

Albumin and mammalian cell culture: implications for biotechnology applications

Geoffrey L. Francis

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Abstract Albumin has a long historical involvement in design of media for the successful culture of mammalian cells, in both the research and commercial fields. The potential application of albumins, bovine or human serum albumin, for cell culture is a by-product of the physico-chemical, biochemical and cell-specific properties of the molecule. In this review an analysis of these features of albumin leads to a consideration of the extracellular and intracellular actions of the molecule, and importantly the role of its interactions with numerous ligands or bioactive factors that influence the growth of cells in culture: these include hormones, growth factors, lipids, amino acids, metal ions, reactive oxygen and nitrogen species to name a few. The interaction of albumin with the cell in relation to these co-factors has a potential impact on metabolic and biosynthetic activity, cell proliferation and survival. Application of this knowledge to improve the performance in manufacturing biotechnology and in the emerging uses of cell culture for tissue engineering and stem cell derived therapies is an important prospect.

Keywords Human serum albumin · Bovine serum albumin · Mammalian cell culture · Biotechnology

Introduction

Mammalian cell culture has played an important part in the advancement of the life sciences over many decades and more recently has been a key factor in the application of biomedical research to the development of new therapeutic strategies. Cell culture was a crucial element in the discovery of numerous hormones, growth factors and other biological factors essential for understanding physiological processes at the cellular level and in the whole animal (McKeehan et al. 1990). In the last decade the benefits of mammalian cell culture have become increasingly important for the production of new protein biopharmaceuticals, including monoclonal antibodies and cytokines, as well as providing new strategies for vaccine production (Merten 2006). Tissue engineering and therapies arising from advances in stem cell science are broadening the applications for cell culture technology.

Historically serum has been a crucial component of cell culture methodology, as a provider of complex biological molecules such as hormones, growth factors, attachment factors as well as numerous low molecular weight nutrients. As an essential medium component it underpinned the successful growth of a variety of cell types and in the development of permanent cell lines. Initially, the requirement for serum-free media was to allow the culture of primary cells where the characterization of the biological molecules present in blood and other biological fluids

G. L. Francis (✉)
Applied R&D, Novozymes Biopharma AU Ltd,
28 Dalglish Street, Thebarton, SA 5031, Australia
e-mail: geof@novozymes.com

was possible; and to allow cell processes, such as differentiation, to be investigated in a more defined medium background (Barnes and Sato 1980). The emergence of industrial scale mammalian cell culture for the production of protein pharmaceuticals presented a new challenge for cell culture medium design, where the question of quality control arose from the use of foetal bovine serum (FBS). The issues of reliability of supply and variability in performance and the risk for biological contaminants (mycoplasmas and viruses), created serious safety concerns for regulatory agencies. In more recent years, the emergence of prion related diseases, specifically bovine spongiform encephalomyelitis, led to an increased demand for defined non-animal sourced medium components to replace both serum and medium supplements purified from animal sources, such as insulin, transferrin and albumin (Merten 2002).

Early in the development of serum-free media, bovine serum albumin (BSA) was often incorporated as an essential component of most media. As the major protein in FBS, albumin was seen as a desirable factor associated with successful outcomes from attempts to grow many cell types and cell lines in the absence of serum. Often the basis for this result was never examined, and only rarely was the role it played in the successful growth of cells established (Ham 1963; Nilausen 1978). These early developments and the requirement to address the regulatory issues surrounding animal-sourced medium components resulted in the opportune progression towards recombinant proteins replacing animal-derived components (Keenan et al. 2006).

Albumin is the major protein in serum and is present typically at around 50 mg/ml, where it makes up around 60% of the total protein. Approximately 60% of total body albumin is in the extravascular space, including within the interstitial space of tissues, which infers an important role in the physiological well-being of cells (Ellmerer et al. 2000). However, its physiological actions and the molecular mechanisms involved are not well understood (Quinlan et al. 2005; Ahn et al. 2008). Despite this the main functions of albumin have been summarized to include (1) maintenance of blood oncotic pressure and pH (2) binding and transport of physiologically important ligands, including lipids, metal ions, amino acids and other factors, and (3) antioxidant functions, but mainly from the perspective of its role in the

circulation. Clearly these basic functions of the albumin molecule also apply to the interaction between albumin and cells in animal tissues, or importantly for this review, cells grown in culture, whether in a research laboratory or commercially at large-scale.

BSA has been more widely used in the development of serum-free media than human serum albumin (HSA) until recently, often due to issues of cost and supply, rather than for any advantageous functionality (Keenan et al. 1996). In this review of albumins in cell culture both bovine and human serum-purified albumins are considered, as well as more recent work with recombinant human serum albumin (rHSA) (Keenan et al. 1997). The actual source of albumin will be described in each case, although where appropriate the general term 'albumin' will be used. Applications for albumin are a product of the physico-chemical, biochemical and cell-specific properties of the molecule. Consequently, this review will address each of these properties in the context of its potential use as a cell culture medium supplement. This includes a consideration of (1) structure and biochemical characteristics of albumin, (2) extracellular actions in the culture medium—including interactions with other substances in the medium, (3) uptake of albumin into the cell and the processes involved—including specific receptors and binding sites, and the fate of internalised albumin, (4) binding and carrier functions of albumin—the transport of lipids, metal ions, and other factors into the cell, (5) cellular actions of albumin—including anti-oxidant action and possible roles in metabolic activity, cell proliferation and survival, and (6) the use of albumin in industrial cell culture and biopharmaceutical manufacturing processes.

Structure and biochemical properties influencing albumin function

To enable a clearer appreciation of the basis for a biological function in cells and an application in cell culture for albumin the unique physico-chemical properties of the molecule are important. The crystal structure of HSA reveals a heart-shaped molecule with 585 amino acids in a single chain that displays 67% α -helix with no β -pleated sheet (He and Carter 1992). The equivalent structures of serum and rHSA

by X-ray crystallography have gone a long way to explaining ligand binding observations for albumin (Carter and Ho 1994). The dominant feature of the albumin structure is the three homologous domains (I, II and III) each made up of two sub-domains (A and B) with each of these composed of 6 and 4 helices, respectively, each helix being connected by flexible loops (Fig. 1). The nine loops created by 17 disulphide bridges make up this pattern of three α -helical domains and one free cysteine, Cys34, which is buried in an approximately 10Å deep crevice, which plays a critical role in the redox properties of albumin. The repeating domain structure of HSA belies the asymmetry in the binding sites of different classes of molecules, including lipids and metal ions, and the importance of this will be addressed later in relation to its interactions with cells.

HSA and BSA have been the subjects of extensive comparative studies for a variety of purposes from ligand binding to models of protein unfolding and refolding (Carter and Ho 1994; Hritz et al. 2002; David et al. 2008). There is no definitive molecular structure for BSA from X-ray crystallography or a solution structure from nuclear magnetic resonance

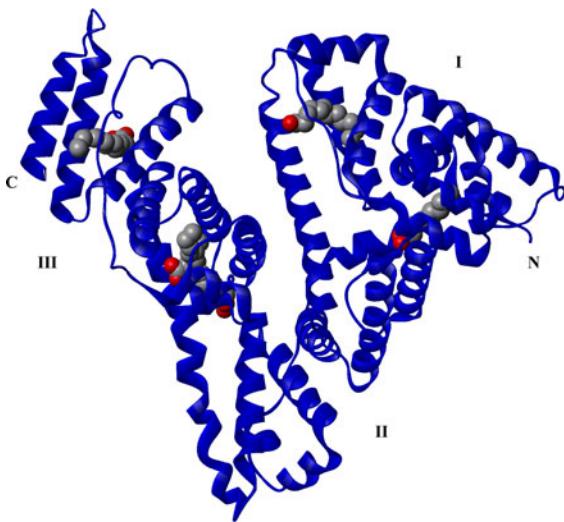


Fig. 1 Crystal structure of recombinant HSA expressed in yeast and complexed with the fatty acid myristic acid. The protein secondary structure is shown schematically (domains numbered I–III) with the bound myristate molecules shown in space-filling representation (carbon, grey; oxygen, red); N and C represent the N- and C- terminus, respectively. Figure modified from Curry et al. (1998)

spectroscopy (NMR), but detailed spectroscopic, chemical cross-linking and other studies have been invaluable in trying to determine the 3-D structure of BSA modelled on the crystal structure of HSA (Huang et al. 2004). Both albumins share 76% sequence identity allowing for some differences in specific physico-chemical properties, although conservation of many, but not all residues characterised as important for binding various ligands is a feature. Despite the asymmetric charge distribution within the primary structure both albumins have approximately 19 negative charges at physiological pH which are distributed relatively evenly across the surface. The strong evolutionary conservation of the albumin sequence across different species infers a critical role in physiological processes that extends beyond the functions so far described. Other aspects of the unique structure of the albumins will be raised where it influences their interactions with cells in culture.

Extracellular actions of albumin in the culture medium

Antioxidant properties of the albumin molecule

The biochemical properties of albumin are responsible for several important interactions with substances present in the culture medium that have a direct or indirect impact on the cell. A key interaction with cells involves the antioxidant properties of albumin, and these are relevant to the extracellular environment of the cell as well as the intracellular compartment. In many ways the role of albumin in the circulation of mammals, both as an antioxidant and a transporter of biologically important ligands, serves as a model for its potential relevance in cell culture. In the cell culture vessel oxidative stress is created by reactive oxygen species (ROS) generated by the interaction of a high oxygen tension, various medium components and general cell metabolism (Halliwell and Whiteman 2004). The extracellular environment of the cell surface as well as the intracellular compartment is important in terms of risk from damage due to ROS, especially as it may lack the regenerating antioxidant mechanisms of the latter (Roche et al. 2008).

The antioxidant potential of albumin usually focuses on the sulphur containing amino acids

cysteine and methionine. As mentioned above there are 35 cysteine residues in albumin with all but one involved in disulphide linkages, leaving, Cys-34, as a free reduced thiol. While it represents the major source of free thiols in plasma the overall anti-oxidative capacity of albumin goes beyond this single residue. It is now recognized that the antioxidant capacity of albumin relates largely to its ability to bind metal ions and to scavenge free radicals as a substitute substrate, in place of other critical protein, nucleic acids or lipid moieties. In this role the relative contribution of Cys-34 and the surface exposed methionine residues have been assessed (Bourdon et al. 2005). These authors concluded that in model systems the antioxidant effect of Cys-34 works largely through its action as a scavenger of free radicals, while Met acts mainly as a metal chelator to reduce subsequent generation of ROS. Other than these two amino acid residue types, most amino acids are susceptible to oxidation in the presence of ROS, and particularly for metal-catalysed oxidation of proteins, which plays an important role in oxidative stress (Guedes et al. 2009). Another oxidative product of Cys-34 involves nitrosylation, a reaction involving the intracellular messenger nitric oxide (NO) or other reactive nitrogen species (RNS) generated by the reaction of NO with superoxide radicals (e.g. O_2^-) (Roche et al. 2008). NO is a vital modulator in most cellular functions as well as a potential mediator of cellular damage where excess RNS can oxidize lipids, DNA and proteins, sometimes leading to cell death via necrosis or apoptosis (Pacher et al. 2007). One RNS, peroxynitrite, is generated within the cell and readily crosses cell membranes, interacts with target molecules in the medium or cells up to two cell diameters away (Liaudet et al. 2009). Mostly the role of RNS in cellular damage has been considered in the context of intracellular metabolic events, even though it has the capacity to act beyond the site of generation to affect other cells.

It is widely acknowledged that albumin provides protection against lipid peroxidation propagated by ROS generated by aerobic metabolism, both in vivo and in vitro (Oettl and Stauber 2007). During this protective action thiol oxidation at Cys-34 occurs, indicating the major source of antioxidant protection afforded by albumin in physiological systems. Bioreactors in large scale mammalian cell culture are a likely environment for generation of oxidative

damage to cells and particularly at the cell surface interface with the external culture medium. The presence of dissolved oxygen or of sparged air bubbles together with medium components, such as transition metal ions including cuprous (Cu^{+1}) or ferrous (Fe^{+2}) ions, can give rise to damaging ROS. When bound to proteins such as HSA or BSA these metals are much less able to participate in the catalytic generation of ROS leading to cell damage. For example, albumin is able to regulate the redox activity of copper ions by virtue of a high-affinity site for copper at the N-terminal; if free in solution copper ions can catalyse the production of free radicals from oxygen. Moreover, fatty acid binding causes conformational changes to HSA and facilitates the oxidation of Cys-34. The effect of this is increased redox-cycling of bound Cu, resulting in an effective conversion of the Cu/HSA complex from an antioxidant to a pro-oxidant, such that it may act itself as a source of oxidative stress (Gryzunov et al. 2003). However, in blood, albumin is the second largest carrier of Cu and co-responsible with ceruloplasmin for its safe transport and prevention of any detrimental pro-oxidant activity. As a component in culture medium albumin would provide the same capability of protecting against detrimental actions of free Cu and potentially facilitate its transport into cells. In a similar manner HSA has the potential to limit extracellular cell damage caused by redox active metal ion contaminants in media such as vanadium, cobalt and nickel (Quinlan et al. 2005).

Few detailed studies exist on the issue of extracellular mediated oxidative stress in bioreactors at any scale, and the results are often equivocal for the few evaluating the impact on production of recombinant proteins in industrial cell lines (Dunster et al. 1997). In fact most of the support for the anti-oxidant role of albumin comes from studies on the circulation in health and disease, including development of atherosclerosis, inflammation, diabetes and kidney disease, using animal and cell culture models to understand the mechanisms involved (Quinlan et al. 2005; Oettl and Stauber 2007). In this context there is evidence for the role of albumin as an anti-oxidant, scavenging ROS and RNS generated in the circulation, probably this also applies to the interstitial spaces of tissues where albumin concentrations are relatively high compared to blood (Ellmerer et al. 2000). The time frames in bioreactor production runs

are incredibly short by comparison with those that apply to development of disease in humans, so it is unlikely that exactly the same processes are involved in creating cell stress in the culture of mammalian cells. Even though the situation for cultured cells is potentially different, the generation of ROS or RNS may impact dramatically on cell physiology, including topics such as genetic stability or cell viability, as discussed later.

Effects of albumin mediated by modifying the extracellular physical environment

The physical environment of the industrial scale bioreactor in terms of hydrodynamic shear forces and the possible detrimental impact on mammalian cell survival or productivity has been widely discussed. However, these questions still remain largely unanswered; although numerous studies have been completed that provide evidence for understanding the impact of hydrodynamic forces generated in agitated and sparged bioreactors (Al-Rubeai et al. 1995; Ma et al. 2002). Recently these and other studies examining the effect of bubble burst in sparged reactors have been compared and, indicates that energy dissipation from typical bioreactor agitation is well below the threshold that can cause acute cell death, although it may lead to sub-lethal changes in cell processes that influence product composition (Mollet et al. 2007; Godoy-Silva et al. 2009). Moreover, bubble rupture following air sparging is a likely source of cell damage or death, as that event generates highly damaging stresses at the cell surface (Chisti 2001; Koynov et al. 2007).

A protective role for albumin against physical damage in airlift or sparged bioreactors is mentioned in many publications, but often without any direct evidence of the exact mechanism involved in cell death or protection (Hülscher et al. 1990; Kunas and Papoutsakis 1990; Smith and Greenfield 1992). It is clear that mammalian cells differ markedly in their susceptibility to the hydrodynamic stress in the bioreactor environment and the ability of albumin and other additives to provide a protective effect (Zhang et al. 1995; Chisti 2001). Mammalian cell damage in sparged bioreactors and the strategies used to control this damage have been examined by Chisti (2000). While evidence exists that protein additives provide protection to animal cells in sparged culture

systems, the exact mechanism by which these additives protect cells is not clear. For example, in a well characterised production process using recombinant BHK-21 cells, in both a 2.5L bubble-free membrane-aerated bioreactor and a 30L pilot airlift bioreactor, the addition of 1 g/l albumin (along with 10 mg/l transferrin and insulin) compared to protein-free medium, leads to a significant reduction of cell lysis, measured as release of lactate dehydrogenase into the medium (Hesse et al. 2003). Furthermore, these cell protective effects of added protein were seen in the presence of the “shear protectant” Pluronic F68 and an anti-foaming agent. The concept of utilizing albumin as a protective agent against the physical damage arising in a sparged bioreactor appears hard to sustain, as reported protective effects occur at protein concentrations that are largely independent of the effects on fluid turbulence. However, the relative contributions of physical and physiological effects are difficult to resolve; despite a recent retrospective on the topic stating that the protection by medium additives is now widely accepted to be physiochemical rather than biological in nature (Papoutsakis 2009), the boundary between the two is largely conceptual. While this apparent distinction may apply to acute lethal cell damage, the situation with apoptotic processes and sub-lethal modification of cell physiology is undoubtedly unresolved. It is still far from clear that protein additives, including albumin, would not have a beneficial influence on cells in the bioreactor environment, however, any increase in productivity gains would have to stand up to cost-benefit analysis.

Physical interaction of albumin with the cell surface

One aspect of an extracellular role for albumin requires direct binding of albumin to the cell surface. At the macroscopic level this has been studied in the biomaterials field for medical device applications where the cell adhesion properties of albumin are of interest. The switching between non-adhesion and adhesion of fibroblasts could be achieved by UV exposure of the albumin coated surface (Yamazoe et al. 2008; Yamazoe and Tanabe 2008). Other cell types have shown similar non-adherence to albumin-coated surfaces, which is at odds with the proposition that albumin “protects” cells by its non-specific

adsorption to the cell surface. This is conceptually different to the specific interactions that have been described at the molecular level between albumin and cells as discussed below.

Uptake of albumin into the cell and its fate

Pathways controlling the entry of macromolecules including albumin into cells

Several pathways for entry of macromolecules into the cell have been described and endocytosis is the basic cellular process that facilitates this transport. To understand the potential of albumin, and its bound ligands, to influence cell physiology requires an understanding of the transport processes in different cell types. Large particles can be taken up by phagocytosis in specialized cells, and bulk fluid by macropinocytosis from the surrounding medium, both of which are relatively major plasma membrane events involving actin-mediated remodelling of the plasma membrane. Macropinocytosis is a signal dependent process stimulated by growth factors which results in actin-driven formation of ruffled membrane protrusions that fuse with the plasma membrane resulting in the large endocytic vesicles ($\geq 1\text{--}2\ \mu\text{m}$); these intracellular vesicles either fuse with lysosomal vesicles for degradation of their cargo, or are recycled back to the plasma membrane surface and released into the medium (Lim et al. 2008). Proteins in the extracellular medium, including albumin, are potential cargo for these and other transport processes that involve endocytosis by clathrin-dependent or independent mechanisms, here the membrane vesicles formed are much smaller in size than in the former modes of entry (Mayor and Pagano 2007). The complexity of the various endocytic pathways is well illustrated by the latter authors who proposed a broad classification framework for the clathrin-dependent and independent mechanisms based on their dependence on the large multi-domain GTPase, dynamin. Further sub-division is by the presence of other components including the small GTPases, CDC42, RhoA or ARF6. The complexity is further highlighted by the fact that the same protein cargo, such as albumin, can be internalised by different mechanism in different cell types, or may

switch pathways within a single cell type under different conditions.

One widely described endocytic process involves specialized membrane structures, caveolae; these membrane domains are involved in clathrin-independent endocytosis and contain the proteins caveolin-1 and caveolin-2, which are essential for this dynamin-dependent pathway. These structures are found widely in many cell types, such as fibroblasts, endothelial cells and smooth-muscle cells. Originally they were thought to be absent in cells of the immune system, including B-lymphocytes, but expression of caveolin-1 in this cell type suggests they are likely to be present, and therefore probably also in hybridoma cells (Medina et al. 2006). It is beyond the scope of this review to discuss the different endocytic processes in depth other than where it reveals details about the intracellular transport of albumin. Whatever the cargo selection process, and the mode of vesicle formation, the endocytosed cargo is usually transferred to the early endosomes where preliminary sorting occurs. Subsequently, the pathway can follow a recycling route back to the plasma membrane, passage to the trans-Golgi network or to late endosomes and lysosomes for degradation.

Cargo selection mechanisms for transport into cells

Cargo stimulated signalling pathways are important in inducing endocytosis, and albumin often employed as a model substrate, binds to and clusters its receptor, gp60, into caveolae to activate G α and Src kinases, triggering caveolae endocytosis (Conner and Schmid 2003). Caveolae are widespread in many cell types and albumin is internalized via caveolae in human skin fibroblasts and endothelial cells. Although in CHO cells albumin is internalized by a clathrin-independent and dynamin dependent process, it involves a Rho A (GTPase)-dependent mechanism, rather than involving caveolin-1/2 (Cheng et al. 2006). In other cell types, such as kidney cell lines or astrocytes, albumin endocytosis involves the membrane glycoprotein megalin, a member of the low-density lipoprotein receptor family that operates via a clathrin-dependent process (Caruso-Neves et al. 2006; Bento-Abreu et al. 2008).

The whole question of cargo selection as part of the uptake process into cells is not well understood in

the different clathrin-independent endocytic pathways. In contrast to this are the clathrin-dependent processes in which specific adaptor molecules are involved, often these are well characterised receptors for ligands such as iron-loaded transferrin, epidermal growth factor, insulin-like growth factor-I and cholesterol carrying low-density lipoprotein. These high-affinity receptors and their bound ligands are sequestered into coated pits assembled around clathrin which invaginate into the cell as endocytic vesicles (Conner and Schmid 2003; Pucadyil and Schmid 2009). Such well-defined specific receptors and adaptor proteins to facilitate entry into the cell have not been described for clathrin-independent processes, including those involving caveolae, and the details remain controversial (Keyel et al. 2006; Parkar et al. 2009).

One recently characterized receptor for albumin transport is the neonatal Fc receptor (FcRn) which was originally characterised as a transport receptor involved following uptake of maternal IgG, providing protection against degradation by selective vesicle recycling back to the cell surface. In a similar fashion albumin is taken up in a pinocytotic mechanism whereupon FcRn bound albumin in the acidified endosomes is recycled to the cell surface, where the higher physiological pH causes release of the albumin (Chaudhury et al. 2003; Andersen et al. 2006; Andersen and Sandlie 2009). The rate of albumin uptake in certain cell types has been measured and in the epithelial cell line RLE-6TN, where both high and low-affinity transport systems operate via apparent clathrin-dependent endocytosis, the transport V_{\max} values were 6.5 and 46.9 μg albumin per mg cell protein per hr, respectively (Tagawa et al. 2008). These revelations leave largely unanswered the broader question as to which uptake processes are important in the different cell types and specifically for the cell types utilized for biotechnology applications.

Fate of endocytosed albumin within different cell types

The pathways following uptake of albumin by the various endocytic mechanisms are not well characterised; the intracellular sorting processes and final destination of endocytosed albumin for most cell types has yet to be established. In the Madin-Darby kidney cell line (MDCK) the mechanism of albumin

uptake, clarified using fluorescently labelled albumin and clathrin, indicated the co-localization of both labels within clathrin-coated pits (Erkan et al. 2005). It is generally accepted that MDCK cells internalize proteins by both clathrin-dependent and independent pathways. Moreover, endocytosis via clathrin-coated pits deliver their cargo to early and late endosomes that are then transferred to lysosomes for degradation to amino acids, to be utilized for energy metabolism or protein synthesis. The fate of albumin in this cycle is not established except in a few cell types. In the RLE-6TN alveolar type II epithelial cell line, albumin transported by a non-caveolae transport mechanism, predominantly a clathrin-dependent process, was tracked to lysosomes and the rate of degradation measured (Yumoto et al. 2006). In the latter study the apparent uptake of fluorescently labelled albumin was approximately 1,100 ng/mg cell protein/60 min, in general agreement with their other measurements in primary type II and type I rat alveolar epithelial cells of between 800 and 6,000 ng/mg cell protein/60 min; the latter is equivalent to 0.2–0.7 pg/cell/60 min (calculated from: Ikehata et al. 2008; Ikehata et al. 2009). Moreover, fluorimetric analysis after SDS-PAGE indicated that during this continuous uptake process approximately 16% of the albumin was degraded after 60 min. In a cell of different origin, rat liver endothelial cells, the uptake of a denatured albumin via gp-18 and gp-30-receptor mediated caveolae-related endocytosis was of a similar order (551 ng/mg cell protein/120 min), and also approximately 20% of the fluorescently labelled albumin was degraded during this period (Bitto et al. 2005). Furthermore, it is interesting to note that the rate of endocytosis of albumin in these different cell systems is similar to the maximum export rates of recombinant proteins by exocytosis in cell lines used in biotechnology processes (≈ 0.4 – 2.0 pg/cell/60 min). This poses the question as to whether there is any common limiting factor in these two different processes involving generation and movement of lipid membrane vesicles within the cells lysosomal/ER-Golgi network. Certainly proteomic and genomic analysis of the performance of cell culture processes has provided support for a rate limiting role for the exocytic process as a whole (Kuystermans et al. 2007).

While the detail of the pathways followed by albumin taken up by various endocytic mechanisms is

unclear, most likely there is considerable redundancy in the capability for albumin transport. Despite this, the existence of many of the known endocytic pathways has been established in cells of industrial relevance including, Chinese Hamster Ovary, mouse myeloma, hybridoma, PER.C6, the various kidney derived cell lines, HEK293, MDCK, Vero, Cos, and BHK-21 cells, to name the most important cell types.

Binding of ligands and carrier-function of albumin in culture media

Serum albumin has a unique capability to bind a wide range of endogenous and exogenous ligands, covalently or non-covalently, and this is one of the most important properties of the molecule in physiological terms (Carter and Ho 1994). The fact that albumin binds so many different compounds, such as fatty acids, metal ions, steroids, amino acids and a variety of drugs, and combined with its high concentration in blood and tissues, makes it very important in health and disease (Peters 1996; Ellmerer et al. 2000). Pertinent to this review is that at the cellular level the ability of albumin to interact with the cell surface, and subsequently the intracellular space, means it also has the potential to influence a wide range of cell processes by the nature of its associated ligand cargo. Consequently, a discussion of some of the more important ligands that bind albumin is warranted. Furthermore, addition of these ligands to the culture medium or pre-bound to added albumin is relevant to the discussion of a role for albumin in the bioreactor performance of industrial cell lines.

Lipid binding to albumin and the effect on cells in culture

Albumin has long been known as the main carrier in the circulation for delivery of unesterified fatty acids (FA) into and from tissues. *In vivo* it increases the solubility of long-chain fatty acids (LCFA) by binding between 0.1 and 2 FA molecules per HSA molecule in blood. Albumin possesses multiple binding sites and a total of seven binding sites in HSA for medium chain FA, and saturated, monounsaturated or polyunsaturated long-chain FA molecules, have been mapped by crystallographic studies (Petitpas et al. 2001). A recent nuclear magnetic resonance (NMR) study determined the

relative affinities of all seven heterogeneously distributed binding sites that provide insights into the potential binding interactions of FA and other ligands binding HSA (Simard et al. 2006). Fatty acid binding stabilizes the native and intermediate unfolded states of HSA during urea and acid induced unfolding, and this indicates the change in conformational properties of albumin that arise from its key physiological role in FA transport.

The molecular mechanisms of FA transport across the plasma membrane are still largely undefined and it is likely the predominant transport mechanisms will be different in different cell types. While the importance of albumin in the transport of FA has been known for some time, more recently an active role rather than a passive role for HSA in the cellular uptake of LCFA has been promoted with evidence for direct albumin interaction with the cell surface (Trigatti and Gerber 1995; Abumrad et al. 2000). Moreover, this process involves sequential transfer with initial dissociation of FA from albumin in conjunction with membrane bound FA-binding proteins including FAT/CD36 (Ehehalt et al. 2006). The latter are associated with cholesterol/sphingolipid dependent lipid rafts on the plasma membrane that facilitate the uptake process into the cell (Stremmel et al. 2001; Ehehalt et al. 2006). It is also proposed that these proteins mediate the dissociation of FA from albumin; moreover, these proteins facilitate the translocation in association with endoplasmic and mitochondrial enzymes catalysing FA esterification involving acyl-coA synthetases (Milger et al. 2006; Ehehalt et al. 2008).

The function of serum albumin in cholesterol transfer between fibroblast cells and lipoproteins is well studied, as it is for endothelial cells, due to its central role in coronary vascular disease and atherosclerosis (Zhao and Marcel 1996; Ha et al. 2003). This is an important function for albumin in many cell types and particularly for cholesterol dependent cell lines such as NS0 myeloma and derivative hybridoma cells. The association of steroids with albumin has not been extensively studied, possibly due to the charge neutrality of many endogenous steroids which as a result have low affinity for albumin, with binding constants (K_d) of between 10^{-6} and 10^{-4} , although this is increased after binding the FA, palmitate. Furthermore, albumin may modulate the biological action of steroids on cells (Amundsen and Sirén 2007).

The evidence for albumin bound lipids as modulators of cell physiology is widely available and the importance of this for industrial cell culture in the development of serum-free media has been long recognized (Merten 2002). Depending on the cell type, a differential response in cell growth may be observed with media containing either recombinant HSA, serum-derived fraction V or fatty-acid free serum-derived HSA. Many studies looking at the role of albumin have employed these various sources of albumin, which due to different production processes and hence impurities or modifications of the albumin, often makes it difficult to draw firm conclusions (Castro et al. 1996). The complexity of this issue is seen with the recent identification of 141 different proteins in commercial human serum albumin preparations using a proteomics approach (Gay et al. 2010). However, a few of the many examples of cell responses to albumin with bound lipid follows:

- (i) Human serum-borne albumin lipids promote cellular survival after apoptosis induction (Schiller et al. 2008)
- (ii) Human serum albumin mediates cholesterol efflux from cultured endothelial cells (Ha et al. 2003)
- (iii) Recombinant HSA and fatty acid-free HSA show slight stimulation of some cell lines but not others (Keenan et al. 1997)
- (iv) Albumin-associated lipids regulate human embryonic stem cell self-renewal (Garcia-Gonzalo and Belmonte 2008)
- (v) The anti-apoptotic activity of albumin for endothelial cells is independent of free radical scavenging and bound lipid but acts via a G-coupled PI3 K-dependent mechanism (Bolitho et al. 2007)
- (vi) Stimulation of kidney cell apoptosis by albumin-bound fatty acids mediated by the peroxisome proliferator activated receptor- γ (Arici et al. 2003)
- (vii) Effects of endogenous ligands on the biological role of human serum albumin in S-nitrosylation (Ishima et al. 2007)
- (viii) Separation of growth-stimulating activity of BSA fraction V from the bulk of albumin using Heparin Sepharose Chromatography (Keenan et al. 1996)

Albumin has enormous potential to modulate the functioning of cells via its capacity to bind lipids and facilitate entry into the cell. It is not clear from the literature how this property could be best utilized to improve the biomedical and manufacturing applications of mammalian cells. However, it does provide some strong research directions to pursue for potential applications utilizing the lipid binding characteristics of HSA in the industrial bioreactor, tissue engineering and stem cell production processes.

Metal ion binding and intracellular actions

Two high-affinity metal binding sites on albumin have been characterized by a variety of spectroscopic and chemical means (Carter and Ho 1994). The location for binding of Cu^{+2} and Ni^{+2} involves the first three residues of the N-terminus, Asp-Ala-His, with His being critical for the observed high-affinity binding for Cu^{+2} , $K_d = 1 \text{ pM}$ (Rózga et al. 2007). The second metal-binding site involves the free sulphhydryl of HSA, Cys-34. In this case the cysteine thiol is essential for high-affinity binding of Cd, Au, Hg and Ag. Weaker binding affinities exist for Ca, Mn, Zn and Fe ionic species, with affinities decreasing in that order. The two contrasting aspects of metals in biology are the requirement for metals as essential co-factors in numerous biological systems and their toxic effects when exposed to sensitive biological processes. Interaction of albumin with various metals means it can play a dual role in both these situations, and some beneficial aspects of a role for albumin in cell culture are considered here.

Although there is considerable interest in the potential role of Cu^{+2} ions in human health, such as neurodegenerative conditions, little is known about the delivery of copper into mammalian cells and the role albumin may play in these processes. Most of the studies on albumin-copper interactions are in cell culture models of human diseases. A recent publication investigated the uptake of copper bound to two human serum-derived proteins, HSA and α_2 -macroglobulin, by human hepatic and mammalian epithelial cell lines. Uptake kinetics were more complex for Cu-albumin and differed for both cell types studied, suggesting more than one transport mechanism, and these did not include either the ubiquitous copper

transporter CTR-1, or the well characterized divalent cation transporter, DTM1 (Moriya et al. 2008).

HSA is also the main zinc binding protein in plasma where 98% of the available zinc in the body is bound to serum albumin. Although binding with lower affinity than Cu^{+2} , it still has an equilibrium binding constant of $K \approx 10^7 \text{ M}^{-1}$, which indicates the potential importance of Zn in biological systems where it is an essential metal co-factor required for numerous extracellular and intracellular proteins and enzymes. On the basis of NMR studies, site-directed mutagenesis and molecular modelling, the major Zn binding site on albumin is located at the interface of domains I and II, and is formed by the side chains of His67 and Asn99 of domain I in conjunction with His247 and Asp249 of domain II (Blindauer et al. 2009). Various studies have identified an involvement of albumin binding in the transport of Zn across the plasma membrane of cells, including endothelial cells, where early studies supported the existence of two major pathways for transcytosis, a receptor mediated endocytosis involving recognition of the Zn-albumin complex and co-transport involving macropinocytosis (Tibaduiza and Bobilya 1996). More recently the same authors examined the uptake into endothelial cells of Zn and albumin, where endocytosis in a 1:1 stoichiometry suggested co-transport with albumin via receptor-mediated endocytosis as the physiological route for Zn delivery (Rowe and Bobilya 2000). Zinc has been proposed as a replacement for the growth factor insulin in hybridoma, myeloma NS0 and Chinese hamster ovary cell culture, to create a more chemically defined cell culture medium (Wong et al. 2004, 2006). The Zn binding and transport capabilities of albumin were not utilized in these latter studies and may further improve the effectiveness of Zn to stimulate cell growth and productivity in these industrially relevant cell lines.

Other metal interactions with albumin are of special interest, including vanadium and vanadium-containing pharmaceutical compounds. Vanadium compounds are known to have insulin-mimetic properties and have been trialled for the treatment of type II diabetes. The mechanism of action is not entirely clear, but recent studies suggest an effect on membrane lipids involved in insulin-receptor signalling, and the inhibition of protein-kinase phosphatases that enhance signalling from the insulin receptor substrate (IRS)-1, phosphatidylinositol 3-kinase (PI3-K) and phosphokinase B (PKB/Akt) cascades (Huyer et al. 1997; Mehdi

et al. 2006; Roess et al. 2008). In cultured cells and in vivo models of vanadium action on these pathways, albumin binding and co-transport was an important factor (Makinen and Brady 2002; Liboiron et al. 2005). This suggests that further use could be made of the known value of vanadium as a trace element in cell growth.

Selenium is widely used as an essential growth promoting agent in cell culture media e.g. insulin/transferrin/selenium (ITS). Despite some early studies that suggest plasma transport of selenium is facilitated by albumin, whereas its intracellular transport is mediated by specific seleno-binding proteins, the full details of the selenium biological cycle are not known (Sani et al. 1988). Recently albumin-mediated selenium transfer by a selenotri-sulphide relay mechanism into cells suggests a potential role for optimizing the beneficial actions of selenium in combination with human serum albumin in medium design (Haratake et al. 2008).

A potential role for albumin in facilitating the uptake and action of essential metals in cell growth leading to improvements in both cell-based applications, and the production of recombinant proteins from mammalian cells is clearly supported.

Cellular actions of albumin

Antioxidant actions of albumin in cells

Few studies have examined the intracellular redox processes that occur in which protein thiolation and dethiolation reactions potentially interact with the established glutathione/glutaredoxin system (Di Simplicio et al. 2005). Most of the studies in this area of redox chemistry have been carried out in vitro in model systems using albumin as the archetypal protein to clarify the mechanisms that may be involved within the cell or in plasma (Summa et al. 2007). As already discussed, very little exact information exists about the intracellular fate of albumin, except in the case of the FcRn receptor, consequently, any intracellular redox role for albumin, comparable to its extracellular role in the circulation, is unknown.

Direct action of albumin on cells in culture

Albumin or modified albumins have an important role in relation to disease mechanisms in human health

including inflammation, diabetes, kidney failure, and heart disease. Cell culture models of the mechanisms by which albumin may play a role in development of these conditions provide an opportunity to gain insight into the possible actions of albumin on cells from a biotechnology perspective. This is especially so when the commercially relevant cell lines share the same tissue or embryonic developmental origin. For example, kidney derived cells such as MDCK, Vero, HEK-293 and BHK-21 cells have been used extensively for vaccine or recombinant protein expression and some of these cells, particularly MDCK cells have been widely used as a model for studying aspects of kidney disease. In studies using MDCK cells as a model for distal tubule cells of the kidney, human serum albumin stimulated net cell proliferation; specifically, total cell turnover was stimulated i.e. both cell proliferation and late onset apoptosis (Erkan et al. 2005). Of interest in the same study with HKC-8 cells of proximal kidney origin, albumin incubation induced marked cytotoxicity, which was closely related to the fatty acid content of the albumin as observed with other proximal kidney derived cell lines (Takase et al. 2008). Likewise there have been a number of studies on the effect of albumin on endothelial cell lines due to the potential importance of albumin mediated effects in vascular disease in humans. In contrast to the often reported apoptotic effects in some kidney cell lines, albumin is anti-apoptotic in endothelial cells of vascular origin, and the latter effects appear independent of its ROS scavenging properties or bound lipid (Bolitho et al. 2007). One issue in many studies on both kidney and vascular endothelial cells is the effect of glycation of albumin, as occurs mostly in diabetes for example, and which universally results in greater cytotoxicity of albumin. As little as one glycol moiety per molecule was sufficient to dramatically increase the toxicity of albumin. The implications are, whether the albumin is sourced from serum or made by a recombinant process, for industrial and biomedical cell culture applications the presence of carbohydrate adducts should be avoided.

The beneficial effects of albumin in primary cell culture and stem cell culture are often mentioned. Improved functionality for cultured mouse and human pancreatic islets was observed when incubated with bovine or human serum albumin, respectively (Barbaro et al. 2008; Zawalich and Zawalich 2008).

There are beneficial effects of albumin in cell culture resulting from its interactions with other biological factors such as insulin, epidermal growth factor (EGF) or as a carrier of the intracellular signalling molecule, nitric oxide (Ishima et al. 2008). In retinal precursor cells albumin augmented their proliferation in association with EGF and this suggests the commercial cell line Per.C6TM, derived from human embryonic retinal cells, may benefit from media containing a combination of these factors (Yang et al. 2009). In the case of stem cells, cell-based tissue engineering, and mammalian cell produced protein therapeutics, the requirement for a recombinant form of human serum albumin may offer culture performance benefits, and certainly would address many of the regulatory concerns arising from the use of animal sourced medium components (Carpenter et al. 2009). For example, cell microencapsulation for biomedical applications requires approved materials, and it has been concluded that HSA may be a possible substitute for FBS to support cells in alginate–polyL-lysine-alginate capsules prior to implantation (De Castro et al. 2006). Moreover, medium design for stem cell and tissue-engineering applications is undergoing rapid development and rHSA is a valuable supplement in this endeavour.

The most recent examples of the use of albumin in cell culture come mostly from stem cell applications, such as providing a crucial ingredient in media to support the reproducible differentiation of human embryonic stem cells (hESC) in response to added growth factors. In this role, recombinant HSA, added at 5 mg/ml, is a more reliable and uniform medium component than serum-purified BSA and provides a robust platform for hESC differentiation (Ng et al. 2008; Hatzistavrou et al. 2009). The culture of hESCs in serum-free, feeder-cell-free conditions requires the use of albumin in the medium, and recombinant HSA meets both this need and the regulatory requirements essential for future clinical applications (Manton et al. 2010). Similarly, there is an increasing interest in albumin for use in culture media in tissue repair and engineering applications; particularly for recombinant HSA, which can provide the platform for serum-free media, such as in the development of keratinocyte and fibroblast co-culture in skin equivalent strategies (Mujaj et al. 2010). It is widely recognized that the development of large scale processes for the generation of hESCs, induced

pluripotent stem cells and adult stem cells, or their derivatives, will be necessary before stem cell-based therapies become widespread in the clinic (Kehoe et al. 2010). Consequently, the availability of reliable sources of high-quality recombinant albumin appears to be an important requirement for achieving these goals.

Applications in large-scale bioreactors

Accounts of albumin use at industrial scale in the literature are limited and lacking detailed analysis. Indeed most of the thinking in this area has centred on finding replacements for serum-derived bovine or human albumin and to provide some of the “benefits” delivered by these preparations; as the former retained “unknown factors” from blood in their final purified forms (Keenan et al. 1997; Merten 2002). Historically the inclusion of albumin in serum-free media was viewed as desirable, but insufficient evaluations have been undertaken to establish a clear benefit for its inclusion. This was partly illustrated in the report of Hesse et al. (2003) discussed earlier, they employed BHK-21 cells and chemically-defined protein-free media versus media containing 1 mg/ml albumin. Although improvements in cell stability were observed at the 2.5 and 20 L scale, no significant improvement in cell specific production of interleukin-2 was observed in the presence of the protein additives at all scales, including at the 1,000 L scale, although only one run was completed at the larger scale.

Growth and monoclonal antibody production by hybridoma cells is stimulated by the addition of 0.5 mg/ml to 5 mg/ml of serum-purified albumin to serum-free media, and albumin is included in a list of recommended components for commercial hybridoma media (Glassy et al. 1988). Although, during the following decade the availability of only serum-purified HSA or BSA, rather than a recombinant source, was an important reason for non-inclusion of albumin in medium design, due to the regulatory and other constraints associated with serum-sourced albumins. Proliferation and production of monoclonal antibody by Sp2/0 hybridoma cells in both a 50 ml tube scale-down model and 2.5 L bioreactor system are stimulated equally by the addition of either native purified BSA or recombinant HSA (L. Chirkova and

K. Bertram personal communication). Commercially, three marketed monoclonal antibodies produced in hybridomas, Erbitux and Remicade, in Sp2/0 cells, and Zenapax in NS0 cells, further indicate that in principle an opportunity exists for recombinant HSA containing media (Stadlmann et al. 2008). While the development of serum-free media for viral vaccine production has involved lengthy consideration of the need to replace the benefits gained from inclusion of HSA or BSA in medium formulations, the answers to this challenge are complex (Merten 2002).

Concluding remarks

Albumin has been widely used in cell culture over the last few decades as a component in serum free media, mainly due to its role as an important carrier of ‘serum-derived’ substances that support mammalian cell growth. These include lipids, amino acids, hormones, peptides, metals and other undefined low molecular weight molecules. Is this carrier function essential or advantageous for most cell types and specifically those employed in biomedical and biopharmaceutical manufacturing? Currently the answer in most cases is that it is not essential, but in many situations it may offer improved cell performance, based on the properties discussed here. Furthermore, the situation may be more encouraging if the emerging cell based therapies in tissue engineering, including use of stem cells, are considered. There is considerable scope to utilize albumin as a carrier of bioactive small molecules, or co-factors, including metal ions. The role of albumin in the function of these bioactive molecules in improving cell growth and performance is unlikely to be absolute; however, it has been shown that albumin contributes to the efficacy of many of these molecules in improving cell growth and survival. At this stage, it must be remembered that most of the effects of albumin have been carried out in small scale cell culture, rather than in large-scale bioreactors or more valid scaled models. It is well established that it is at industrial scale, where cells are producing large quantities of recombinant protein, they are under most physical and metabolic stress. Hence, it is in the industrial scale situation where the beneficial role of albumin would be expected to be seen. Currently, there is a strong move towards chemically-defined and even

protein-free media, including the avoidance of animal-free but complex hydrolysates. This position impacts on how albumin is perceived, despite its production to similar process standards as the recombinant products being manufactured for the clinic. The wide-spread inclusion of recombinant albumins in media for large-scale recombinant production will depend on the specific application, where cost-benefit analysis is favourable, and is more likely to be included in cell-based vaccine production. The situation is more encouraging for the production of cells or related products for tissue engineering and in stem cell–cell therapy, where the cost-structures are different, and some of the beneficial properties of albumin described in this review may be critical to developing a successful therapeutic product.

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