

## STIMULATION OF DNA SYNTHESIS AND CELL DIVISION IN VITRO BY SERUM FROM CHOLESTEROL-FED SWINE

R. A. FLORENTIN, B. H. CHOI, K. T. LEE, and W. A. THOMAS. From the Department of Pathology, Albany Medical College, Albany, New York 12208

### INTRODUCTION

The most prominent feature of early atherosclerosis induced in swine by ingestion of cholesterol is the focal accumulation of smooth muscle cells (SMC) in arterial intimas that results from increased rates of DNA synthesis and cell division without corresponding increases in rates of cell death (Florentin et al., 1968; Thomas et al., 1968). The nature of the interactions between the cells and their environment that account for the increases is unknown.

The purpose of the experiments reported here was to determine what effects serum from hypercholesterolemic (HC) swine has on DNA synthesis and the division of cells in tissue culture as compared with the effects of serum from controls with "normal" cholesterol levels (NC). A stimulatory effect occurred when HC serum was added at the

20% level, with cell division rates increasing approximately twofold. The *in vitro* demonstration of an effect that parallels *in vivo* responses does not necessarily mean that the underlying cellular dynamics are the same. However, it seems reasonable to accept as a working hypothesis that the dynamics are similar, and to exploit the *in vitro* approach presented herein, with the expectation that this approach will provide insight into cellular mechanisms involved in cholesterol-induced atherosclerosis.

### MATERIALS AND METHODS

The basic design of the experiments is indicated in Table I. For each culture four 2 mm<sup>2</sup> intima-media strips taken from the aortic arch were sandwiched between two coverslips and placed in Leighton tubes to which were added a mixture of Eagle's basal medium (BME) (Eagle, 1955) and HC or NC serum in

TABLE I  
Basic Information on Material Used for Study

	Experiment		
	1	2	3
No. of swine	2	2	2
Type of swine	Pitman-Moore	Yorkshire	Pitman-Moore
Time on diets	42 days	100 days	37 days
Weight of swine, initial/final			
HC swine, kg	4.7/9.9	6.4/23.6	5.0/9.5
NC swine, kg	4.5/10.7	8.0/25.9	4.0/7.5
Serum cholesterol level, mg HC/NC	248/91	888/96	265/73
No. of explants taken	202	180	72
Days original explant removed	7	7	7
Tissue-serum combination	N <sub>t</sub> N <sub>s</sub> , N <sub>t</sub> H <sub>s</sub> H <sub>t</sub> N <sub>s</sub> , H <sub>t</sub> H <sub>s</sub> *	N <sub>t</sub> N <sub>s</sub> , H <sub>t</sub> H <sub>s</sub>	N <sub>t</sub> N <sub>s</sub> , N <sub>t</sub> H <sub>s</sub>
<sup>3</sup> H-TdR labeling			
in 20% serum on	8, 10, 14 days	8, 11 days	15 days
in 40 or 80% serum on	—	1, 4 days†	—
Mitotic indices on	—	8, 11 days	—
LMx§ on live cultures on	daily	daily	daily
LMx on fixed cultures on	8, 10, 14 days	8, 11 days	15 days
EM study on	14th day	—	—

\*Code: N<sub>t</sub>N<sub>s</sub> = NC tissue in NC serum (autologous); N<sub>t</sub>H<sub>s</sub> = NC tissue in HC serum; H<sub>t</sub>N<sub>s</sub> = HC tissue in NC serum; H<sub>t</sub>H<sub>s</sub> = HC tissue in HC serum (autologous).

† 1 or 4 days in 40 or 80% autologous serum after growing in 20% autologous serum for 7 days.

§LMx = light microscopy; EM = electron microscopy.

proportions indicated in Table I. Cultures were maintained at 37.5°C and media were changed every 2 days. When daily inspection by phase-contrast microscopy revealed that new cell growth in most cultures exceeded twice the area of original explant, the latter was removed, leaving only new cells. The pH of media was monitored by phenol red indicator and further checked on occasion by pH meter. pH was maintained in the range of 7.2 to 7.4 in all cultures, except for two measurements which deviated not more than 0.3 pH unit.

Blood for serum was obtained by cardiac puncture and kept at 4°C until used. For light microscopy, cells were fixed with absolute methanol and stained with May-Grünwald-Giemsa stain or fixed with paraformaldehyde and stained with Oil Red O. From many cultures (50 plus) the fluid removed at time of changing was centrifuged at 1,000 rpm for 5 min, and the sediment was stained with methyl violet or eosin y and examined for detached cells. Eight cultures each from HC and NC groups of Experiment 1 were used for chromosome preparations made by the method of Brown and Porter (1968).

For electron microscopy, cultures were fixed *in situ* for ½ hr with 2.5% glutaraldehyde followed by Dalton's osmium fixative for 1 hr. Cells were embedded in Epon, sectioned, and stained with uranyl acetate and lead citrate.

For <sup>3</sup>H-thymidine radioautography, cells were placed in tubes containing media to which had been added 1.0 μc per ml of <sup>3</sup>H-thymidine of specific activity 13 c per mmole. After 30 min at 37.5°C, coverslips containing cells were transferred to tubes containing 10 ml of 0.01 M nonradioactive thymidine and kept for 5 min at 4°C, then washed in Hanks' solution. Cells were fixed in 95% ethanol for 15 min followed by 80% ethanol for 24 hr. Coverslips were dried in air, dipped in Ilford K2 nuclear emulsion, exposed for 5 days, developed, and stained with hematoxylin and eosin.

Both HC and NC diets fed to swine consisted, by calories, of 40% fat, 24% protein, and 36% carbohydrate, with appropriate minerals and vitamins. The only difference between the two diets was that the HC diets also contained 8 g of cholesterol daily. Caloric intakes of the two dietary groups were approximately the same.

## RESULTS

### Observations on Cells Exposed to 20% Serum

The cells grew in monolayers and by both light and electron microscopy appeared healthy. The various groups were indistinguishable in general appearance except that lipid droplets, which were

TABLE II  
Data on Cells in Experiment 1 Growing in 20% Serum and Labeled with <sup>3</sup>H-Thymidine

Days after explant	Group*	Total cells counted	Cells labeled with <sup>3</sup> H-thymidine	Labeled	Significance of selected comparisons by chi square
		No.	No.	%	
8	N <sub>t</sub> N <sub>s</sub>	10,183	2,189	21.5	N <sub>t</sub> N <sub>s</sub> < N <sub>t</sub> H <sub>s</sub> p < 0.001
	N <sub>t</sub> H <sub>s</sub>	6,607	2,029	30.7	N <sub>t</sub> N <sub>s</sub> < H <sub>t</sub> H <sub>s</sub> p < 0.001
	H <sub>t</sub> N <sub>s</sub>	3,315	710	22.5	H <sub>t</sub> N <sub>s</sub> < H <sub>t</sub> H <sub>s</sub> p < 0.001
	H <sub>t</sub> H <sub>s</sub>	6,170	1,943	31.4	N <sub>t</sub> N <sub>s</sub> vs H <sub>t</sub> N <sub>s</sub> p N.S. ‡ N <sub>t</sub> H <sub>s</sub> vs H <sub>t</sub> H <sub>s</sub> p N.S.
10	N <sub>t</sub> N <sub>s</sub>	7,220	681	9.4	N <sub>t</sub> N <sub>s</sub> < N <sub>t</sub> H <sub>s</sub> p < 0.001
	N <sub>t</sub> H <sub>s</sub>	5,659	819	14.5	N <sub>t</sub> N <sub>s</sub> < H <sub>t</sub> H <sub>s</sub> p < 0.001
	H <sub>t</sub> N <sub>s</sub>	5,066	610	12.0	H <sub>t</sub> N <sub>s</sub> < H <sub>t</sub> H <sub>s</sub> p < 0.001
	H <sub>t</sub> H <sub>s</sub>	7,191	1,119	15.6	N <sub>t</sub> N <sub>s</sub> < H <sub>t</sub> N <sub>s</sub> p < 0.001 N <sub>t</sub> H <sub>s</sub> vs H <sub>t</sub> H <sub>s</sub> p N.S.
14	N <sub>t</sub> N <sub>s</sub>	4,086	473	11.6	N <sub>t</sub> N <sub>s</sub> < N <sub>t</sub> H <sub>s</sub> p < 0.001
	N <sub>t</sub> H <sub>s</sub>	3,449	715	17.8	N <sub>t</sub> N <sub>s</sub> < H <sub>t</sub> H <sub>s</sub> p < 0.001
	H <sub>t</sub> N <sub>s</sub>	4,476	627	14.0	H <sub>t</sub> N <sub>s</sub> < H <sub>t</sub> H <sub>s</sub> p < 0.001
	H <sub>t</sub> H <sub>s</sub>	1,828	336	18.4	N <sub>t</sub> N <sub>s</sub> < H <sub>t</sub> N <sub>s</sub> p < 0.001 N <sub>t</sub> H <sub>s</sub> vs H <sub>t</sub> H <sub>s</sub> p N.S.

\* For code see Table I. All cells were grown in the same type of serum throughout the experiment.

‡ N.S. = not significant.

Smaller numbers were counted as the study progressed and as it became apparent that counting larger numbers would not materially affect results.

TABLE III  
Data on Cells in Experiment 2 Growing in 20, 40 or 80% Serum and Labeled with <sup>3</sup>H-Thymidine

Days after explant	Group*	Total cells counted	Cells labeled with <sup>3</sup> H-thymidine	Labeled	Significance of selected comparisons by chi square
		No.	No.	%	
8	N <sub>t</sub> N <sub>s</sub> 20	3,054	671	22.0	H <sub>t</sub> H <sub>s</sub> 20 > N <sub>t</sub> N <sub>s</sub> 20 p < 0.0001
	H <sub>t</sub> H <sub>s</sub> 20	2,192	629	28.7	H <sub>t</sub> H <sub>s</sub> 80 vs N <sub>t</sub> N <sub>s</sub> 80 N.S.
8 (1) ‡	N <sub>t</sub> N <sub>s</sub> 40	1,741	268	15.4	H <sub>t</sub> H <sub>s</sub> 20 > H <sub>t</sub> H <sub>s</sub> 80 p < 0.0001
	H <sub>t</sub> H <sub>s</sub> 40	3,928	1,039	26.5	
8 (1)	N <sub>t</sub> N <sub>s</sub> 80	2,811	537	19.1	N <sub>t</sub> N <sub>s</sub> 20 > N <sub>t</sub> N <sub>s</sub> 80 p < 0.05
	H <sub>t</sub> H <sub>s</sub> 80	2,562	499	19.5	
11	N <sub>t</sub> N <sub>s</sub> 20	5,272	697	16.3	H <sub>t</sub> H <sub>s</sub> 20 > N <sub>t</sub> N <sub>s</sub> 20 p < 0.001
	H <sub>t</sub> H <sub>s</sub> 20	5,268	891	20.9	
11 (4)	N <sub>t</sub> N <sub>s</sub> 40	2,258	446	19.7	H <sub>t</sub> H <sub>s</sub> 80 < N <sub>t</sub> N <sub>s</sub> 80 p < 0.0001
	H <sub>t</sub> H <sub>s</sub> 40	1,914	398	20.8	H <sub>t</sub> H <sub>s</sub> 20 > H <sub>t</sub> H <sub>s</sub> 80 p < 0.0001
11 (4)	N <sub>t</sub> N <sub>s</sub> 80	2,254	389	17.3	
	H <sub>t</sub> H <sub>s</sub> 80	2,594	23	0.9	N <sub>t</sub> N <sub>s</sub> 20 < N <sub>t</sub> N <sub>s</sub> 80 N.S.

\* Code same as in Table I except for the number after "s" which indicates the percentage of serum in the media.

‡ Numbers in parentheses refer to days in 40 or 80% autologous serum after growing in 20% autologous serum for 7 days.

TABLE IV  
Data on Mitoses of Cells in Experiment 2 Growing in 20, 40 or 80% Serum

Days after explant	Group*	Total cells counted	Cells in mitosis	Cells in mitosis	Significance of selected comparisons by chi square
		No.	No.	%	
8	N <sub>t</sub> N <sub>s</sub> 20	9,703	163	1.7	H <sub>t</sub> H <sub>s</sub> 20 > N <sub>t</sub> N <sub>s</sub> 20 p < 0.001
	H <sub>t</sub> H <sub>s</sub> 20	7,844	260	3.3	H <sub>t</sub> H <sub>s</sub> 80 < N <sub>t</sub> N <sub>s</sub> 80 p < 0.001
8 (1)‡	N <sub>t</sub> N <sub>s</sub> 40	5,257	125	2.4	
	H <sub>t</sub> H <sub>s</sub> 40	3,928	119	3.0	H <sub>t</sub> H <sub>s</sub> 20 > H <sub>t</sub> H <sub>s</sub> 80 p < 0.001
8 (1)	N <sub>t</sub> N <sub>s</sub> 80	9,110	174	1.9	N <sub>t</sub> N <sub>s</sub> 20 vs N <sub>t</sub> N <sub>s</sub> 80 N.S.
	H <sub>t</sub> H <sub>s</sub> 80	5,139	59	1.2	
11	N <sub>t</sub> N <sub>s</sub> 20	14,273	144	1.0	
	H <sub>t</sub> H <sub>s</sub> 20	12,521	230	1.8	H <sub>t</sub> H <sub>s</sub> 20 > N <sub>t</sub> N <sub>s</sub> 20 p < 0.001
11 (4)	N <sub>t</sub> N <sub>s</sub> 40	5,218	51	1.0	
	H <sub>t</sub> H <sub>s</sub> 40	7,790	94	1.2	H <sub>t</sub> H <sub>s</sub> 80 < N <sub>t</sub> N <sub>s</sub> 80 0 < 0.001
11 (4)	N <sub>t</sub> N <sub>s</sub> 80	6,459	62	1.0	H <sub>t</sub> H <sub>s</sub> 20 > H <sub>t</sub> H <sub>s</sub> 80 p < 0.001
	H <sub>t</sub> H <sub>s</sub> 80	15,159	14	0.1	N <sub>t</sub> N <sub>s</sub> 20 vs N <sub>t</sub> N <sub>s</sub> 80 N.S.

\*Code same as in Table I except for the number after "s" which indicates the percentage of serum in the media.

‡Numbers in parentheses refer to days in 40 or 80% autologous serum after growing in 20% autologous serum for 7 days.

present in some cell in all cultures, appeared more common in HC groups. As shown by electron microscopy, many cells had some recognizable myofilaments and a few cells had interdigitations suggestive of endothelial cells. Most cells had no specialized features and were not identifiable as to type. (A rapidly growing atherosclerotic lesion shows all of these cellular forms, but a greater proportion has a few myofilaments and a smaller proportion is unidentifiable [Thomas et al., 1968].)

The sediment from the centrifuged culture media samples contained only a few intact cells and consisted principally of amorphous debris. The various groups were indistinguishable. Chromosome counts on 40 metaphases revealed the diploid mode of 38 chromosomes and no abnormalities.

Results of the radioautography studies and the mitotic indices for Experiments 1 and 2 are presented in Table II, III, and IV. In Experiment 3, 13% of cells from NC tissue in NC serum were labeled as compared with 35% from NC tissue in HC serum.

#### Observations on Cells Exposed to 40% and 80% Sera

Seen by light microscopy 24 hr after exposure, cells in 40% and 80% sera were similar to those

in 20% serum. By 96 hr numerous cells exposed to 80% HC serum were degenerate or dead, but those exposed to 80% NC serum were indistinguishable from those in 20% serum. Numerical data are given in Tables III and IV.

#### DISCUSSION

In vivo homeostasis of cell populations is maintained by delicately balanced control mechanisms, about which little is known. The rate of production of new cells is kept at levels necessary for replacement of loss and for appropriate increases during growth.

In experiments described in this report cells were removed from the body and placed in an in vitro environment suitable for growth. This procedure results in considerable dedifferentiation and an enormous increase (30-fold or more) in the number of cells synthesizing DNA and dividing (Florentin et al., unpublished observations).

HC serum at 20% levels results in greater numbers of cells synthesizing DNA and a greater rate of cell division than does NC serum. One possible explanation is that HC serum is supplying some rate-limiting growth factor in greater quantities than NC serum. For example, cholesterol per se has been shown to be required for optimum growth in

HeLa cells kept in media containing serum apparently depleted of cholesterol by dialysis (Sato et al., 1957). However, only a few micrograms of cholesterol were required for optimum growth, and the quantity in NC serum far exceeded this amount. Also, if cholesterol were a limiting factor, the addition of NC serum at 40 and 80% levels would have been expected to increase division rates, but it did not.

A more likely explanation for observed increases with HC serum is that something is present in HC serum that provides a signal for rates of cell division greater than those produced by NC serum. Control mechanisms involved in DNA synthesis and cell division are not well understood in any tissue. They are being actively investigated in vivo in a number of special situations, including regenerating livers after partial surgical extirpation, isoproterenol-treated salivary glands, and various tissues that respond to hormones by altering rates of cell division (Barka, 1965; Bullough, 1965). One fact that appears to be emerging from these diverse studies is that many mature cells with specialized functions synthesize molecules that suppress DNA synthesis and cell division. Elements in the environment may trigger cells to synthesize DNA and divide by interfering with the production or with the activity of postulated suppressor molecules. The primary aortic culture model presented in this report should be suitable for search for such a suppressor.

In vivo evidence for arterial cell damage in HC swine has been obtained in addition to evidence for cell multiplication (Thomas et al., 1968). This aspect was difficult to evaluate in vitro. It was not possible to clearly distinguish between cells grown in 20% HC and those grown in NC serum, except that perhaps more fat droplets were present in cells grown in HC serum. Examination of culture fluid sediment after centrifugation revealed very few detached cells in any culture, although some debris was present that may have originated from disintegrated cells.

We thought that possible damaging effects of HC serum might become apparent at higher concentrations of serum, so we used concentrations 40 and 80% in one experiment. Only at 80% did we demonstrate toxicity of HC serum as compared

to the corresponding NC serum. Even at this level, differences were not present in all comparisons. The 80% level far exceeds minimum required levels of serum for optimum growth as demonstrated with NC serum in this experiment, and the results with 80% HC serum may be unrelated to hypercholesterolemia. However, in pharmacological studies it is well known that many drugs produce one type of physiologic response at one level and the opposite type at a higher level. Perhaps a similar phenomenon is involved in the current experiment.

We are postulating that HC serum contains some element that under appropriate circumstances helps to trigger DNA synthesis and cell division in primary aortic tissue cultures. The proposed element could be cholesterol per se (which was the only dietary constituent that was different between HC and NC groups) or an entirely different molecule produced in response to the HC diet. It may be possible to identify the postulated element in further studies with the same system.

This work was supported by United States Public Health Service Grant No. HE-7155.

Received for publication 9 September 1968, and in revised form 10 January 1969.

#### REFERENCES

- BARKA, T. 1965. Stimulation of DNA synthesis by isoproterenol in the salivary gland. *Exp. Cell Res.* **39**:355.
- BROWN, C. D., and I. H. PORTER. 1968. In *Methods in Cytogenetics*. Zeiss-Mitteilungen. **4**:333.
- BULLOUGH, W. S. 1965. Mitotic and functional homeostasis. *Cancer Res.* **25**:1683.
- EAGLE, H. 1955. Nutrition needs of mammalian cells in tissue culture. *Science.* **122**:501.
- FLORENTIN, R. A., and S. C. NAM. 1968. Dietary-induced atherosclerosis in miniature pig. *Exp. Mol. Pathol.* **8**:263.
- SATO, G., H. W. FISHER, and T. T. PUCK. 1957. Molecular growth requirements of single mammalian cells. *Science.* **126**:961.
- THOMAS, W. A., R. A. FLORENTIN, S. C. NAM, D. N. KIM, R. M. JONES, and K. T. LEE. 1968. Pre-proliferative phase of atherosclerosis in swine fed cholesterol. *Arch. Pathol.* **86**:621.