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Structural and Chemical Characterization of the Inorganic Deposits in Calcified Human Aortic Wall

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Chemical and crystallographic characterization of the inorganic deposits present in the human aortic wall is reported. No evidence of calcification has been found in the adventitia layer of the aortic wall, while two different phases have been identified in the intima and media layers. The inorganic deposits localized in the atherosclerotic plaques of the intima layer have been identified as hydroxyapatite. The inorganic phase uniformly deposited in the human media aortic wall is highly crystalline β-TCMP with a magnesium content which depends on the calcification degree.

Introduction

Calcification of human aortic wall occurs commonly [1, 2]. It begins in the youth and increases progressively with age. Although pathogenesis is not clear, calcium deposition appears to be associated with accumulation of lipids [2, 3], while recent evidence suggests that 'aging' changes of the connective tissue of the aortic wall may play a role in the mechanism of calcification [4]. Atherosclerotic plaques which occur exclusively in the intima layer of the aortic wall are the most evident culminative trauma of atherosclerotic disease [5, 6].

X-ray diffraction studies [7–10] established that the main crystalline constituent of the atherosclerotic plaques in the aortic wall is the polycrystalline form of hydroxyapatite. Many authors [9, 11–13] identified as hydroxyapatite the inorganic deposits observed in the aortic media layer by electron diffraction studies. Kim [13] described these deposits as large, calcified bodies containing needle-shaped, rodly arranged apatite crystals.

Taylor and Little [14] described the presence of β-tricalcium phosphate in abnormal calcified abdominal aorta and in a variety of pathological human calcifications.

Le Geros *et al.* [15] suggested that the presence of magnesium promotes the formation of an amorphous calcium phosphate in visceral calcification of patients affected with chronic renal failure.

The effect of magnesium on the conversion of calcium phosphate to hydroxyapatite (HA) has been described [16–19]. Since amorphous calcium phosphate is believed to be the precursor of bone apatite, the magnesium content of the biological calcifications is of great importance.

The aim of this research is to establish the chemical composition and crystalline nature of the inorganic phases present in the intima and media layers of the human aortic wall. We have put particular care to evaluate the magnesium content and the Ca/Mg molar ratio of the inorganic deposits of the aortic wall not only to explain the replacement of magnesium to calcium ions in calcium phosphates, but also to investigate the role played by magnesium concentration on the aortic wall calcification. Furthermore we have examined the composition and structure of these inorganic phases as a function of the calcification degree of the artery and of the distance from the aortic arch.

Experimental

Materials

Samples of calcified aortae were obtained at autopsy from forty 19–80 years old human subjects and stored at −30 °C until used. The adventitia, media and intima tonacae were hand-dissected. Ten media layer specimens 1 cm wide were excised within the upper 10 cm of the descending artery. Atherosclerotic plaques were stripped from the intima layer within the examined arterial segment. The ashes of the tissues were obtained by heating at 700 °C.

Synthetic amorphous calcium phosphates containing magnesium were synthesized after Le Geros [19] by addition of calcium–magnesium acetate solutions (Ca/Mg molar ratio ranging from 32.3 to 0.7) into a phosphate solution.

Synthetic hydroxyapatite was prepared by addition of phosphoric acid into a solution of $Ca(OH)_2$.

TABLE I. Chemical Composition of Samples of Media Layer and Atherosclerotic Plaques. Two values of the ionic contents corresponding to the lowest and highest calcification degree are given for media layer. Average values are reported for atherosclerotic plaques.*

	Media layer		Atherosclerotic plaques
Ca^{2+}	$(0.78 \pm 0.02)\ 10^{-4}$	$(10.0 \pm 0.2)\ 10^{-4}$ [a]	$(9.5 \pm 0.1)\ 10^{-3}$ [b]
Mg^{2+}	$(1.56 \pm 0.03)\ 10^{-5}$	$(9.7 \pm 0.2)\ 10^{-5}$ [a]	$(5.2 \pm 0.2)\ 10^{-4}$ [b]
PO_4^{3-}	$(0.73 \pm 0.02)\ 10^{-4}$	$(6.5 \pm 0.1)\ 10^{-4}$ [a]	$(5.94 \pm 0.04)\ 10^{-3}$ [b]
Ca/P	1.1	1.5	1.6
Ca/Mg	5.0	10.2	18.3
(Ca + Mg)/P	1.3	1.7	1.7

*The analytical data are expressed (a) in mol per gram of dry tissue and (b) in mol per gram of inorganic deposit.

Chemical Analysis

Calcium and magnesium contents were determined using an atomic absorption spectrophotometer (Perkin Elmer 373). Ashed tissue was diluted to an appropriate volume with 10% lanthanum in 50% HCl.

Phosphorus was determined spectrophotometrically as molybdovanadophosphoric acid [20].

Infrared Absorption Analysis

Infrared spectra were used to determine the carbonate content. About 1 mg of the powdered sample was mixed intimately with 300 mg of KBr (infrared grade) and pelletized under vacuum. The pellets were analysed using a grating spectrophotometer (Perkin Elmer 390 IR), ranges 4000 to 400 cm^{-1}, normal slit, scanning speed 50 min.

The quantitative determination of the carbonate ion in the calcium phosphates present in the ashed media layer and in the ashed atherosclerotic plaques was obtained by an analysis of the absorption bands characteristic of phosphates and carbonates. We have correlated the ratio of the absorbances of carbonates and phosphates (measured from the areas of the two absorption bands at 870 and 960 cm^{-1}) to the carbonate content of the material, through the comparison with the ratio of carbonate and phosphate absorption bands of synthetic mixtures of hydroxyapatite containing different amounts of calcium carbonate.

X-ray Diffraction Analysis

X-ray diffraction patterns were recorded by means of a powder diffractometer unit (Philips Norelco). The 2θ diffraction range covered was from 10° to 65° at a scanning speed of 0.5°/min. Patterns were interpreted by comparing them to the american Society of Testing Materials (ASTM) index cards.

X-ray photographs of wet or air-dried tissue samples were recorded using a flat or a Debye–Scherrer camera (diameter 57.3 mm). All the diffraction patterns were obtained with nickel-filtered CuK radiation.

The lattice constants were determined by a least squares refinement with the angular values of at least 12 reflections for each sample.

Results

Chemical Composition

The results of the chemical analysis carried out on samples of aortic media layer and on atherosclerotic plaques are shown in Table I. The degree of calcification of the samples of media layer dried for 72 hours at 110 °C to constant weight ranges between 0.78×10^{-4} and 10.0×10^{-4} mol of calcium per gram of dried tissue. The calcium, phosphorus and magnesium contents of the media layer depend on the degree of calcification of the aortic wall and range between the lowest and highest values reported in Table I, while no significant change in the ionic content of the examined atherosclerotic plaques has been observed. The analytical data for atherosclerotic plaques reported in Table I are the average values of measurements performed on about fifty different samples.

In the media layer the Ca/P and Ca/Mg molar ratios increase from 1.1 to 1.5 and from 5.1 to 10.4 respectively, with the increase of the calcification degree. The replacement of calcium with magnesium is higher when the calcification degree is low, as can be see in Fig. 1 where the Ca/Mg molar ratio is plotted against the calcium content. No correlation between the calcificiation degree of the sample and its distance from the aortic arch was found.

The Ca/P molar ratio of the atherosclerotic plaques is 1.6, slightly lower than the stoichiometric value of hydroxyapatite. The relative content of magnesium of the atherosclerotic plaques is constantly lower than that obtained for media layer.

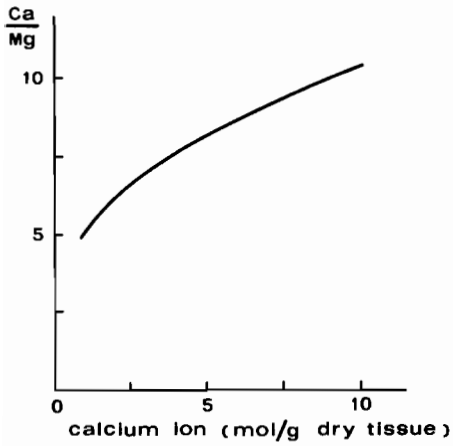

Fig. 1. Ca/Mg molar ratio of the ashed media layer plotted against the degree of calcification expressed as mol of calcium ion per gram of dry tissue.

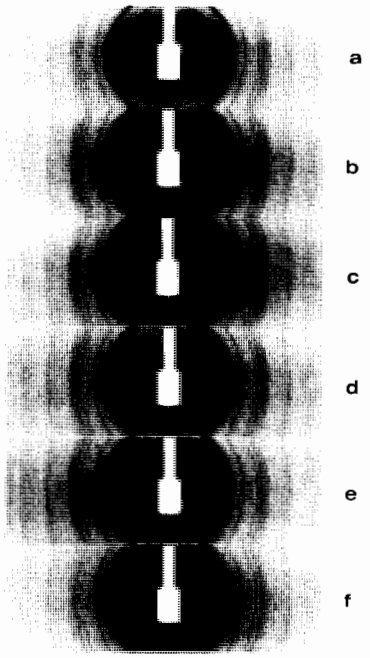

Fig. 2. X-ray diffraction patterns of a powdered atherosclerotic plaque annealed at different temperatures: 150 °C (a), 300 °C (b), 450 °C (c), 600 °C (d), 750 °C (e), 800 °C (f), 900 °C (g). Debye–Scherrer camera, 57.3 mm diameter.

X-ray Diffraction Analysis

From the Debye–Scherrer patterns of the inorganic deposits of the atherosclerotic plaques it is possible to identify this material as hydroxyapatite. After heating of the samples for 10 hours at different temperatures it is possible to see a clear sharpening of the Debye–Scherrer lines with increasing annealing temperature up to 900 °C (Fig. 2). The X-ray diffraction pattern does not change after heating at temperatures from 900 to 1200 °C. From the diffraction maxima characteristic of the hexagonal form of hydroxyapatite it is possible to calculate the lattice constants a = 9.417(2) Å, c = 6.886(2) Å, very close to the ASTM data [21].

TABLE II. Lattice Constants of the Synthetic β-TCMP as a Function of the Ca/Mg Molar Ratio in the Mother Solution (a) and in the Solid Phase (b).

Ca/Mg[a]	Ca/Mg[b]	*a*-axis (A)	*c*-axis (A)
32.3	46.4	10.442(6)	37.35(3)
13.3	29.3	10.406(6)	37.27(2)
4.0	16.4	10.370(4)	37.20(2)
3.0	11.4	10.342(1)	37.15(5)
1.5	4.4	10.332(2)	37.11(1)
0.7	3.6	10.334(3)	37.09(2)

Standard deviations, in units of the last significant figure, are given in parenthesis.

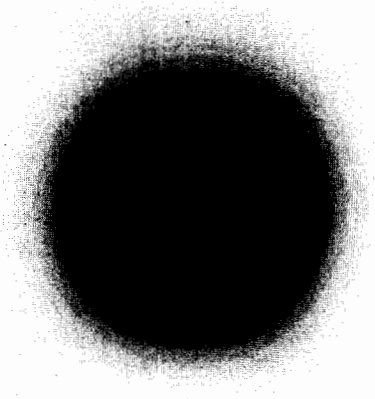

Fig. 3. X-ray diffraction pattern of a sample of defatted air-dried media layer. Flat camera, sample to film distance 40 mm.

The X-ray diffraction pattern of samples of media layer after lipids elimination by treatment with methanol–chloroform (1:3, v/v) for 22 hours allows to identify the mineral phase present in the tissue as highly crystalline β-tricalcium phosphate (β-TCP) (Fig. 3). The heat-treatment of the sample does not produce changes in the shape of the diffraction peaks but reduces the background because of the elimination of the organic matrix which disappears completely by heating at 600–700 °C for 10 hr. The lattice constants of the mineral phase of the aortic media calculated from the angular values of the reflections obtained for samples annealed at 700 °C are a = 10.345(4) Å and c = 37.05(2) Å and are shorter than those of β-TCP (a = 10.429 Å, c = 37.38 Å) [22]. The shortening of the a- and c-axes can be ascribed to the magnesium substitution according to the contraction of the lattice constants measured for

synthetic magnesium substituted β-TCP (β-TCMP). The Ca/Mg molar ratio and the lattice constants of the synthetic β-TCMP annealed at 700 °C for 10 hours are reported in Table II together with the Ca/Mg molar ratio of the mother solution. The variation of the lattice constants with the magnesium content shows some discrepancies with the data reported by Rowles [23]. However, this is not surprising in view of the fact that the same Author reported different values of the lattice constants for samples prepared by sintering at 1100 °C or from aqueous solutions. When the Ca/Mg molar ratio of the synthetic β-TCMP is within the values obtained for the biological material the lattice constants agree well with those of the crystalline inorganic phase of the media layer. Thus this phase can be identified as magnesium substituted β-TCP.

Spectroscopic Analysis

The IR spectrum of ashed atherosclerotic plaques gives absorption bands corresponding to vibration modes of the hydroxyl and phosphate ions in agreement with the identification of this material as hydroxyapatite. However the absorption peaks at 1450, 1410 and 870 cm^{-1} reveal also the presence of the carbonate ion.

The IR spectrum of the media layer heated at 700 °C shows absorption bands which suggest the presence of phosphate and carbonate groups. No evidence for hydroxyl group has been found. This is in agreement with the identification of this material by X-ray diffraction analysis as a non-hydroxyapatitic form of calcium phosphate.

The carbonate content of the inorganic phase of the media layer ranges from about 4% to about 6% wt. as a function of the calcification degree. The carbonate content of the atherosclerotic plaques is about 3% wt. and no appreciable differences have been revealed as a function of the calcification degree of the artery and of the localization of the atherosclerotic plaques in the aortic segment.

Discussion

The adventitia layer of the human arterial wall does not show any evidence of calcification during the ontogenetic process, while inorganic deposits are present in the media and intima layers. The intima layer shows a deposition of mineral salts localized mainly in the atherosclerotic plaques. On the contrary, no evidence of preferential localization of the inorganic phase has been revealed in the media tonaca. Furthermore two distinct inorganic phases have been found. The inorganic phase of the atherosclerotic plaques is hydroxyapatite, while the inorganic deposit of the media layer is β-tricalcium phosphate with a partial replacement of calcium with magnesium (β-TCMP).

The heat-treatment of the atherosclerotic plaques for 10 hours at 700 °C produces the elimination of the organic matrix and an increase of the crystallinity, while no phase variation is evident up to 1200 °C. The complete removal of the carbonate ion after annealing at 1200 °C observed by infrared analysis, does not produce any change in the X-ray diffraction pattern. Since the carbonate content of 3% wt. in the inorganic phase does not produce any change in the lattice constants of hydroxyapatite, a replacement of phosphate or hydroxyl with carbonate in the crystal lattice can be excluded. This is in agreement with the IR spectrum which shows the characteristic absorption bands of calcium carbonate at 1450, 1410, 870 cm^{-1}, while the carbonate apatite with a replacement of carbonate to hydroxyl or to phosphate shows absorption bands respectively at 1465, 1534, 883 and 1430, 1455, 864 cm^{-1} [24].

The chemical analysis of the inorganic deposits in the media layer has shown that the magnesium content in these deposits is a function of the degree of calcification. In the last stages of calcification the Ca/Mg molar ratio is about twice that in the first stages. The inorganic phase is uniformly distributed within the media layer and its chemical composition is practically the same along the first ten centimeters of the descending thoracic aorta. The X-ray diffraction patterns indicate that the crystalline inorganic phase is magnesium substituted β-TCP. The values of the lattice constants are in agreement with those obtained for synthetic β-TCMP with a magnesium content in the same range as that of biological samples. The variation of the lattice constants within this range is too small to appreciate a lattice contraction passing from the more to the less calcified samples. On the other hand as regards this comparison it must be noted that magnesium substituted β-TCP from different sources can show differences in lattice constants due to defective structure or to different conditions of formation [23].

Pure β-TCP is transformed to apatite by heating at 900 °C [15]. However the crystalline β-TCMP found in media layer resisted the transformation to apatite, suggesting that the substitution of magnesium to calcium with a molar ratio Ca/Mg = 10 is enough to stabilize the β-TCP structure. This is in agreement with the results obtained by Le Geros *et al.* [15] for a pathological calcification associated with uremia.

At high calcification degree of the arterial wall the Ca/P molar ratio in the media layer is 1.5, in agreement with the stoichiometric value of β-TCP, but undoubtedly greater than the value calculated of 1.35 and 1.20 for β-TCP with a replacement of one and two magnesium ions respectively every ten calcium ions. The (Ca + Mg)/P molar ratio of 1.7 is clearly greater than the stoichiometric value. At the

first stage of the calcification process the Ca/P molar ratio is lower than the stoichiometric value calculated for β-TCP and β-TCMP. This holds true also for (Ca + Mg)/P molar ratio. The presence of carbonate and possible structural defects can be invoked to explain the non-stoichiometry.

The Ca/P molar ratio of 1.6 obtained from the analysis of the inorganic phase present in the atherosclerotic plaques is very close to the stoichiometric value calculated for hydroxyapatite, in agreement with the observed low ionic substitution in the mineral phase.

The results, besides confirming the presence of hydroxyapatite in the atherosclerotic plaques [8], show that the unique inorganic phase present in the aortic media layer is highly crystalline β-TCMP with a magnesium content which depends on the calcification degree. Although the ability of magnesium to promote the formationof β-TCMP found in a variety of abnormal human calcification has been previously demonstrated [15, 17, 18, 25], chemical and crystallographic characterization of crystalline β-TCMP uniformly deposited in the human media aortic wall is reported here for the first time.

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References

1 H. T. Blumenthal, A. I. Lansing and P. A. Wheeler, Calcification of Media of the Human Aorta and its Relation to Intimal Arteriosclerosis, Aging and Discasc, *Am. J. Pathol., 20,* 665 (1944).
2 R. Ross and J. A. Glomset, Atherosclerosis and the Arterial Smooth Muscle Cell, *Science, 180,* 1332 (1973).
3 S. Cell and R. E. Scully, Aging Changes in the Aorta and Mitral Valves. Histologic and Histochemical Studies, with Observations on the Pathogenesis of Calcific Aortic Stenosis and Calcification of the Mitral Annulus, *Am. J. Pathol., 46,* 345 (1965).
4 J. Hallgrimsson, U. Friberg and J. H. Burke, Change with Age in the Methabolism of Sulfated Glycosaminoglycans in Guinea Pig Cardiac Valves, *Exp. Molec. Path., 12,* 70 (1970).
5 C. Moses, 'Atherosclerosis, Mechanism as a Guide to Prevention', Lea and Febiger, Philadelphia, 1963.
6 R. J. Jones, 'Evolution of the Atherosclerotic Plaque', Univ. of Chicago Press, Chicago, 1963.
7 W. F. De Jong, La Substance Minérale dans les Os, *Rec. Trav. Chim., 45,* 445 (1926).
8 D. Carlstrom, B. Engfeldt, A. Engstrom and N. Ringertz, Studies on the Chemical Compositcon of Normal and Abnormal Blood Vessel Walls: I. Chemical Nature of Calcified Deposits, *Lab. Invest., 2,* 325 (1953).
9 A. Serafini-Fracassini, Electron Microscope and X-ray Crystal Analysis of the Calcified Elastic Tissue, *J. Atheroscler. Res., 3,* 178 (1963).
10 M. Spector, E. M. Krokosky, M. Sax and J. Pletcher, Atherosclerotic Plaque: X-ray Diffraction Investigation, *Science, 165,* 711 (1969).
11 K. M. Kim and B. F. Trump, Amorphous Calcium Precipitations in Human Aortic Valve, *Calcif. Tiss. Res., 18,* 155 (1975).
12 M. B. Gardner and D. H. Blankenhorn, Aortic Medial Calcification, *Arch. Path., 85,* 397 (1968).
13 K. M. Kim, Calcification of Matrix Vesicles in Human Aortic Valve and Aortic Media, *Federation Proc., 35,* 156 (1976).
14 T. K. F. Taylor and K. Littlc, Calcification in the Intervetebral Disk, *Nature, 199,* 612 (1963).
15 R. Z. Le Geros, S. R. Contiguglia and A. C. Alfrey, Pathological Calcification Associated with Uremia, Two Types of Calcium Phosphate Deposits, *Calcif. Tiss. Res., 13,* 173 (1973).
16 N. C. Blumenthal, F. Betts and A. S. Posner, Stabilization of Amorphous Calcium Phosphate by Mg and ATP, *Calcif. Tiss. Res., 23,* 245 (1977).
17 O. R. Trautz and R. Z. Le Geros, Effect of Magnesium on Various Calcium Phosphates I (abstract), *40th General Meeting of the IADR, 101* (1962).
18 O. R. Trautz and R. Z. Le Geros, Effect of Magnesium on Calcium Phosphates II, *J. Dent. Res., 43,* 17 (1964).
19 R. Z. Le Geros, W. P. Shirra, M. A. Miravite and J. P. Le Geros, Amorphous Calcium Phosphates: Synthetic and Biological, *Coll. Intern. C.N.R.S., No 230,* 105 (1975).
20 K. P. Quinlan and M. A. De Sesa, Spectrophotometric Determination of Phosphorus as Molybdovanadophosphoric Acid, *Anal. Chem. 27,* 1626 (1955).
21 X-ray Powder Data File, rcf. card 13–404, American Society for Testing and Materials, Philadelphia.
22 X-ray Powder Data File, ref. card 17–404, Amerıçan Society for Testing and Materials, Philadelphia.
23 S. L. Rowles, The Precipitation of Whitlockite from Aqueous Solutions, *Bull. Soc. Chim. Fr.,* 1797 (1968).
24 G. Montel, Conceptions Actuelles sur la Structure et la Constitution des Apatites Synthétiques Comparables aux Apatites Biologiques, *Coll. Intern. C.N.R.S., No 230,* 13 (1975).
25 E. Hayer and H. Newesely, Über die Existenz von Tricalciumphosphat in wässriger Lösung, *Monatsh. Chem., 89,* 88 (1958).