requires adhesion to an appropriate substratum, and contributes to the maintenance of cell viability. Furthermore, epidermal growth factor appears able to regulate cell volume and cell survival under conditions of cell attachment to the substratum. The enhancement of cell survival, associated with cell spreading, by tumor-promoting phorbol ester and vitronectin[7] also seems to be related to the regulation of cell volume, since stretching of the cells by spreading might lead to activation of ion flux and regulation of cell volume[19]. The volume of the cells is transiently increased in the absence of calf serum[13], and growth factors stimulate ion flux and glucose transport[8-10]. It is possible that a disturbance in ion transport may be related to the increase in volume and disruption of the integrity of cells in the absence of serum. However, further studies are necessary to clarify the mechanism of promotion of cell survival through regulation of cell volume.

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Identification of hemolytic granules isolated from human myocardial cells

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Summary. Human myocardial cells from fresh autopsy material contained granules which possessed hemolytic activity against guinea pig and rabbit erythrocytes. The hemolytic granules, which had a density of 1.02 and a diameter of 200–300 nm, were recovered as a microsome fraction from subcellular homogenates of human myocardial cells by differential centrifugation in 300 mM sucrose containing 0.1 mM PMSF and 10 mM EDTA. The membrane lesions caused by the granules were ring-like structures with an internal diameter of about 10–17 nm, analogous to that caused by perforin- and complement-induced lysis. However, the requirement for divalent cation differed from that for perforin-induced lysis, since the microsome-mediated lysis occurred in the presence of EDTA.
Key words. Myocardial cells; microsome granules; hemolysis; membrane lesion.

Cytotoxic T lymphocytes and natural killer lymphocytes are known to lyse a variety of target cells in the presence of calcium ions by contact-dependent mechanisms. The membrane lesions formed by large granules of these effector cells have been demonstrated as ring-like structures resembling complement-mediated transmembrane channels[1,2]. Recent studies have indicated that murine and human cytolytic T lymphocytes contain several proteins which are possibly associated with the killing of various target cells[3]. Perforin and serine protease specific for the synthetic substrate BLT are present at high levels in the large granules of cloned cytolytic T lymphocytes and are implicated in the lytic process of these effector cells[4]. Recently, regulatory mechanisms which protect the organism's own cells from cytolytic proteins have been reported[5,6]. Although perforin and C9-related

proteins have been found only in a subset of T lymphocytes, it is possible that such proteins may be masked and undetected in tissue cells because of their possible co-existence with regulatory substances. On the other hand, it has been suggested that the complement activation system may play a role in damage occurring in the ischemic myocardium[7,8]. In our studies on the localization of the membrane attack complex(C5b-9), possibly formed in necrotic myocardium as a result of myocardial infarction, we found that a microsome fraction isolated from apparently normal myocardium exerted a hemolytic activity against guinea pig and rabbit erythrocytes, and that the hemolysis seemed to be caused by a novel mechanism unrelated to the complement activation system. We report here on the nature of the hemolytic granules obtained from myocardial cells and the membrane lesions produced by them on guinea pig erythrocytes.

Materials and methods

Human ventricular myocardium of an autopsied subject preserved below 4 °C for 4 h after death was washed with heparinized saline and stored at − 80 °C. A block of frozen material was minced with a microtome razor and homogenized with a teflon homogenizer in 300 mM sucrose containing 10 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were fractionated by differential centrifugation to separate microsomal granules as described[9]. Microsomal granules were suspended in 10 mM piperazine-N,N′-bis(2-ethanesulfonic acid), pH 6.8, containing 100 mM KCl, 3.5 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP and 1.25 mM EGTA (relaxation buffer), layered on the top of a percoll (Pharmacia) discontinuous gradient (10 ml of 90%, 5 ml of 60% and 8.75 ml of 39% percoll) and centrifuged at 30,000 × g (Beckman 50.2 Ti rotor) for 15 min at 4 °C. Hemolytic assay was performed by incubating 0.1 ml of sample with 0.4 ml of erythrocytes (2.5×10^7/ml) suspended in veronal buffered saline, pH 7.4, containing 0.2% gelatin and 10 mM EDTA(EDTA-GVB). The reaction was stopped with 3 ml of cold saline and after centrifugation the optical density of the supernatant was measured at 413 nm.

Serine esterase activity was measured as reported[4]. Briefly, the reaction mixture consisted of 0.1 ml of granules solubilized by 3% NP 40 and 0.9 ml of substrate containing 2×10^{-4} M benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT), 2.2×10^{-4} M nitrobenzoic acid and 0.2 M tris, pH 8.1. After an incubation of 30 min, the optical density of the reactant was determined at 412 nm. Protein content was quantitated with BCA protein assay reagent (Pierce) using bovine serum albumin as a standard.

Transmission electron microscopy was performed according to a method described previously[10]. For negative staining, guinea pig erythrocytes (gpE) were incubated at 37 °C for 90 min with granules at concentrations resulting in 5 times 100% hemolysis in EDTA-GVB. Membranes were washed with 5 mM EDTA and digested with 200 µg/ml trypsin for 2 h at 37 °C. After two washes in isotonic Tris, pH 7.4, the membranes were mounted on grids and negatively stained with 2% uranyl acetate. As a control, membranes were prepared from gpE lysed by human serum with an activated complex of factor B and cobra venom factor in the presence of EDTA[11].

Results

Identification of hemolytic granules. The microsome fraction collected from myocardial cell homogenates by differential centrifugation contained granules with a diameter of 200–300 nm. Some of the granules contained amorphous material bounded by a typical membrane structure (fig. 1). The granules lysed gpE in a dose- and temperature-dependent manner, and the lysis was enhanced by the addition of EDTA (fig. 2). Electron mi-

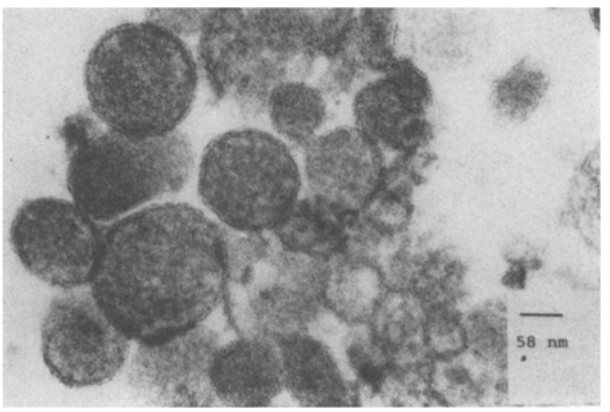

Figure 1. Ultrastructure of microsome granules.

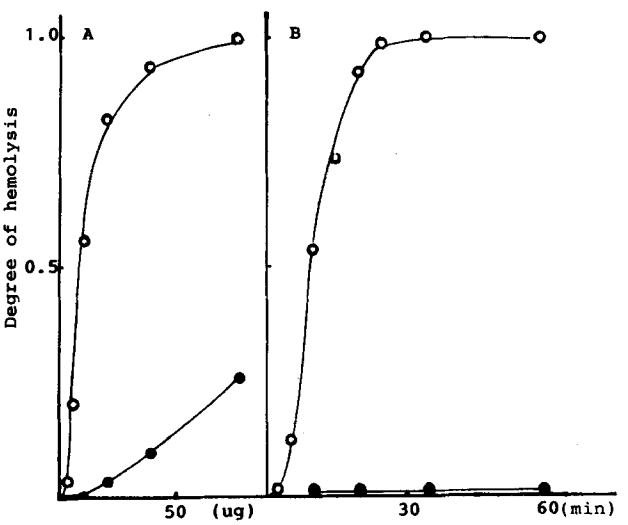

Figure 2. Hemolysis of gpE by microsome granules. *A* gpE (1×10^7) suspended in 0.4 ml of EDTA-GVB (○) or GVB (●) were incubated with 0.1 ml of various amounts of granules suspended in relaxation buffer at 37 °C for 90 min. *B* gpE were mixed with 77 µg microsomes at 37 °C (○) or 4 °C (●).

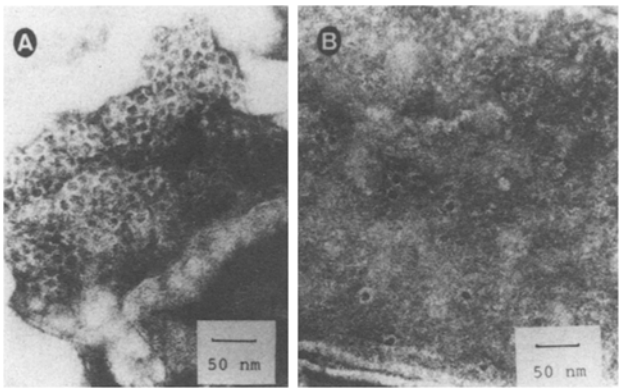

Figure 3. Ultrastructure of membrane lesions produced on gpE membranes. gpE (5×10^7) were lysed (5 times 100% hemolysis) in the presence of EDTA by microsome granules (*A*) and by human serum with activated complex of factor B and cobra venom factor (*B*).

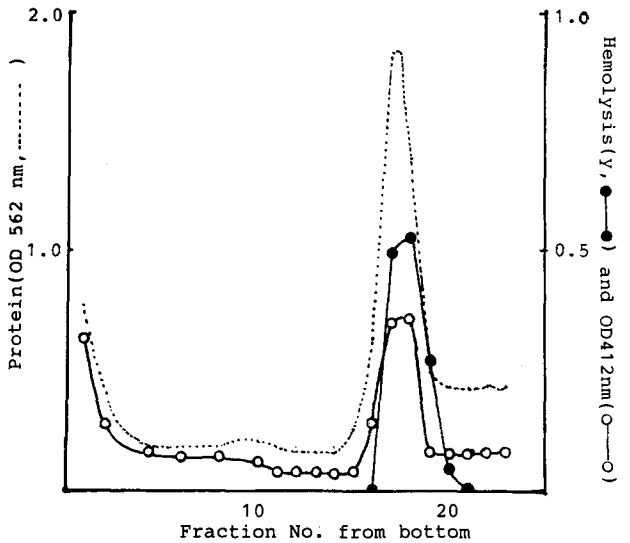

Figure 4. Separation of microsome granules in percoll density gradient. A dotted line indicates protein content and solid lines show hemolytic activity (●) and BLT-specific serine esterase activity (○).

croscopy showed that a ring-like structure with an internal diameter of 10–17 nm was formed on membranes of gpE ghosts which had been attacked by the microsome granules (fig. 3A). These pores resembled the transmembrane channel of lesions formed by human complement with an activated complex of factor B and cobra venom factor in the presence of EDTA (fig. 3B). However, the lesion was different from complement-mediated lysis in that it consisted of a cluster of circular structures irregularly distributed on the membranes. Percoll density gradient centrifugation (fig. 4) showed that hemolytic activity was associated with granules with density of 1.02–1.026, but the maximum activity was invariably detected in the lighter granules ($\sigma = 1.02$, fraction number 18). The heavy particles in the same peak ($\sigma = 1.026$, fraction number 16) inhibited hemolysis mediated by the hemolytic granules (fraction number 18). Figure 4 also shows the presence of BLT-specific serine esterase in granules with the same density as hemolytic granules. Among all the target-erythrocytes tested, guinea pig erythrocytes were most sensitive. Rabbit erythrocytes were less sensitive to the lytic granules. Human, mouse and chicken erythrocytes were not lysed by the granules.

Discussion

It has been shown that the first component of complement and the membrane attack complex accumulate in ischemic myocardial cells[7,8], and it is inferred that the complement activation system plays some role in the development of necrosis in ischemic myocardium. We have examined whether functionally active complement components required for cobra venom factor-dependent hemolysis are present in human myocardial cells and/or in their subcellular fractions. These studies have failed to find such components so far, but unexpectedly we found that human myocardial cells contained hemolytic granules with a relatively low density ($\sigma = 1.02$) in their microsomal fraction. Morphologically defined granules were successfully isolated from samples from autopsied subjects preserved below 4 °C for maximum 6 h after death, but could not be separated in cases where autolytic reactions had proceeded.

Membrane lesions formed by these granules were apparently similar to those formed by human complement and large granules of cytotoxic T lymphocytes[12]. However, the lytic granules differed from large granules of CTL in that they were able to induce lysis in the presence of EDTA, and their targets were limited to guinea pig and rabbit erythrocytes, although in preliminary tests U937 cells were killed (about 20% killing) by a large excess of the granules. The possibility was considered that the lysis of gpE was caused by contamination with lysophosphatide, a catalytic product of phospholipase A_2. If this had been the case, erythrocytes from all species should have been equally sensitive to the granules. Lysolecithin did in fact lyse all eryhtrocytes to the same extent, and pancreatic phospholipase A_2 was not hemolytic against gpE.

The reason why only guinea pig and rabbit erythrocytes are sensitive to the granules is unknown. Considering the similarity with complement-induced lysis, it is suggested that an active substance, probably localized in the membranes of the granules, would polymerize and form transmembrane channels in those erythrocyte membranes which carry receptors, as with cholesterol for streptolysin O[13] and C5678 complex for C9, and that EDTA possibly enhances exposure of the receptor site on target membrane. Another explanation would be that an inhibitory substance is present in the membranes of insensitive target cells. We detected granules which inhibited the process of lysis by the hemolytic granules, suggesting that hemolytic factor and its inhibitor co-exist in the granules of myocardial cells. It is as yet unknown whether such hemolytic granules are specific for myocardium or are also present in other tissues, whether they

are formed solely as a result of post-mortem reactions. If such hemolytic granules and their inhibitor could be demonstrated in laboratory model animals, the role of these factors in tissue injury and their distribution could be clarified. Isolation of lytic factor from human granules and experiments using laboratory animals are in progress.

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Comparison of retinal pigment epithelium cell preparations from the bovine eye

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Summary. Retinal pigment epithelium (RPE) cells were collected from bovine eyes using a new method. The cells were harvested by vortexing the RPE and underlying choroid in 0.05 M citrate phosphate buffer, pH 5. RPE cells recovered by this method were compared to a standard method by microscopic examination of cell integrity, estimation of total protein, and assay of 11-*cis* and all-*trans* retinyl ester hydrolase (REH) activities. Results suggest that this method collects RPE cells of good integrity and with a significantly higher protein yield than the conventional method. Additionally, a much higher retinyl ester hydrolase activity was noted. Therefore we propose that this procedure offers a new and convenient method in the collection of RPE proteins for certain purposes such as enzyme purification.
Key words. Retinal pigment epithelium; retinyl ester hydrolase; vitamin A; bovine.

The retinal pigment epithelium (RPE) is a darkly pigmented single layer of hexagonally shaped cells situated between the choroid and the photoreceptor cells of the retina. RPE cells are joined together by tight intracellular junctions allowing little extracellular space [1]. The RPE is one of the most important cell layers in the visual system and its anatomical arrangement is uniquely adapted to its many diverse functions such as the storage, transport, and metabolism of vitamin A, the nurturing and renewal of the photoreceptor cell layer, and the absorption by melanin granules of scattered light which improves the resolution of images [2]. In addition, it possesses 'glia-like' functions to control water and metabolite fluxes via regulation of extracellular potassium [3]. Therefore it is important to study different methods in which to collect RPE cells.

In the present study, a new method was evaluated to collect RPE cells from the bovine eye. Quantitative recovery of RPE cells was monitored by a) microscopic examinations of cell integrity; b) estimation of recovered protein using the Lowry protein assay; and c) evaluation of enzyme activity by retinyl ester hydrolase (REH) assay. Our results show that this new approach offers a convenient method to recover metabolically active RPE cell proteins in significantly larger amounts than the conventional method (brushing and dislodging RPE cells using a camel-hair brush).

Methods

Dissection of bovine eyes. Bovine eyes were obtained from a local meat packing company (Allstate Packing Co., San Antonio, Tx). The eyes were kept at 0 °C within 1–2 h of enucleation. The anterior portion (anterior to ora serrata) was removed and the vitreous was detached from the posterior segment by tilting the eye. The posterior eye cup consisted of a blue-green region (which was the amelanotic RPE overlying the tapetum lucidum) and a black region (which was the melanotic RPE). Overlying the entire region was the translucent retina consisting of branching blood vessels. With blunt forceps, the retina was teased and gently pulled away from the RPE in one piece. It was then collected at the optic disk and detached with iris scissors.

Collection of RPE cells. In the first method (Method 1), the surface of the exposed posterior eye cup was rinsed