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Observations Suggesting a Possible Link Between Gammacarboxyglutamic Acid and Porcine Bioprosthetic Valve Calcification

Joseph A. Helpern, MA,* Stephen J. McGee, MS,* and Jeanne M. Riddle, PhD**

Observations that link gammacarboxyglutamic acid (Gla) peptides with ectopic calcification are accumulating in the literature and may be summarized as follows: 1) Gla peptides selectively bind calcium and hydroxyapatite. 2) The presence of detectable levels of Gla in calcified tissue is concurrent with the onset of mineralization. 3) In an animal model, osteocalcin (a Gla-containing protein) accounts for more than 90% of all the Gla found in the resulting subcutaneously implanted calcified leaflet.

Most inorganic chemical complexes associated with the process of mineralization contain calcium. Calcification normally occurs in specific areas, such as those associated with bone growth and development as well as the formation of enamel matrixes. However, calcification can also occur in areas not normally associated with the deposition of inorganic calcium complexes. This is referred to as ectopic calcification. Little is known about this process including the causes. This paper, therefore, serves to introduce this complex subject and discusses the possible role of gammacarboxyglutamic acid in the calcification process of the implanted bioprosthetic heart valve.

Calcification Process - Background Information Formation of hydroxyapatite

A detailed account of the calcification process, both ectopic and nonectopic, can be found in reviews by Boskey (1) and Anderson (2). Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is the most prominent inorganic calcium complex found in calcified tissue. Despite its abundance, the initiating events responsible for its formation and its derivatives remain unknown. Available information suggests that the formation of hydroxyapatite crystals is an energy-dependent process that requires precise concentrations of phosphate and calcium ions, the correct local tissue pH, and a surface on which crystalli-

4) Vitamin D stimulates osteocalcin synthesis in cultures of osteosarcoma cells, and in vitamin D deficient rats subcutaneously implanted valve leaflets are not calcified. 5) Gla content and the degree of calcification in degenerated porcine bioprosthetic valves removed from humans are positively correlated. 6) Porcine bioprosthetic valves implanted in children are calcified more rapidly than those of adults, and the normal Gla levels in the urine of children are more than twice those of normal adults.

zation may occur (Fig. 1). The formation of crystals, which is referred to as the seeding process, has been the primary focus of most investigations studying the mineralization process. However, studies into the origin of hydroxyapatite, which would reveal the steps required for the seeding process, are complicated because the crystalline structure and composition of the inorganic chemical complexes that form are changed with removal of mineralized tissues from their in vivo environment.

Some constituents that may be involved in the seeding process include matrix vesicles, proteoglycans, lipids, and proteins. Whether seeding is a result of one or a combination of constituents, cells adjacent to the calcification site seem to influence or play a direct role in the process.

Gammacarboxyglutamic acid (Gla) and Gla-containing proteins

Gla, which is synthesized by postribosomal vitamin K-dependent carboxylation enzymes (Fig. 2), was first

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isolated and characterized in 1974 by Stenflow, et al (3) and independently by Nelsestuen (4). Gla-containing proteins are plasma proteins, predominantly associated with blood coagulation. These proteins include Factor II (prothrombin), Factor VII (Hageman factor), Factor IX (Christmas factor), and Factor X (Stewart factor). Proteins of less well-known function that contain Gla are osteocalcin, atherocalcin, and proteins C, M, S, and Z (5). Gla-containing proteins have been detected in bone (6), kidney (7), lung (8), and blood (3,4). They have also been found in association with various types of ectopic calcification, including renal stones (9), atherosclerotic plaques (10), surfaces of artificial heart pump sacs (11), skin lesions of scleroderma (12), and degenerated porcine bioprosthetic valves (13). A common feature of Gla-containing proteins is their ability to bind calcium. Several blood clotting factors also bind to hydroxyapatite (14).

Since both free and protein-bound Gla are excreted quantitatively in urine (15), abnormally high urinary levels of Gla may indicate abnormal calcium deposition. Indeed, patients with subcutaneous calcific deposits related to dermatomyositis and scleroderma do have higher than normal urinary levels of Gla (12,16). Also, since Gla synthesis is vitamin K-dependent, urinary Gla levels can be used to monitor vitamin K-inhibiting drug therapy.

Calcification of Porcine Bioprosthetic Valves

Gla levels in an animal model

Calcification is frequently a prominent feature of the degenerated glutaraldehyde-treated porcine bioprosthetic valve. In the animal model developed by Levy, et al (17), glutaraldehyde-treated porcine valve leaflets were implanted subcutaneously into rabbits or rats. They found that the protein, osteocalcin, accounted for almost all of the Gla content of the calcified valve leaflets (18). Quantitative analysis of the calcified leaflets after they were removed from the implantation site demonstrated a positive correlation between the degree of calcification and the concentration of Gla (17). Levy also demonstrated that a similar quantitative relationship existed in degenerated, calcified porcine bioprosthetic valves removed from humans and further showed that the first detectable levels of Gla in calcified valves were correlated with the onset of mineralization (13). Additional studies have also demonstrated that valve leaflets subcutaneously implanted into vitamin D deficient rats failed to calcify (Levy RJ, personal communication). This observation may be particularly relevant since it has been reported that osteocalcin synthesis in cultured

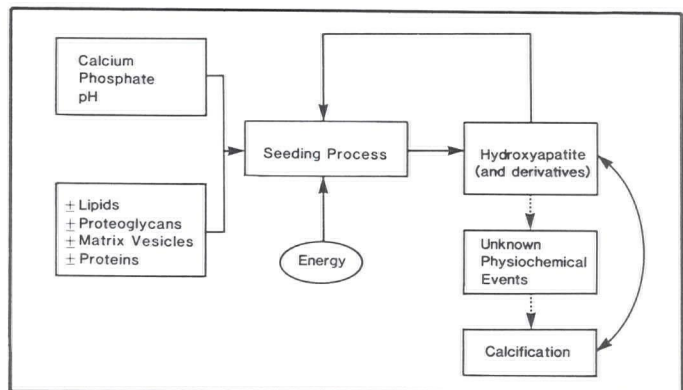


Fig. 1

Schematic drawing of possible contributing factors in the calcification process.

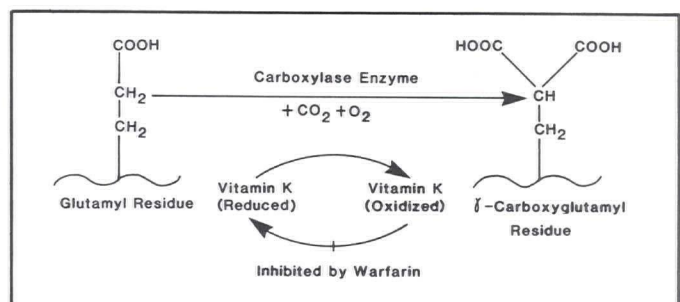


Fig. 2

Enzymatic vitamin K-dependent carboxylation of glutamyl residues to γ -carboxyglutamyl residues.

osteosarcoma cells is increased six-fold when physiological concentrations of $1,25(\text{OH})_2$ -vitamin D_3 are added to the culture medium (18).

These findings suggest a role for osteocalcin in the calcification process, at least in this animal model. However, the environmental differences between valve leaflets implanted subcutaneously in rats and porcine bioprosthetic valves implanted into the heart of a human are substantial. For example, in the animal model the implanted valve leaflets are bathed with tissue fluid and do not directly contact flowing blood, as do bioprosthetic valves inserted into the heart. However, it is difficult to study the ectopic calcification process in degenerated porcine bioprosthetic valves removed from human recipients, because most (up to 90%) of the protein-bound Gla cannot be extracted, so that its origin is difficult to establish. The simplicity of the animal model and the fact that the calcification process does involve a Gla-containing protein make the animal model an

important, but limited, means of studying the process of porcine bioprosthetic valve calcification.

Gla levels in children

Normal children excrete more than twice as much Gla in urine per day as normal adults (19). Between 10 and 15 years of age, urinary levels of Gla in children fall to adult levels. Interestingly, calcific degeneration of porcine bioprosthetic valves progresses more rapidly in children and young adults than in older individuals (20). These findings suggest that perhaps the increased levels of Gla (free or protein-bound) may expedite the process of ectopic calcification within the implanted porcine bioprosthetic valve. The validity of this relationship remains to be determined.

As a result of these various observations, it seems reasonable to hypothesize that gammacarboxyglutamyl peptides may play a significant role in the calcification process of an implanted porcine bioprosthetic valve. These peptides are clearly available from the blood that continuously contacts the valve. The currently unanswered question is to what degree, if any, they are involved in the deposition of the inorganic calcium complexes. Are they a necessary part of the seeding process and/or proliferation of crystallization, or are they only secondarily associated with the deposits because of their availability and affinity for calcium?

Possible relationship between structural components and calcification

Histologically, glutaraldehyde-treated porcine bioprosthetic valve leaflets are mostly composed of a collagen framework, with a somewhat open texture (21,22). Matrix vesicles are not observed, but a small amount of lipids can be present, and both of these constituents may be important in the process of calcification. After

degeneration, the morphology of the leaflets changes. Platelets may adhere to the collagen framework along with leukocytes, and the collagen framework itself is structurally altered. The result of this degeneration is a progressive accumulation of blood components (cells and plasma proteins) as well as the presence of remnants of damaged fibroblasts and altered collagen fibers. The modified blood constituents, particularly activated platelets and cellular debris, may serve as focal niduses for the calcification process.

A possible route for preventing the calcification process - Vitamin K-inhibiting drugs

Since Gla synthesis is vitamin K-dependent, administration of vitamin K-inhibiting drugs such as warfarin might delay the development of ectopic calcification. Such studies have been reported by Pierce, et al (11), who administered warfarin, aspirin, and dipyridamole to Holstein-Freisan calves with implanted artificial hearts. At autopsy, the pump sacs were found to be essentially free of calcification. However, when the experiment was repeated without warfarin, the pump sacs were extensively mineralized. Other studies investigating this relationship need to be performed.

Conclusions

Not all of the biochemical factors involved in controlling ectopic calcification have been determined. No direct evidence available to date demonstrates that Gla-containing proteins initiate or extend the calcification process. Gammacarboxyglutamic acid peptides and Gla-containing proteins may be implicated only because of their availability and affinity for calcium. However, the evidence seems to warrant further study of the function of Gla-containing calcium-binding proteins, vitamin K, and vitamin D in the ectopic calcification process.

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