald Aurbach, PTH; Dr. J. W. Bastian, calcitonin; Dr. Bates, Czereszko and murine TSH; Dr. Brown M. Dobyns, EPS; Dr. Harold Edelhoch, Condliffe-Bates TSH; Dr. W. P. Koella, rumalon; Dr. J. M. McGuire, insulin; Dr. Frank Morgan, purified HCG; Dr. J. G. Pierce, TSH; and Dr. Leo Reichert, Jr., LH, LER 1373. The control cell lines were donated by Drs. Paul Price, F26-10C; Alan S. Rabson, VERO; and Gary Williams, FTRL-4. Michael D. Sokoloff programmed the statistical calculations, Walter G. P. Seewald made the photomicrographs, and Sister Olga Hernandez gave technical assistance.

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