Structure of asteroid bodies in the vitreous of galactose-fed dogs

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Purpose: Asteroid hyalosis is a condition in which white spherical particles (asteroid bodies) are suspended in the vitreous, usually in the dependent part of the vitreous. These particles seldom cause serious visual symptoms; however, their presence can be a source of irritation. It has been suggested, but not confirmed, that asteroid hyalosis may be associated with systemic diseases such as diabetes, hyperlipidemia, or hypertension. Studies indicate that these particles are composed of lipid material and calcium; however, the specific composition and structure of asteroid bodies remains unknown. We have observed that asteroid hyalosis occurs in galactose-fed dogs, and this represents the first animal model which consistently forms this vitreal condition. The purpose of this study was to identify the main structural component of the asteroid bodies present in the vitreous of these dogs.

Methods: Vitreous humor containing asteroid bodies was collected and frozen from long-term galactose-fed beagles and from age-matched normal controls where asteroid bodies were absent. A portion of the frozen vitreous was sent out for elemental analysis. Thawed vitreous samples were sonicated with HPLC grade water and the aqueous layer was extracted three times with chloroform and then three times with n-butanol. The three organic layers from each extraction were combined and the solvents removed in vacuo. The residue from each extraction was re-dissolved in methanol and analyzed by electrospray ionization mass spectrometry (ESI-MS).

Results: Vitreous-containing asteroid bodies had significantly higher levels of calcium and phosphorus. Negative mode ESI-MS analysis of the n-butanol extracts from vitreous samples with and without asteroid bodies were similar with both containing a predominant peak with a mass to charge ratio (m/z) of 538.4. However, similar analyses of the chloroform extracts indicated that three peaks with m/z values of 547.1, 690.5, and 1430.6 were present only in vitreous samples containing asteroid bodies. Subtraction analysis indicated that the m/z of 690.5 peak corresponded to the main component present. This peak was identified and confirmed to be the quasimolecular ion of 1,2-dipalmitoyl-glycero-3-phosphoethanolamine (DPPE).

Conclusions: Based on the current belief that asteroid bodies are composed of lipid-calcium complexes, we propose that the main component of asteroid hyalosis in the galactose-fed dog is a quasimolecular ion of DPPE in which two molecules of DPPE are complexed through their phosphates with calcium.

Asteroid hyalosis is a benign condition of the vitreous in which whitish spherical bodies are dispersed [1]. These particles are more common in the dependent part of the vitreous humor. Clinically, the presence of asteroid “bodies” in the vitreous can be a source of irritation, but they rarely cause changes in visual acuity, even in cases of severe vitreous involvement. However, vitrectomy may be indicated when asteroid bodies cause poor visualization of the fundus which makes the diagnosis, follow-up and treatment of retinal diseases difficult [2,3]. Vitrectomy may also be required for accurate dioptric power determinations.

The etiology of asteroid hyalosis is not well understood. A number of reports suggest that the formation of asteroid bodies is linked with diabetes mellitus (DM), hyperlipidemia, or hypertension [4-10]. These links, however, have not been confirmed [11,12]. In the general human population, asteroid hyalosis occurs with a frequency of up to 0.5%, is most often unilateral, and affects all races with a male to female ratio of 2:1. The prevalence of asteroid hyalosis has been observed to significantly increase with age.

Asteroid bodies stain positive for fat (oil-red-O) and calcium (von Kossa). This indicates that asteroid bodies are composed of a lipid-calcium complex. Energy-dispersive x-ray analysis also demonstrates that phosphorus along with calcium are the main elements detectable in asteroid bodies. Similarly, electron spectroscopic imaging reveals that calcium, phosphorus, and oxygen are homogeneously distributed. Structure analysis using electron diffraction also indicates that calcium hydroxyapatite and, possibly, other forms of calcium phosphate crystals are present in asteroid bodies [13-16]. The presence of hydroxyapatite has also been confirmed by electron energy loss spectra [15]. While the specific composition of asteroid bodies remains unknown, the large quantity of complex lipids and calcium present in asteroid bodies suggest that they may be derived from sources exogenous to the vitreous especially from retinal diseases.

Asteroid hyalosis has also been reported in dogs, chinchillas, and one cat [17-19]. Moreover, in hypercholesterolemic rabbits, abnormal blood flow, resulting from experimental vascular occlusions, gives rise to an exaggerated deposition of extravascular lipid material that has been suggested to be relevant to such human conditions as drusen, lipoid deposition in the choroid and cornea, and the formation of asteroid...
hyalosis [20]. In our studies with long-term galactose-fed beagles, an animal model that develops diabetes-like ocular changes that range from background retinopathy to the proliferative stage [21-24], we have observed the development of asteroid hyalosis [25]. Here, we report the identification of the major lipid complex contained in the asteroid bodies from the vitreous of galactose-fed dogs.

METHODS

Dogs: Male beagles obtained from Marshall Farms USA, Inc., (North Rose, NY) at two to nine months of age were fed a daily diet containing either 30% galactose (Bio-Serve, Frenchtown, NJ) or normal control diet as previously described in the literature [26]. All dogs were housed individually in 3x9 foot runs. Experiments on all dogs conformed to The Guiding Principals in the Care and Use of Animals (DHEW Publications, NIH 80-23). After enucleation, the anterior segment was removed and approx. 0.5 ml aliquots of vitreous were obtained during the subsequent dissection for retinal studies.

Analysis of calcium and phosphorus: Elemental analysis of vitreous-containing asteroid bodies from four 72-month galactose-fed dogs and normal vitreous from three age-matched controls was conducted using flame atomic absorption spectroscopy by Galbraith Laboratories, Inc. (Knoxville, TN).

Extraction of vitreous: All procedures were conducted in duplicate. The anterior segment from enucleated eyes from galactose-fed dogs was removed, and vitreous samples containing predominantly asteroid bodies were carefully collected and frozen. Similar amounts of vitreous were collected from the enucleated eyes of age-matched controls and frozen. Frozen vitreous containing predominantly asteroid bodies from two dogs fed galactose for 72 months were thawed and combined. Similarly, vitreous from two age-matched dogs without asteroid bodies were thawed and combined. HPLC-grade water (2 ml) was added to each of the combined fractions and the samples were homogenized with a microprobe Sonicator® ultrasonic processor (Misonix Inc. Farmingdale, NY) for 1-2 min. Each sample was then extracted three times with two ml of HPLC grade CHCl3, and then separated by centrifugation for 30-60 min in a glass centrifuge tube at 10,000 rpm. The three chloroform fractions were combined. The aqueous layer was then similarly extracted three times with two ml of HPLC grade n-butanol and the n-butanol, fractions were combined. Solvents from both the CHCl3 and butanol fractions were removed in vacuo, and the residues from each of the fractions were dissolved in 0.5 ml HPLC grade methanol for mass spectrometric analysis.

Mass spectrometry: electrospray ionization mass spectrometry (ESI-MS) was conducted in the negative mode on a Finnigan LCQ™ mass spectrometer (ThermoQuest; San Jose, CA). Samples were directly infused into the heated capillary of the detector at 1.5 µl/min. The spray voltage was 5 kV and the temperature of the capillary was 275 °C; capillary voltage and tube lens offset voltage were both set up at -30 V. The mass range was 150-2000.

Statistics: Statistics were obtained using the independent two sample t-test found in Origin 7 (OriginLab Corporation, Northampton, MA).

RESULTS

Asteroid hyalosis developed in all male beagles fed a daily diet containing 30% galactose for 42-71 months (Figure 1). Dog vitreous is viscous and attempts at separating asteroid bodies from vitreous by centrifugation without vitreal contamination were unsuccessful. Therefore, in order to identify the major component(s) present in asteroid bodies, vitreous from dogs with asteroid hyalosis and vitreous from age-matched dogs not containing asteroid bodies were similarly extracted with chloroform and butanol. The extracts were then analyzed by ESI-MS conducted in the negative mode through direct infusion.

Analysis of the n-butanol extracts revealed no distinct difference between the vitreous components of dogs where asteroid bodies were present or absent. In contrast, similar analysis of the chloroform extracts revealed the distinct presence of a mass to charge ratio (m/z) peak of 690.5 in the asteroid bodies extracts (Figure 2). In addition to the m/z 690.5 peak, the difference spectra between asteroid and nonasteroid bodies containing vitreous extracts revealed the presence of two additional minor peaks of m/z 547.1 and m/z 1430.6 (Figure 3). Data base searches indicated that the major m/z 690.5 peak corresponded to the quasimolecular ion of 1,2-dipalmitoyl-glycero-3-phosphoethanol-amine (DPPE). No matches were
Figure 2. Comparison of spectra from negative ion electrospray mass spectrometry. Negative ion electrospray mass spectrometry spectra of chloroform extracts from vitreous from age-matched dogs without asteroid bodies (A) and with asteroid bodies (B). The data presented illustrates the distribution of the ions with the most abundant ion assigned a relative intensity value of 100. Note the presence of the mass to charge ratio (m/z) 690.6 peak in the asteroid bodies-containing spectrum.

Figure 3. Difference spectra from negative ion electrospray mass spectrometry. The spectrum was obtained by subtracting the total amounts of ionized ions in the methanol extract from normal vitreous from that obtained from the methanol extract of asteroid bodies containing vitreous.
obtained for the m/z 547.1 and m/z 1430.6 peaks. The identity of the m/z 690.5 ion as DPPE was confirmed by tandem mass spectrometry (MS/MS) analysis using a relative collision energy of 34 eV and comparing these results to a similar MS/MS analysis of standard DPPE (Sigma Aldrich, St. Louis, MO). As illustrated in Figure 4, MS/MS of the experimentally observed m/z 690.5 ion and the DPPE standard both resulted in the formation of identical daughter ions at m/z 452.3, 434.3, and 255.2, which correspond to $[C_{21}H_{43}O_7PN]^{-}$, $[C_{21}H_{41}O_6PN]^{-}$, and $[C_{16}H_{31}O_2]^{-}$.

Elemental analysis was also conducted on vitreous-containing asteroid bodies taken from the galactose-fed dogs and similar vitreous without asteroid bodies obtained from age-matched controls dogs. As summarized in Figure 5, samples containing asteroid bodies demonstrated significant increases in calcium and phosphorus. Based on the aforementioned identification of DPPE, the presence of calcium and phosphorus in asteroid bodies [13-16], and the suggested structural and elemental similarity of the asteroid body with hydroxyapatite [14,15], we propose that the major component of asteroid bodies is composed of a complex between two molecules of DPPE and calcium as outlined in Figure 6.

**DISCUSSION**

Studies on the chemical composition of asteroid bodies in vitreous have been hampered by sample size and the difficulty of obtaining asteroid bodies free from vitreous contamination. To circumvent these problems, the present studies were conducted using ESI-MS which is a powerful analytical tool that can accurately obtain the molecular weights of biomolecules extracted from nonderivatized cell samples through ion formation through a combination of solvent evapo-

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**Figure 4.** MS/MS spectra of the major constituent found in asteroid bodies and DPPE. Comparison of MS/MS spectra of the major constituent found in asteroid bodies and standard 1,2-dipalmitoylphosphoethanolamine (DPPE). Identical, corresponding peaks were obtained from the MS/MS analysis of the DPPE standard (A) and the chloroform extract (B), both having a mass to charge ratio (m/z) of 690.5. C: Structures formed during the MS/MS analysis are summarized.
ration and low-energy ion-molecule processes. Moreover, ESI-MS data obtained from extracts from vitreous samples containing asteroid bodies from galactose-fed beagles were directly compared to ESI-MS data from clear vitreous samples not containing asteroid bodies from age-matched control beagles. Using subtraction analysis, any differences between the two groups should be directly attributed to the presence of asteroid bodies. ESI-MS of the chloroform extract from vitreous with asteroid bodies contained a major peak with a m/z of 690.5, which was subsequently identified as DPPE. This was confirmed by MS/MS analysis, a technique in which multiple rounds of mass spectrometry are conducted on an initial ion that is “trapped” in the instrument in a quadrupole ion trap. As select energy is applied to the trapped ion at each step, a reproducible unique set of ions is formed. MS/MS of the m/z 690.5 ion produced a set of fragments that were identical to fragments produced by standard DPPE. The present results are also identical to those of Utzmann and Lederer, who identified a similar m/z 690.5 peak to be the quasimolecular ion of DPPE [27]. Elemental analysis indicated that vitreous-containing asteroid bodies possessed increased levels of phosphorus and calcium. This is in agreement with previous reports that calcium and phosphorus are present in asteroid bodies [13-16]. Based on the elemental analyses and the suggested structural and elemental similarity of the asteroid bodies with hydroxyapatite [14,15], we propose that the major component of the asteroid bodies may be composed of a complex that is formed between two molecules of DPPE and calcium (Figure 5). This complex contains a hydroxyapatite component and is consistent with the proposal that asteroid bodies are not true crystals but rather are liquid crystals of phospholipids contained within the vitreous humor [28].

In addition to the m/z 690 peak, two additional minor peaks at m/z 547.1 and 1430.6 in the extract from asteroid hyalosis-containing vitreous were observed. Their presence, however, was only observed in difference peaks between vitreous containing and not containing asteroid bodies. Both peaks are independent of the m/z 690.6 peak and their structures to date have not been identified.

Figure 5. Phosphorus and calcium present in vitreous from normal and galactose-fed dogs. Percent elemental composition of calcium and phosphorus in vitreous from galactose-fed dogs containing asteroid bodies (AB) and vitreous from age-matched controls not containing AB. (n=3-4), Asterisks indicate AB containing vitreous had statistically significant larger fractions of calcium and phosphorus (p<0.05). The error bars represent the standard deviation.

Figure 6. Chemical complex containing DPPE. Proposed chemical complex containing DPPE identified in asteroid bodies from galactose-fed dog vitreous. Chemical structure of the major component contains 2 molecules of DPPE complexed with calcium. The dashed box identifies the hydroxyapatite-like structure.
Little on the specific composition of asteroid bodies has been reported. Earlier studies using thin layer chromatography have reported that asteroid bodies from humans contain a number of lipids including relatively high amounts of cholesterol (21%), sphingomyelin (38%), and ceramide dihexoside (23%) along with low amounts of cholesteryl esters (5%), cerebroside (10%), and trace amounts of lecithin [29]. In our present studies, subtraction analysis did not show specific peaks corresponding to any of these compounds. This suggests that these lipids may not be exclusive to asteroid bodies, that the lipid composition of asteroid bodies may vary according to the underlying cause of asteroid hyalosis, or that there may be species differences in the composition of asteroid bodies.

No specific reports on the presence of DPPE in the eye have been published. However, palmitic acid is a major saturated free fatty acid in serum, and it comprises 10% of the total fatty acids in rod outer segment phosphatidylethanolamines [30]. Palmitic acid is also associated with the induction of apoptosis in retinal microvascular cells [31,32]. Glycerophosphorylthanolamine is present in the human retina [33] and diabetic lens [34] and 1-palmitoyl-sn-glycerophosphoethanolamine is present in the developing chick retina [35]. Protein kinase C (PKC) activity which has been linked to retinopathy is also associated with diacylglycerols such as dipalmitoylglycerol [36,37]. The retinal presence of glycerophosphorylthanolamine, the relationship between PKC activity and retinopathy, and the observation that palmitate incorporation into diacylglycerol increase in both diabetes and galactosemia [38] suggest that the DPPE observed in the asteroid bodies of the galactosemic dogs may be derived from the retina and linked to the formation of retinal changes in these dogs.

To date, the etiology of asteroid hyalosis remains unknown and research into their formation, composition, and potential pathophysiological relevance has been hampered by the lack of animal models that adequately demonstrate the formation of asteroid bodies. While the clinical prevalence of asteroid hyalosis has been reported to increase to approximately 3% with age, the prevalence of asteroid hyalosis in animals has not been specifically reported. However, asteroid hyalosis has been reported to occur in 26 out of 595 dogs examined (4.4%) and in 2 of 14 chinchillas (15%) from a specific colony [18,19]. In contrast to these studies, we have observed asteroid hyalosis in all dogs fed galactose diet from periods greater than 42 months. This suggests that the long-term galactose-fed dog not only is an excellent model for the development of retinal changes associated with diabetic retinopathy [21-24], but also a potential animal model to investigate the etiology and physiological significance of asteroid hyalosis.

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