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Short Review

Extracellular vesicles and biomineralization

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Introduction

At the end of the last millennium the concept of the cell membrane had been modified from that of a container and physical barrier to include the function of a regulating and transducing organelle. Initially it was the impact of electrophysiology that changed the approach [1] but then the universality of calcium signalling was recognised [2] and the observation emerged that evolution had adapted the positively charged calcium cation and the negatively charged phosphate anion as the two primary signalling elements of cells. Calcium alone was found to bind thousands of different proteins, with consequent effects on the understanding of cell functions.[3] That was quite a revolution but it is certainly not the only one.

In 1946 a young woman presented herself with a slight bleeding problem at the local hospital where they were studying the clotting time of blood [4]. In treating her they discovered that if a sample of plasma was centrifuged, the time taken for it to clot was extended. It appeared that something was being spun out of the supernatant fluid and whatever it was could be returned by adding the small pellet of debris in the centrifuge tube. Several decades later it became apparent that there was a large selection of smaller and smaller vesicles that occurred not only in the plasma but also with in virtually all cells and their extracellular fluids. Since then it has been a continual revelation as to what functions these structures might encompass.

Exosomes and Endosomes

Perhaps one of the simplest systems to be exposed was the- process whereby the cell membrane could invaginate and bud off vesicles that were able to carry materials into the cell where they could be processed by other organelles. The process was called endocytosis and the vesicles involved could fuse with other membrane components to form an endosomal pathway from the outside to the inside of the cell. The opposite activity, whereby cellular products would be carried to the out- side of the cell in vesicles, was called exocytosis. Between these two processes a variety of vesicles and membrane types could communicate and encompass a diversity of metabolic processes.

It was suggested that the process of exosome formation was initiated when the invagination of a region of a cell membrane fused with a so-called early endosome membrane system (figure 1). This progressed to produce a late endosome where intraluminal vesicles developed to produce a multivesicular body (MVB). Within this organelle are regions where the intraluminal bodies form exosomes by means of a process using an 'endosomal sorting complex required for transport' or an ESCRT. It was originally thought that these multivesicular bodies contained materials that would simply be broken down and digested in lysosomes but it is now clear that is only one of its functions. There is also a pathway from the multivesicular body where additional membrane invaginations form the exosome bodies that are transported to the plasma membrane and released into the body fluids. The decision as to which pathway was used appeared to be determined by the ESCRT process.

It is important to note that during the formation of the early endosome the external layer of the plasma membrane becomes

infolded to form the inner layer of the late endosome. This then turns inside out again to produce the intraluminal vesicles with the original membrane orientations restored. These manipulations produce the intralumenal vesicles that will in turn form exosomes in the multi-vesicular body. Presumably these membrane movements relate to the loading of the biochemical 'cargo' in the exosomes that may then be released into the body fluids. It is this formation and loading of exosomes and their incorporation into a signalling pathway between the donor and recipient cells that has stimulated the current interest in vesicles [5].

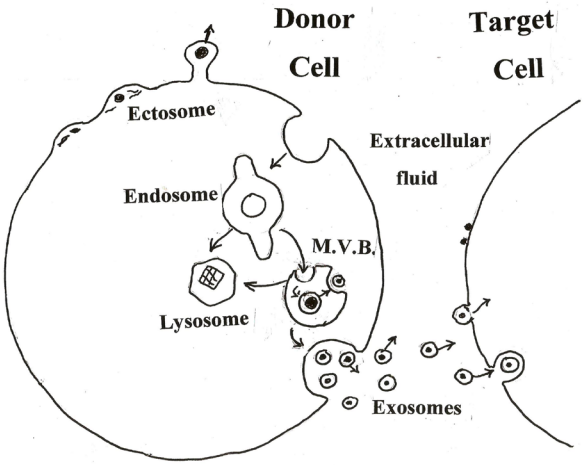

Figure 1. Simplified diagram showing the exosome and ectosome vesicles involved in intracellular and extracellular routes between donor and recipient cells (MVB refers to multivesicular bodies.)

Exosomes are the most common vesicles that are found in the extracellular fluids but they are not the only type. A second type of vesicle called the ectosome is formed simply by the outward budding of 'cargo' deposits from within small domains of the plasma membrane [6].

With this discovery of different vesicle types it soon became apparent that some attempt was necessary to classify them. The operational approaches to this challenge used the shape, volume, viscosity and centrifugal time involved for the separation of three main types of membrane materials. These are microvesicles (100-1000 nm diameter) apoptopic blebs, released from cells with programmed death (50-500 nm diameter) and exosome vesicles (30-100 nm. diameter). The exosomes possess a density of 1.13-1.19 g/ml and require a very high sedimentation force of 100,000g to isolate them [7]. It was generally agreed that separating the cell samples by these procedures resulted in a somewhat heterogeneous mixture of vesicles. It is, however, a workable approach that can be further exploited by using named tissues with protein markers to identify transport and fusion membranes, lipid domains, heat shock proteins, and other indicator molecules [8]. It needs to be recognised however that the components of an exosome membrane may also differ from the rest of the cell membrane that was involved in their origin [9].

The details of this communication system, using vesicles that pass between donor and recipient cells appear to involve some of the same properties as a hormonal system although all sorts of cells may be involved in the process of shedding vesicles into their surrounding extracellular fluids.. The ability of one cell type to release exosomes into a wide range of body fluids (blood, urine, cerebrospinal fluid etc) from where they may transfect an equally wide range of other cell types has been extensively studied [10]. It should also be noted that the number of exosomes available at any one time may involve a remarkable concentration of 10^{10} exosomes per ml of body fluid. As a result the exosome system has been advocated as providing not only a basis for signalling pathways between different cell types [9] but also within intercellular communications [11,12].

The potential applications for delivering molecular 'cargos' were further enhanced when it was shown that genetic material such as mRNA and microRNAs [13] or DNA sources [14,15] could also be transferred in exosome vesicles to provide functional ways of introducing additional genetic material into cells. There were, however, two difficulties that had not been fully resolved. The first was that most of the experimental work in this area was based on *in vitro* tests [9] and the second was somewhat alarming when it was discovered that there could also be communications between hosts and parasites via exosomes if these techniques were involved in clinical situations [16,17].

Exosomes and Calcium

In the past few years exosomes have revealed the potential for their use as a remarkable new tool for diagnosing medical problems by simply analysing minute samples of the patient's body fluids. It has also raised the possibility of a new approach to medication if the pharmaceutical industry can develop a model exosome for dispensing the treatments. If this potential development is realised it will clearly be a revolutionary advance.

Beneath all this excitement there is however, another group of people scratching their heads. Are there some worked examples of what might be achieved theoretically and practically in the use of these vesicles. If calcium is the second messenger in virtually all cells could this be used to demonstrate the practicality of these new revelations? If the first sensation in life is a calcium pulse in the membrane of a fertilised egg and your final reaction is when an uncontrolled flux of calcium passes through your cell membranes and causes cell death; what will be going on between those two dramatic events ? You can read the article 'Calcium and cell death' [18] although it doesn't explain much about the practicalities of how to progress in the

vesicle world if calcium is also involved in the activities of vesicles?

A recent study found that exosome release was regulated by a calcium-dependent mechanism while the RAB proteins activated the docking and fusion of the multi-vesicular bodies within a similar system [20]. If vesicles are critical components of intercellular signalling can they be used in a therapeutic manner i.e, will they have to be integrated into other cell and organ functions [21]. Clearly exosomes are going to become an increasing component in the understanding of cell biology.

Biomineraliztion

Recently a very large group of investigators [22] published the results of a study on the genome of inbred oysters (Crassostrea gigas). This genome is highly polymorphic and rich in repetitive sequences but transcriptosome analyses revealed a large set of genes responding to environmental stress which they interpreted as due to "a highly stressful intertidal environment and the complexity of shell formation." In order to study the proteins involved in shell deposition they identified 259 proteins and found that the majority (84%) were not classical secreted materials of the type that might have been expected if protein secretion was a major contributor to shell formation. Instead they identified the proteins in the shell as being mainly indicative of cellular activity with 61 of the 259 proteins (i.e, 24%) matching the exosome data base. This raised the questions of whether, or how, exosomes might be involved in calcification processes.

A follow-up study [23] used the same population of oysters to study eight groups. One group was used as controls and the other seven had the left hand shells damaged and then sampled at fixed times of up to 21 days of repairing the shell. What they discovered was that the mantle tissue in the region of damage contained elevated levels of shell and exosome derived proteins. The 'punch-line' however, was that all the other tissues such as muscle, digestive glands, labial palps and gonads showed similar enhanced responses i.e all these visceral organs were reacting to the localised damage by producing shell proteins. This suggested that most of the cells in the mollusc actually responded to exosomes and assisted in producing shell repair proteins. That would seem to imply that these exosomes induced a 'whole body' response and that possibility clearly needs further study.

A considerable range of suggestions have been made over recent years as to the biochemical basis of bone and cartilage mineralization but they have been centred on two models. One involves the role of collagen and its ability to act as a crystal nucleating site and the other has concentrated on the ability of osteoblasts and osteocytes to bud off vesicles at sites of mineral deposition. It is now generally accepted that both bone and cartilage forming cells produce matrix vesicles that initiate the formation of hydroxyapatite [24] .It was interesting therefore to read the article entitled 'Matrix vesicles: are they anchored exosomes? [25]. The detailed studies of [26] have pointed out that bone vesicles do not have the apoptopic or programmed death vesicles and the best fit with the study of other vesicle types would be ectosomes rather than exosomes (Figure 1). This cellular pathway omits the normal exosome system with its multivesicular bodies and its ESCRT system and instead develops a vesicle by expansion of an area beneath the plasma membrane until it eventually detaches [7].

It appears, therefore, that there may be substantial links between vesicles and calcification and that these may deserve further study of the.role of ectosomes in the mineralisation of bone and cartilage.

Exosomes may not be solely involved in intellectual communications. They may affect calcification and the pathology of cardiovascular mineralization [27].

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