densation (concentration) of the secretory product. While this difference in concentration cannot be ruled out as the source of the observed asymmetry, it does not seem the most likely mechanism at present.

Since filipin binds to cholesterol, a possible interpretation of the change in polarity of filipin-induced deformations between the condensing vacuole and the zymogen granule membrane is that it is caused by a change in cholesterol partitioning between the two leaflets accompanying the transformation of condensing vacuoles into zymogen granules. If this is so, filipin may be the first morphological marker of membrane lipid asymmetry, and the relation between structural polarity and cholesterol partitioning polarity should be established.

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6. Condensing vacuoles represent a station at which secretory pancreatic enzymes migrating from their site of synthesis (the rough endoplasmic reticulum) to their site of release (the plasma membrane) start to be concentrated; zymogen granules, deriving from condensing vacuoles, are intracellular storage sites for enzymes until they are released by exocytosis [reviewed in G. Palade, *Science* **189**, 347 (1975)].
7. Fragments of rat pancreas were minced into very small pieces (< 1 mm), fixed in 2 percent glutaraldehyde in 0.1*M* cacodylate buffer, *p*H 7.4, for 60 minutes, and further incubated overnight at room temperature in the same buffer containing filipin (0.2 mg/ml; provided by J. E. Grady, Upjohn Co.) dissolved in dimethyl sulfoxide (final concentration of DMSO, 1 percent). After exposure to filipin, the pieces of pancreas were washed in 0.1*M* cacodylate buffer, soaked in the same buffer containing 30 percent glycerol (by volume) for 2 hours, and freeze-fractured at −110°C in a Balzers BAF 301 apparatus. Replicas were cleaned with sodium hypochlorite followed by dimethyl formamide, rinsed in distilled water, and recovered on Parlodion-coated copper grids (150 mesh). Freeze-fracture replicas were examined in a Philips EM 300 electron microscope.
8. We evaluated 36 micrographs of replicas showing fracture faces of zymogen granule and condensing vacuole membrane at a suitable magnification. The surface area and number of deformations (pits and protuberances) on regions of fractured membrane that appeared to be roughly perpendicular to the viewing axis were recorded on a graphic tablet (Tektronix, type 4953) connected to a microprocessor (IMSAI, type 8080) programmed to calculate the number of protuberances (or pits) per square micrometer of membrane.
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Stimulation of DNA and Collagen Synthesis by Autologous Growth Factor in Cultured Fetal Rat Calvaria

Abstract. *Conditioned medium derived from organ or cell cultures prepared from 19- to 21-day fetal rat calvaria stimulated the incorporation of [^{3}H]proline into collagen and of [^{3}H]thymidine into DNA in organ cultures of the same tissue. Addition of cortisol enhanced the effect on collagen but not on DNA synthesis. These effects appeared to be due to a nondialyzable and heat-stable growth factor.*

The regulation of bone remodeling is incompletely understood. The importance of local factors is suggested by the close linkage between resorption and formation during remodeling and by skeletal responsivity to deforming forces. Since nonskeletal tissues and cells elaborate growth factors when maintained in culture in vitro (*1*, *2*), we have searched for such regulatory factors in bone. We report that conditioned medium obtained from fetal calvaria in organ or cell culture contains a growth factor which stimulates the incorporation of [^{3}H]proline into bone collagen in the presence of cortisol and increases the incorporation of [^{3}H]thymidine into DNA both in the presence and absence of cortisol.

The growth factor was obtained from the incubation medium of fetal rat calvaria maintained in organ or in cell culture according to techniques previously reported (*3*, *4*). In the organ culture model, half-calvaria from 21-day fetal rats were cultured in BGJ_b medium (*3*) without serum or albumin for periods of 96 hours in a shaking incubator. The medium was changed every 24 hours, pooled, and stored at 4°C. In the cell culture model, cells from 19- to 21-day-old fetal rat calvaria were dispersed by incubation in crude collagenase (Worthington Biochemical) after we removed the periosteum. Cells were washed and cultured at an initial density of 10^5 cells per square millimeter of surface area in modified BGJ_b medium without serum or albumin (*5*). The medium was replaced at 24 hours of culture and every 2 to 4 days thereafter. The medium from 6- to 12-day-old cultures was pooled and stored at 4°C.

Conditioned medium was dialyzed exhaustively against distilled water or 0.05*M* acetic acid at 4°C and lyophilized. The conditioned medium from organ culture was further purified by gel filtration chromatography. Ten milligrams of lyophilized, dialyzed, conditioned medium containing 5 mg of protein (*6*) were obtained from 100 ml of medium and chromatographed on a column of Sephadex G-75 (Pharmacia, Uppsala, Sweden) in 1*M* acetic acid at room temperature. Eluates were lyophilized before assay. Conditioned medium from cell cultures was purified by ion exchange chromatography by a modification of the method described by Dulak and Temin (*1*). Medium (500 ml) adjusted to *p*H 6.3 at room temperature was loaded onto a Dowex 50W-X8 column (4.5 by 30 cm; Bio-Rad), equilibrated with 0.15*N* NaCl, and eluted with a sequential gradient of 0.1*N* sodium carbonate and 0.15*N* NaCl (*p*H 9), 0.1*N* ammonia and 0.15*N* NaCl (*p*H 11), and 1*N* NaOH and 0.15*N* NaCl. The eluates were dialyzed and lyophilized before assay.

The effects of the growth factor on bone formation were studied in organ culture as previously described (*3*). Half-calvaria from 21-day fetal rats were cultured in modified BGJ_b medium supplemented with bovine serum albumin (1 to 4 mg/ml; Reheis Chemical), 1 m*M* proline, and 0.1 m*M* thymidine under 5 percent CO_2 in a shaking incubator at 37°C for 24 hours. Collagen and noncollagen protein synthesis were studied by adding [2,3-^{3}H]proline (5 μCi/ml; specific activity 20 to 30 Ci/mmole; New England Nuclear) for the last 2 hours of the culture period. The calvaria were extracted with trichloroacetic acid, acetone, and ether;

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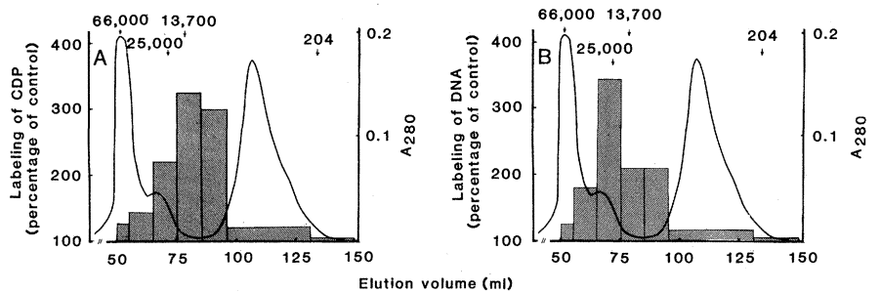

Fig. 1. Gel filtration chromatography of the growth factor derived from cultured calvaria. The solid line represents absorbance at 280 nm. Shaded areas represent the stimulation of the incorporation of (A) [^{3}H]proline into collagenase-digestible protein (*CDP*) and (B) [^{3}H]thymidine into acid-insoluble residues (*DNA*). Chromatographic fractions were tested in the presence of cortisol, 100 n*M*, and data are expressed as percentages of control. The arrows at the top of the chromatograms represent the peak elution volumes of bovine serum albumin (molecular weight 66,000), chymotrypsinogen (molecular weight 25,000), ribonuclease A (molecular weight 13,700), and tryptophan (molecular weight 204).

they were then weighed and homogenized, and the labeled proline incorporated into collagenase-digestible protein (CDP) and noncollagen protein (NCP) was measured (*7*). DNA synthesis was determined by adding [*methyl*-^{3}H]thymidine (5 μCi/ml; specific activity 65 to 80 Ci/mmole; ICN Radiochemicals) during the last 60 minutes of culture and measuring the radioactivity incorporated into acid-insoluble residues. Total DNA was measured by fluorometry (*8*).

Calvaria cultured with conditioned medium obtained from calvaria in organ culture showed increased incorporation of [^{3}H]proline into CDP and, to a lesser extent, into NCP in the presence of cortisol but not in its absence (Table 1, experiment A). The effect of cortisol was maximum at 100 n*M* but could be seen at concentrations as low as 0.1 n*M*. In contrast, conditioned medium stimulated the incorporation of [^{3}H]thymidine in the absence or in the presence of cortisol. The DNA content was increased by conditioned medium from control values of 8.1 ± 0.7 (mean ± standard error; $N = 5$) to 12.4 ± 0.3 μg per half-calvarium ($P < .05$). The stimulatory activity for CDP, NCP, and DNA was released by calvaria after 6 hours of culture and was sustained for 96 hours.

If the conditioned medium from organ culture was dialyzed with a membrane having a molecular weight (MW) cutoff of 3500, or was lyophilized or heated at 56°C for 30 minutes, the stimulatory effects were not lost (data not shown). When we analyzed the conditioned medium by gel filtration chromatography, one peak that showed maximum stimulation of the incorporation of [^{3}H]proline into CDP eluted with and immediately after the protein marker ribonuclease A (MW 13,700) (Fig. 1A). The peak showing maximum stimulation of the incorporation of [^{3}H]thymidine eluted with chymotrypsinogen (MW 25,000) (Fig. 1B). Labeling of CDP and NCP was increased in the absence of cortisol, but the effect on CDP was enhanced in its presence. The effect on the incorporation of [^{3}H]thymidine was similar in the presence or absence of cortisol (Table 1, experiment B). The stimulatory effects on the labeling of CDP and DNA were maintained after treatment with ribonuclease but were lost after trypsinization.

Ion exchange chromatography of conditioned medium from cell cultures revealed two major peaks, which eluted at *p*H 9 and 11. Both stimulated the incorporation of [^{3}H]proline into CDP in the presence of cortisol but had a small inhibitory effect in its absence (Table 1, experiment C). Both fractions increased the incorporation of [^{3}H]thymidine and were more effective in the absence of cortisol.

The present studies indicate that conditioned medium from fetal calvaria in organ or cell culture contains stimulatory activity for bone collagen and, to a lesser extent, for NCP synthesis, which is cortisol-dependent, and for bone DNA synthesis, which is cortisol-independent. We do not know if this activity is due to a single growth factor, or to several factors. The peak of activity for stimulation of collagen and NCP synthesis elutes with proteins of a lower molecular weight than the peak of activity for the stimulation of [^{3}H]thymidine incorporation. Ion-exchange chromatography of cell culture medium is consistent with the presence of two acidic factors that have similar effects on the labeling of CDP, NCP, and DNA. The inhibition of CDP by these two factors in the absence of cortisol could be secondary to contamination by an inhibitor, or could be due to reduced expression of dif-

Table 1. Effect of the growth factor derived from cultured calvaria studied in the presence or absence of cortisol (100 n*M*) on the incorporation of [^{3}H]proline into collagenase-digestible protein (CDP) and noncollagen protein (NCP) and on the incorporation of [^{3}H]thymidine into acid-insoluble residues (DNA) in fetal rat calvaria. In experiment A, the effects of conditioned medium from calvaria in organ culture diluted 1:2 with fresh medium were compared to those of medium obtained from vessels incubated in the absence of bones (control medium). In experiment B, conditioned medium from bones in organ culture was dialyzed, lyophilized, and placed on a column of Sephadex G-75. Fractions were tested at 2.5 to 4 μg of protein per milliliter (*6*). The data represent the effect of fractions with maximum activity on the labeling of CDP, NCP, and DNA which eluted in volumes corresponding to protein markers with molecular weights of 10,000 to 20,000 (CDP and NCP) and of 20,000 to 35,000 (DNA). In experiment C, conditioned medium from cell cultures was dialyzed and partially purified by ion-exchange chromatography. Chromatographic fractions eluted at *p*H 9 and 11 were tested at a concentration of 1 μg of protein per milliliter. Values are means ± standard error for four to five half-calvaria.

Treatment	Radioactivity (dpm/μg dry weight)		
	CDP	NCP	DNA
	Experiment A		
Control	34 ± 3	48 ± 3	4.2 ± 0.2
Conditioned medium	38 ± 1	47 ± 2	9.9 ± 0.2*
Control plus cortisol	39 ± 2	36 ± 1	4.8 ± 0.4
Conditioned medium plus cortisol	67 ± 7*	58 ± 2*	11.9 ± 0.5*
	Experiment B		
Control	39 ± 2	45 ± 2	5.7 ± 0.3
Gel filtration fraction	60 ± 6*	69 ± 1*	13.3 ± 0.9*
Cortisol	37 ± 3	35 ± 3	3.7 ± 0.4
Gel filtration plus cortisol	92 ± 5*	61 ± 3*	14.4 ± 0.3*
	Experiment C		
Control	49 ± 4	53 ± 2	3.7 ± 0.3
*p*H 9 fraction	31 ± 2*	48 ± 4	9.4 ± 0.2*
*p*H 11 fraction	35 ± 1*	60 ± 2	10.5 ± 0.4*
Cortisol	44 ± 5	45 ± 2	3.1 ± 0.1
*p*H 9 fraction plus cortisol	62 ± 2*	61 ± 3*	5.6 ± 0.3*
*p*H 11 fraction plus cortisol	63 ± 3*	64 ± 7*	4.8 ± 0.3*

*Significantly different from respective control, $P < .05$.

ferentiated function during a period of cell replication or to different effects on separate types of target cells.

The effects of the bone-derived growth factor are similar to those previously described for the somatomedins (*9–11*). We found that the factors derived from cell cultures, tested at a variety of concentrations, did not stimulate the incorporation of ^{35}S into costal cartilage as the somatomedins do and did not have detectable somatomedin activity on radioreceptor assay (*12*). The fraction derived from organ cultures which eluted with chymotrypsinogen and stimulated thymidine incorporation did not have detectable sulfation activity. However, the fraction of lower molecular weight increased ^{35}S incorporation in hypophysectomized rat cartilage at 2 to 20 μg/ml and contained 1.1 to 1.4 ng of Somatomedin C per microgram of protein as determined by radioreceptor and radioimmunoassay (*13*). Thus, one of the activities appears to be related to the somatomedins while the others do not. Cortisol seems to play a permissive role in the expression of the effect of conditioned medium on collagen and NCP synthesis. This is consistent with the observation that glucocorticoids can enhance the differentiated function of osteoblasts in organ culture (*14*).

The presence of a growth factor in cultured fetal rat calvaria could provide a mechanism for stimulation of rapid skeletal growth during late fetal life. If similar factors are produced by adult bone, they could play a role in local responses to mechanical stress and in the coupling of new bone formation to prior bone resorption.

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13. Kindly performed by R. L. Hintz using the radioreceptor assay described by J. M. Horner and R. L. Hintz [*J. Clin. Endocrinol. Metab.* **48**, 959 (1979)] and the radioimmunoassay described by R. W. Furlanetto *et al.* [*J. Clin Invest.* **60**, 648 (1977)].
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Retinal Tumor Induced in the Baboon by Human Adenovirus 12

Abstract. *Three of 21 newborn baboons injected intraocularly with human adenovirus type 12 developed an intravitreal mass 12 to 36 months later. Two of the masses were indistinguishable from human retinoblastoma, a retinal tumor that afflicts children. To our knowledge this is the first time a retinoblastoma-like tumor has been induced experimentally by adenovirus type 12 in a nonhuman primate.*

Although the nature and morphology of human retinoblastoma, the most common tumor of the retina in infancy and childhood, are well established (*1*), neither spontaneously occurring nor experimentally induced retinal tumors in nonhuman primates has ever been described (*2*). Because baboons are among the most intensively studied of all nonhuman primates, and because the time sequence of their growth and aging parallels that of humans in many biologic respects (*3*), we attempted to induce retinal tumors in baboons. We used human adenovirus serotype 12 (Ad. 12) because of the results of two earlier studies: the pioneering work of Albert *et al.* (*4*), who demonstrated that young adult hamsters' neural retina cells could be transformed in vitro by Ad. 12; and our own production of retinoblastoma-like neoplasms in rodents with Ad. 12 (*5*).

Twenty-one baboons were each given a single intraocular inoculation of Ad. 12 (Fig. 1, A and B), 0.05 to 0.1 ml of 10^8 $TCID_{50}$ (50 percent tissue culture infective dose) per 0.1 ml in human embryo kidney (HEK) cells, in the right eye through a No. 30 G1/2 hypodermic needle. Their left eyes were not treated. The baboons were inoculated within 24 hours after birth; at this stage, baboons' retinas are well differentiated (Fig. 1C). At approximately 12 months after inoculation, one of the baboons exhibited a peculiar light reflection in its virus-inoculated eye (Fig. 1D). Two more baboons later showed the same reflection: one at 18 months after inoculation, the other at 36 months. Ultrasonographic scanning showed that all three baboons had an intravitreal mass. Twenty-four months after inoculation, a grayish-red mass was removed from the enucleated right eye of the first baboon. Part of the mass was fixed in 2.5 percent glutaraldehyde in phosphate buffer, *p*H 7.4, at 4°C for electron microscopy; part was fixed in Formalin for light microscopy; and part was frozen for tumor (T) antigen detection. Cell impressions on cover slips were also made for the T-antigen test. Paraffin sections 7 μm thick were cut from both glutaraldehyde- and Formalin-fixed specimens and stained with hematoxylin-eosin, thionin, periodic acid–Schiff, Luxol fast blue, and Holmes' silver impregnation.

Under low magnification, the intravitreal mass appeared to be composed of densely packed cells (Fig. 1E). A closer examination revealed dense, uniformly distributed retinoblastoma-like tumor cells with rosettes of various forms (Fig. 1F). In some areas, "Flexner-Wintersteiner" true rosettes predominated, but most transformed cells had the morphologic appearance typical of the neuroblastoma-like cells we observed in tumors induced by Ad. 12 in rodents (Fig. 1G).

Integration of Ad. 12 viral DNA into the transformed retinal cells was demonstrated by the presence of immunofluorescent Ad. 12–specific T antigens (Fig. 1H). Electron microscopy showed that the neoplastically transformed retinal cells had oval nuclei with evenly distributed chromatin and a scanty rim of cytoplasm with poorly organized intracellular organelles (Fig. 1I). Ciliated tumor cells (with a 9 + 0 tubule pattern) were common. These ultrastructural characteristics of the Ad. 12–induced

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