In Vitro Studies of Vitamin D-Induced Aortic Calcification[1] (34148)

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The arterial calcification induced by the administration of large doses of vitamin D has been known and studied for many years. In the course of these studies, a great deal of morphological, histochemical, and biochemical data have accumulated which have been largely descriptive in nature (1–4). More recently, as new data have accumulated on the metabolism of Vitamin D and its mode of action, the manner in which this vitamin exerts its effects on osseous and calcium metabolism has been greatly clarified (5, 6). But with regard to the arterial calcification induced by hypervitaminosis D, two central problems remain. The first is whether the vitamin or one of its metabolites exerts a direct effect on the artery or whether the calcification is entirely a consequence of secondary changes in the composition of the extracellular fluids. The second is what factors are responsible for the peculiar and predictable distribution of arterial disease which hypervitaminosis D has in common with many other arteriopathies including human atherosclerosis (2). One approach to the study of this problem might be to assess the effects of sera of various compositions on arterial segments *in vitro*, isolated from general body metabolism. We here report the results of such a study which disclosed that the serum levels of calcium and vitamin D are both important determinants of the accumulation of calcium by such arteries and that the susceptibility of different arterial segments to calcification *in vitro* paralleled their susceptibility *in vivo* to some extent.

Materials and Methods. Two hundred g male Sprague-Dawley rats were used in all experiments except those involving rachitic animals. Sera were obtained by aortic puncture from animals lightly anesthetized with ether. Hypervitaminosis D was produced by injecting 200,000 units of Vitamin D_3 intraperitioneally three times weekly for 3 weeks. These rats, which were hypercalcemic and had extensive aortic calcification were the source of hypervitaminotic serum. Rachitic rat serum was obtained from 75 g male rats maintained for 3 weeks on Steenbock's rachitogenic diet no. 2 (7). Control sera for these experiments were from pair-fed animals of comparable weight on a stock diet. Two types of experimental systems were used. The first was an organ culture method in which 2-mm thick rings of thoracic aortas of rats were dissected free of surrounding connective tissue and placed on strips of cellulose acetate surgical burn dressing overlying metal rafts in Falcon organ culture dishes containing 2.2 m of a nutrient medium consisting of 40% rat serum and 60% NCTC 109 tissue culture medium supplemented with 1000 units of penicillin and 1000 units of streptomycin/ml. The serum was from normal rats or rats with hypervitaminosis D. The cultures were refed three times weekly and were maintained at 37° in an atmosphere of 95% O_2 and 5% CO_2. After 1 week in culture, some rings were fixed in formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin or the von Kossa reagent. Others were dried to constant weight at 104°, weighed, and ashed in sulfuric acid. Calcium was measured with an atomic absorption spectrophotometer and phosphate by the method of Fiske and Subba Row (8).

Since it soon became apparent that simple incubation of arteries in serum yielded results essentially similar to those obtained with organ culture, the method of Martin *et al.* (9) was used for most of the experiments. In this technique, segments of aortic arch,

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thoracic aorta, or abdominal aorta were dissected free of surrounding connective tissue, weighed and placed in 0.2 ml of serum/mg of wet weight of tissue. The serum contained 1000 units of penicillin and 1000 units of streptomycin/ml. The tubes in which the experiment was done were gassed with 95% O_2–5% CO_2, capped, and incubated at 37°. After the test period was complete the arteries were removed and chemical and histological studies were done in a manner identical to that used for the organ cultures.

Results. Organ cultures. With this technique the aortas appeared to be histologically viable, although small areas of focal necrosis were found even in control specimens, particularly at the sites of intercostal artery origin. Calcification was uncommon in control specimens cultured in medium supplemented with normal rat serum. In specimens cultured in medium supplemented with the serum of rats with hypervitaminosis D, calcification was uniformly much more extensive than in controls and occurred mostly in elastic tissue fibers and later in adjacent smooth muscle cells which were necrotic. Calcification was not observed in viable areas of the explant. There was no significant proliferative cellular response to these calcified areas which were largely in the media, often near the adventitial surface, and particularly advanced at sites of branching of intercostal arteries. This localization was perhaps related to the injury produced by the trauma of dissecting the surrounding periadventitial connective tissue during preparation. In keeping with the histological observations, chemical measurement showed that there was much more calcium in cultures maintained in medium containing serum from rats with hypervitaminosis D than in control cultures (Fig. 1).

Serum incubations. In these preparations histological study indicated that by the second day of incubation, necrosis had begun and after 72 hr was virtually complete. When a timed study of calcium accumulation was done, the greatest increment of calcium accumulation occurred between 48 and 72 hr after incubation was begun if normal rat serum was used. In keeping with the observations of Martin *et al.* (9), calcification was

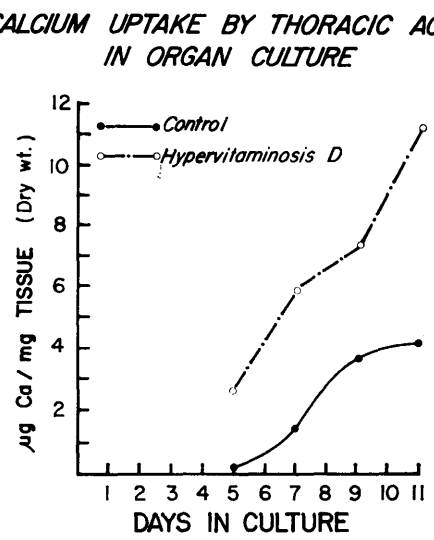

FIG. 1. arteries maintained in organ culture in medium supplemented with serum from rats with hypervitaminosis D accumulate more calcium than those maintained in medium supplemented with normal rat serum.

virtually restricted to elastic tissue lamellae when the vessels were studied histologically. Arteries incubated in serum from rachitic rats accumulated less calcium and phosphate than those incubated in control sera. Table I shows the results of this experiment. Except for experiments using rachitic serum, the results depicted in each Tables I–IV were reproduced in three repeated experiments. The PO_4 values varied similarly to those of calcium. When serum from normal rats and those with hypervitaminosis D were compared, it was found that if incubation was performed in serum from hypervitaminotic rats there was greater accumulation of both calcium and phosphate than with control serum (Table II).

TABLE I.[a]

Serum	No. of samples	Mean[b]	SD[c]
Rachitic	4	0.0019	0.00037
Normal	4	0.0065	0.0022

[a] Thoracic aortas of normal rats were incubated in serum for 72 hours. They accumulated more calcium from normal than from rachitic serum.

[b] (mg of Ca/mg of dry wt).

[c] Standard deviation.

TABLE II.[a]

Serum	No. of samples	Mean[b]	SD[c]
Normal	6	0.0017	0.0011
Hypervitaminosis D	6	0.0073	0.0018

[a] If thoracic aortas of normal rats were incubated for 72 hr in serum from normal rats or rats with hypervitaminosis D, they accumulated more calcium from hypervitaminotic serum.

[b] (mg of Ca/mg of dry wt).

[c] Standard deviation.

When large amounts of Vitamin D_3 (50 or 100 units/ml of serum dissolved in 0.01 ml of EtOH) were added to the serum and these sera compared with serum containing an equal weight of cholesterol dissolved in a similar amount of alcohol, no difference in the amount of calcium accumulated by either thoracic aorta or aortic arch was demonstrable. In agreement with the data of Martin *et al.* (9), addition of sufficient $CaCl_2$ to raise the level of serum calcium by 5 mg/100 ml also did not increase the amount of calcium accumulation. However, when both vitamin D and calcium were added to the serum in these amounts and comparisons made with serum containing equivalent amounts of cholesterol and NaCl, arteries incubated in the serum with added Vitamin D and calcium accumulated more calcium and phosphate than those incubated in control sera (Table III). Here, measurements were made both 48 and 72 hr after incubation was begun and the differences were present at both time intervals.

In experiments in which different segments of the aorta were compared, it was found that when segments of aortic arch, thoracic aorta or abdominal aorta were incubated in either normal serum or serum from rats with hypervitaminosis D and calcium measured in the arteries after 72 hr of incubation, aortic arch accumulated the most calcium, thoracic aorta slightly but not significantly less, abdominal aorta the least (Table IV). Calcium was measured in one set of arteries before incubation and no differences in calcium content of the three segments were found. Since histological studies indicated that calcification was virtually restricted to elastic tissue in these preparations, the amount of elastic tissue was measured in these portions of the aorta. This was done by the method of Hass (10) which uses warm 88% formic acid to digest the tissue elements which are not elastic. The isolated elastic tissue was then blotted dry, weighed, and the value was expressed as a percentage of the wet weight of the tissue. Table IV shows that the amount of elastic tissue in these three aortic segments paralleled the calcium accumulation in this incubation system.

Discussion. Superficially, at least, these data appear to provide clear-cut evidence that the composition of the serum is of primary importance in the pathogenesis of the arterial calcification induced by hypervitaminosis D and that both the hypercalcemia

TABLE III.[a]

	48 hr			72 hr		
	No. of samples	Mean[b]	SD[c]	No. of samples	Mean[b]	SD[c]
Serum + NaCl + cholesterol	6	0.0014	0.002	6	0.0018	0.0007
+ Ca + cholesterol	6	0.0013	0.0016	6	0.0023	0.0005
+ Vit. D + NaCl	6	0.0007	0.0005	6	0.0023	0.001
+ Ca + Vit. D	6	0.0048	0.0005	6	0.0042	0.0007

[a] As described in the text, addition of Vitamin D or calcium alone to serum did not influence the accumulation of calcium by thoracic aortas when incubated in such serum. If both calcium and Vitamin D were added, the aortas bound more calcium after either 48 or 72 hr of incubation.

[b] (mg of Ca/mg of dry wt).

[c] Standard deviation.

TABLE IV.[a]

Segment	Calcium accumulation			Elastica (%)		
	No. of samples	Mean[b]	SD[c]	No. of samples	Mean[b]	SD[c]
Arch	8	0.007	0.0004	5	70.0	6.4
Thoracic	8	0.006	0.0001	5	62.4	7.4
Abdominal	8	0.002	0.0005	5	23.8	6.6

[a] The amount of calcium accumulation by different arterial segments parallels the amount of elastin they contain.

[b] (mg of Ca/mg of dry wt).

[c] Standard deviation.

induced by Vitamin D and a direct local action of the vitamin itself are synergistic in contributing to the development of the lesion. There are, however, several qualifying points which detract from the strength of the data presented here. The first is the nature of the experimental system used. In it, whether simple serum incubation or an organ culture system was used, calcification was virtually restricted to areas of necrosis and even here was found restricted to elastic tissue fibers. In addition, even in the organ cultures, the distribution of calcification was primarily in the deep media in contrast to what is seen *in vivo*, where calcification usually begins in the inner elastic lammelae in major arteries (2). Even *in vivo*, however, calcification of viable smooth muscle cells is an uncommon event in the arteries which calcify under the influcence of Vitamin D and elastic fibers are the first elective site of calcification (3). The differences in the mural pattern of calcification *in vivo* and *in vitro* are perhaps related to hemodynamic factors. Since the rat aorta contains no vasa vasora (11), its nutrition is derived from the luminal surface in an intact animal. In our systems, there was no flow or pressure and the nutrition of the tissue was by diffusion from all its surfaces. The adventitial trauma produced during dissection may also have contributed to the localization of the disease.

The observation that serum from hypervitaminotic animals or normal sera enriched with both vitamin D and calcium induced increased accumulation by the arteries as early as 48 hr after incubation was begun suggests that this was a true metabolic effect rather than something more nonspecific because necrosis was not yet advanced at this time.

The dosages of vitamin D added to the serum in these experiments was massive, and it might be argued that they are too far beyond the physiological range to be meaningful. However, this study was not designed to investigate the normal action of vitamin D but its toxic effects. It is more pertinent to point out that it has now been established that Vitamin D is normally converted in the body to 25-hydrocholecalciferol, a compound which has now been isolated and shown to be much more metabolically active than vitamin D itself *in vivo* (5) as well as in studies of organ cultures of bones (6). If it were used in a system such as this one, it might be possible to demonstrate an effect on arteries in lower dosage, and perhaps even without the addition of excess calcium to the serum.

In vitro, the arteries exhibited much the same sequence of calcification as they do *in vivo*, where calcification begins in the aortic arch and then spreads centrifugally (2). As expected, since the calcification was localized in the elastic tissue, the amount of elastic tissue in the artery paralleled the amount of calcium accumulation. The segmental distribution of many arterial diseases has often been considered to be related to local hemodynamic differences (11). These data, as do some others in the literature in studies of other arterial diseases (11) suggest that part of the hemodynamic effects on the distribution of arterial diseases are not primary, but are related to the morphological modifications

which pressure, flow, or turbulence induce in arteries both during embryonic development and in the adult. For example, elastic tissue develops most entensively in areas of pulsatile flow, and there is experimental data indicating that pulsatile flow through tubes inserted under rat skins can induce alignment of proliferated connective tissue elements into an organized structure having some of the morphological organization of an artery (12).

Summary. Arterial segments incubated in a culture system containing serum from rats with hypervitaminosis D accumulate more calcium than segments incubated in a system containing normal rat serum. If such arterial segments are incubated in serum, they accumulate more calcium from normal serum than rachitic serum and more from serum from rats with hypervitaminosis than from normal serum. Addition of either vitamin D or calcium alone to normal serum does not result in excess calcium accumulation by arteries incubated in such serum, but addition of both does. Different aortic segments accumulate different amounts of calcium, apparently in relation to the proportion of elastic tissue in the segments.

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1. Carlstrom, D., Engfeldt, B., Engstrom, A., and Ringertz, N., J. Lab. Invest. **2**, 325 (1953).

2. Hass, G. M., Trueheart, R. E., Taylor, C. B., and Stumpe, M., Am. J. Pathol. **34**, 395 (1958).

3. Konetzki, W., Hyland, R., and Eisenstein, R., Lab. Invest. **11**, 488 (1962).

4. Eisenstein, R. and Zeruolis, L., Arch. Pathol. **77**, 27 (1964).

5. Lund, J. and DeLuca, H. F., J. Lipid Res. **7**, 27 (1964).

6. Trummel, C. L., Raisz, L. G., Blunt, J. W., and DeLuca, H. F., Science **163**, 1450 (1969).

7. Steenbock, H., Bellin, S. A., and Wiest, W. G., J. Biol. Chem. **193**, 843 (1951).

8. Hiller, A., "Practical Clinical Chemistry" pp. 55, 118. Thomas, Springfield, Illinois (1953).

9. Martin, R. G., Schiffman, E., Blanden, A., and Nylen, M., J. Cell Biol. **16**, 243 (1963).

10. Hass, G. M., Arch. Pathol. **34**, 807 (1942).

11. Wolinsky, H. and Glagov, S., Circulation Res. **20**, 99 (1967).

12. Glagov, S., Rowley, D. A., and Wolinsky, H., Am. J. Pathol. **52**, 25a (1968).

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