The role of recombinant proteins in the development of serum-free media

Joanne Keenan · Dermot Pearson ·
Martin Clynes

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Abstract Early developments in serum-free media led to a variety of formulations in which components normally provided in serum and required for growth (insulin, transferrin, lipid supplements, trace elements) and poorly defined components (extracts, hydrolysates) were added to defined basal media. These additives were mostly animal-derived. Given recent concerns about TSEs (transmissible spongiform encephalopathies) and other adventitious agents, the drive in media formulations must be towards elimination of animal-origin materials while maintaining cell line productivity. The progress made towards removing animal-derived components and the use of recombinant proteins in serum-free media for mammalian cells is reviewed.

Introduction

There are two distinct phases in the development of serum-free media (SFM) for the manufacture of

biopharmaceutical products. In the first phase (15–30 years ago), the desire was to remove serum due to cost, batch variability, undefined composition and problems with downstream processing in an industrial setting (a 10% solution can add 4.8 mg protein/ml medium). The SFM formulations developed in this phase were more defined and less variable than their predecessors, but suffered problems with cost and crucially, the inability of one SFM to fit all the different cellular requirements. They generally contained components derived from serum and/or other animal sources for successful growth and production, and often included animal origin protein hydrolysates.

The second phase in development from about 1990, coincides with the rapid growth of biopharmaceutical products from mammalian cells for therapeutic use. At present, most therapeutic biopharmaceuticals (recombinant proteins, monoclonal antibodies and viral vaccines) are produced from animal cells.

The possibility of contamination with adventitious agents (mycoplasma, viruses, bovine spongiform encephalopathy (BSE)) while always a consideration, became a driving force with the aim of producing cell culture media devoid of animal-derived materials. While biopharmaceutical products have not been implicated in transmission of viruses or TSEs to date, the need for products free from adventitious agents is of utmost concern to the industry, its regulators and patients as most of these substances are administered parenterally.

J. Keenan (✉) · M. Clynes
National Institute for Cellular Biotechnology, Dublin City
University, Dublin, Ireland
e-mail: Joanne.keenan@dcu.ie

D. Pearson
Delta Biotechnology Ltd., Nottingham, UK

Serum supplementation

Fifty years ago, Eagle (1955) reported a chemically defined medium (Eagles modified essential medium) based on extensive studies of cellular requirements for the growth of mammalian cells *in vitro*. The medium needed a small undefined component of biological origin, often serum. This was an important milestone as previous work depended solely on biological fluids added to balanced salts solutions to encourage growth. Serum was the most efficient of the body fluids investigated and thus became the additive of choice for growth of most cells.

To replace serum is however, not straightforward. Its complexity has made it difficult to identify all the components responsible for the growth promotion and support. Serum supplies;

- growth factors and hormones as mitogens (e.g. insulin, PDGF, FGF, EGF)

- transport proteins and detoxifying agents (e.g. albumin and transferrin)

- attachment factors (e.g. collagen, fibronectin, vitronectin)

- protease inhibitors (e.g. trypsin inhibitor)

- shear protective effects (albumin and bulk proteins)

- nutrients and energy source (e.g. glucose)

- purines and pyrimidines

- vitamins (B group, C and E)

- trace elements and inorganic compounds (e.g. Cu, Sn, Mn, Va, Zn and Se)

Supplementation of basal media with additional vitamins, trace elements, glucose and nucleotides such as Ham's F12 (Ham 1984) reduced the additives required, leaving only the protein and lipid components to be added.

Despite numerous efforts over the past 30 years to develop serum-free formulations (Barnes and Sato 1980; Hewlett et al. 1989) most mammalian cells in the research setting are still grown routinely in media supplemented with 5% fetal calf or fetal bovine serum (FCS/FBS). One of the main factors is the lack of a universal replacement for serum that could support the growth of the variety of cells that serum does. Other drawbacks include the time-consuming and complex preparations of SFM that can be as expensive as serum. Many of these serum-free formulations resulted in reduced growth and specific factors required by a particular cell line had to be

determined empirically. While many media were serum-free, ill-defined components (peptone, hydrolysates and bovine pituitary extract) were added to simulate the growth achieved with serum (Saad et al. 1993). Only where there has been particular advantage in using SFM, are cells such as normal human cells and embryonal cells grown routinely in SFM to control growth and differentiation status (Yao et al. 2003; Draper et al. 2004).

SFM can be classified based on the components added to the basal medium. In SFM, all the components are known but some of the components are of animal origin (Johnson et al. 1992; Liu et al. 2001), notably insulin, transferrin and albumin. During early serum-free development, it was not possible to replace these most common animal-derived proteins with recombinant proteins—only recombinant insulin was available. Protein-free media (PFM) are devoid of protein but often contain peptide hydrolysates. Chemically defined media (CDM) using a complex mixture of non-animal derived components (e.g. metals, salts, amino acids) to replace proteins have been achieved for a few cell types (Darfler 1990a; Hesse et al. 2003; Spens and Häggström 2005) but growth and production do not always match that of medium containing FCS.

For anchorage-independent cells (such as hybridomas and myelomas), SFM have been more successful than for anchorage-dependent cells as there are no requirements for attachment factors, anchorage-independent cells are generally less fastidious and can be scaled up easily. This is fortuitous for the biotechnology industry where many of the cell lines used are anchorage-independent (hybridomas, myelomas) or facultative for attachment (CHO, BHK). The murine myelomas (NS0 and SP2) and the hamster cell lines (CHO and BHK-21) account for nearly all recombinant therapeutics (recombinant proteins and monoclonal antibodies) of which at least 50% are produced in SFM (Chu and Robinson 2001).

Viral production under totally serum-free conditions has been problematic (Merten 1999; Brands et al. 1999). While the virus production phase is serum-free, the cell amplification phase is dependent on the cell type being used as host. Primary or diploid cells, due to growth requirements, need a complex SFM and are therefore in most of the cases expanded in serum-supplemented media. However, a number of continuous cell lines such as MDCK, Vero and

BHK-21 already have SFM associated with them and can be expanded in SFM. These include influenza virus production in MDCK (Palache et al. 1997; Brands et al. 1999; Merten et al. 1996); polio virus production in Vero (Cinatl et al. 1993; Merten et al. 1996) and rabies virus production in BHK-21 or Vero (Perrin et al. 1995; Frazzatti-Gallina et al. 2004). In many of these systems the viral yield was equal if not better than the yield in serum-supplemented media (Merten 2002). Despite this, most industrial scale growth phase processes are serum-supplemented (Chu and Robinson 2001).

Other cell lines with therapeutic applications have also been grown in serum-free or protein-free media such as chondrocytes used in tissue regeneration (Kim et al. 2004) and *ex vivo* expansion of hematopoietic progenitor cells (Möbest et al. 1998).

Regulatory concerns

With the growth of the biotechnology sector and the increasing use of mammalian cell cultures for the production of therapeutics, the need for a medium that is chemically defined and free of animal-derived proteins (Castle and Robertson 1998) is of prime importance and is one of the challenges of the future (Kretzmer 2002). The Food and Drug Administration (FDA) in 1993 “recommended against the use of bovine-derived materials from cattle which have resided in or originated from countries where BSE has been diagnosed”. Protein components may be acceptable provided synthetic processes are validated and sourcing is documented. As a result, all FCS and FBS for the biopharmaceutical industry are supplied with documentation on sourcing, age of animals and the absence of known adventitious agents. Such adventitious agents would include HIV, HBV and HCV from human sources; BSE from bovine sources and parvovirus from porcine sources (trypsin). European Union guidelines also focus on sourcing, testing and risk assessment of cross-contamination during slaughtering and preparation. Recently, a requirement to justify the use of bovine material (amongst others) in pharmaceutical production has been included. This is based on a case-by-case risk-benefit evaluation and is, of course, dependent on the existence of known adventitious agents; it is much more difficult to assess the impact on unknown agents

and this also provides a substantial driving force to eliminate animal-origin materials from medicinal product manufacture (Merten 2002a).

The main additives to serum-free media are insulin, transferrin, hydrolysates and to a lesser extent albumin. The options then are either to replace proteins with small molecular weight intermediates, to use recombinant alternatives or to engineer the cells to remove the requirement for protein additives. Two of the most common supplements added to basal media for serum-free development are insulin and transferrin (Fitzsimmons et al. 2004; Barnes 1987).

Insulin

Insulin *in vitro*, acts as a mitogen for most cells and is involved in glucose and lipid metabolism, amino acid uptake and DNA synthesis (Hoffmann et al. 1989; Conover et al. 1985). The mechanism of mitogenic action of insulin appears to be cell-line dependent. Most studies found insulin to exert its mitogenic effect at supraphysiological levels (1–20 µg/ml) through the Insulin-like Growth Factor-1 (IGF-1) receptor rather than the insulin receptor (Lammers et al. 1989; Tartare et al. 1994). Where insulin acts at physiological levels (ng/ml), additional or novel IGF-1 receptors are often involved to generate a mitogenic response e.g. Ins/IGF-1 (Jonas and Harrison 1985; Moss and Livingstone 1993). In some instances, insulin is reported to act through its own receptor at physiological concentrations (Loo et al. 1990; Harvey and Kaye 1992). For a small number of cell lines, the possibility then exists of using insulin at ng/ml levels or replacing it with IGF-1 (Kim et al. 2005; Morris and Schmid 2000). However IGF-1 is not used regularly in SFM. For the most part, bovine-derived insulin has historically been employed in serum-free media.

Insulin mimetics may offer a limited alternative replacement for insulin in SFM (Tang and Shay 2001; Zhang and Robinson 2005). Replacement of insulin with zinc showed no alteration in cell growth, antibody production and glucose and amino acid consumption of hybridoma CRL1606 and myeloma NSO cells (Wong et al. 2004). This effect was cell line specific however, as CHO-K1 grew in insulin-free medium with zinc (marginally slower than with insulin) but the recombinant clone CHO-IFN could not. An alternative

strategy to remove the requirement of insulin was used by Schröder et al. (2004). By weaning CHO-DU-KXB11 cells from SFM with reducing insulin concentrations, a PFM was generated. Growth in the PFM was slower but once in stationary phase, ATIII production was not affected.

Lai et al. (2004), dispensed with addition of insulin or IGF-1 by genetically engineering CHO-dhfr- to express IGF-1, antiapoptotic protein Bcl-2 and vitronectin. The cells were able to grow in an anchorage-dependent format in protein-free medium (IMDM) without affecting Interferon-beta production.

Recombinant insulin has been available since 1982 (Genentech, Eli-Lilly) but was not used to replace animal-derived insulin until later (Gu et al. 1997; Sun et al. 2004). It is the most universal replacement of animal-derived insulin, being suitable for most industrially relevant cell lines and is present in some commercial supposedly 'protein-free' media (e.g. VP-SFM from InVitrogen; ACETM from INCELL; Cambrex and JRH) while others use non-protein compounds instead of insulin (CDM from Invitrogen).

Transferrin

The most commonly used chelator of iron in SFM is transferrin (Barnes and Sato 1980). Iron is required by eukaryotic and prokaryotic cells where it supports metabolic processes (Richardson and Baker 1994) and cell growth (Laskey et al. 1988). While many cell lines depend on an exogenous source of transferrin (Okamoto et al. 1996), some cells produce their own (Vyhlidal et al. 2002).

The addition of iron without a carrier can be problematic. In aqueous solution, non-protein bound iron can lead to free radical formation and toxic effects on cells (Conrad et al. 1994; Gutteridge et al. 1981). Also, the formation of insoluble ferric hydroxides can make the iron biologically unavailable. During initial serum-free developments, the removal of transferrin was desirable as the most widely used source was of human origin (less expensive and more effective than bovine transferrin). A variety of iron chelators were used to replace transferrin for specific cell lines but none could support the universal activity of transferrin (Sanders and Cheung 1988).

The ability of simple ferric compounds (ferrous sulphate, ferric chloride, sodium nitroprusside, ferric

ammonium sulphate) to replace transferrin has been reported for a variety of cells (Laskey et al. 1988; Keenan and Clynes 1996). Further, such replacement did not interfere with monoclonal antibody production (Darfler 1990; Shinmoto et al. 1988). Insoluble iron has even been suggested to stimulate growth in a mouse hybridoma, PLV-01 (Kovar 1990).

Where simple iron salts were insufficient to replace transferrin, more complex synthetic chelators such as Ferric pyridoxal isonicotinoyl hydrazone (Fe-PIH) have been used for a limited number of cell lines (Tsao et al. 1987; Sanders and Cheung 1988).

A complicating factor in the selection of a replacement for transferrin is the necessity to measure growth and production over a number of passages to ensure the replacement is effective without relying on residual transferrin (Keenan and Clynes 1996; Balls et al. 1997). Residual transferrin may be present due to the cyclic nature of iron trafficking and a single screening assay may still contain cycling transferrins that can mediate iron uptake. Kovar and Franek (1987) found ferric citrate could replace transferrin over 30 passages, maintaining growth and monoclonal antibody production.

Commercial SFM use a variety of iron chelators in formulations and sometimes require screening with a kit to obtain the most suitable for a particular cell line (e.g. InVitrogen iron chelator kit). This underlies one problem with replacing transferrin.

Recently, Deltaferrin, a recombinant human transferrin has been developed by Delta Biotechnology (Nottingham, UK). Deltaferrin expressed from *Saccharomyces cerevisiae* was tested for its ability to support growth of a variety of industrially relevant cell lines (MDCK, BHK-21 and Vero) in comparison to human holo transferrin and a range of chemical iron chelators commonly used in cell culture. The recombinant transferrin was indistinguishable in performance from the native human transferrin and consistently supported optimal growth in the cell lines tested. By contrast, none of the chemical iron chelators demonstrated this "universal" ability to support cell growth of all the cell lines tested (paper in press).

Hydrolysates

With the drive towards chemically-defined SFM and PFM, the use of hydrolysates has become popular to

compensate for lower growth and production rates. Hydrolysates offer an alternative to use of growth factors/hormones (recombinant or native). Hydrolysates are non-chemically defined mixtures of enzyme or acid digests of biological material usually of animal source supplying peptides, amino acids, vitamins and trace elements. Hydrolysates were also found to have a beneficial effect on unwanted proteolysis of secreted proteins (Mols et al. 2005). Animal-derived hydrolysates (e.g. Primatone RL, casein hydrolysates) still present a source of adventitious agents and may not be produced to GMP standards (Jayme 1999). Alternatives to animal-derived hydrolysates (including yeast, soy, wheat gluten or rice) are used for therapeutic products with Vero and MDCK cells (Merten et al. 1999), BHK-21 cells (Heidemann et al. 2000) and CHO cells (Sun et al. 2004). However, as sialylation of BHK-21 secreted protein was altered when soy hydrolysate replaced a casein peptone (Gu et al. 1997), care needs to be taken to ensure that the product is unaffected by such supplementations.

Lipid supplementation/albumin

The role of proteins (including recombinant ones) in lipid supplementation is as transporters to overcome problems of poor solubility and toxicity of lipids in cell culture systems. Apart from the limited addition of free fatty acids (Litwin 1992; Bradshaw 1996), most methods of overcoming solubility are protein based: albumin, most commonly bovine serum albumin (BSA), Ex-cyte, cyclodextrins or protein-based liposomes.

BSA has been incorporated into a variety of SFM (Jäger et al. 1988; Liu et al. 2001; Fitzsimmons et al. 2004) as a lipid transport system, source of intermediary metabolites and protection from shear and oxidation. Naturally bound ligands of albumin include bilirubin, fatty acids, vitamins, amino acids, hormones, globulins and metal ions of which, long-chain fatty acids are quantitatively the most important (especially linoleic, arachidonic, palmitic, linolenic and oleic acids). By controlling the release of fatty acids in SFM, albumin prevents toxic effects of free fatty acids on cells in culture. The 'sticky' nature of BSA has resulted in additional factors contributing to growth promoting activity of albumin including

lysophosphatides (Tigyi and Miledi 1992), citrate (Kane 1990) and erythropoietin (Congote 1987).

Recombinant human albumin produced from yeast by Delta Biotechnology Ltd. (Nottingham, UK) offers the possibility of replacing BSA as there are no human or animal-derived materials used in its manufacture. Recombinant albumin has been used as an additive to SFM (Blake et al. 2002), in the production of ReFacto (to control hemorrhagic episodes) from CHO cells by Genetics Institute and in the manufacture of MMR-II vaccine by Merck.

Ex-cyte (Bayer) is a concentrate of cholesterol, lipoproteins and fatty acids that is used in SFM to enhance cell growth and protein production as well as glycosylation status in a variety of mammalian cells including: hybridomas, myelomas, CHO and HEK293 cells (Castro et al. 1995; Gorfien et al. 2000; Savonniere et al. 1996). It is however bovine-derived and is recommended to be used in conjunction with BSA. Replacement of BSA with recombinant albumin and use of non animal-derived components could support a more defined lipid supplement, albeit not entirely animal-origin free.

Plant-derived cyclodextrins (Gorfien et al. 2000) and synthetic low density lipoprotein complexes (Hayari and Halbert 2005) offer an opportunity to replace animal-derived lipid transporters. For some commercial SFM such as VP-SFM (suitable for Vero, BHK-21, Cos, MDCK), the need for albumin is replaced by di and tri-peptides from plants.

Additional animal-derived factors

Other components involved in growing cells in SFM that maybe of animal origin include attachment factors, growth factors/hormones and trypsin. There is the possibility of replacing such animal-derived proteins with recombinant versions.

Attachment factors were originally included in many SFM, not because the cells could not make the factors but to ease the demands on the cells under serum-free conditions. Where attachment is required, the cell itself may be engineered to produce the attachment factor (Lai et al. 2004). Alternatively, synthetic or recombinant attachment factors (e.g. Pronectin, a synthetic fibronectin, recombinant Enactin) or synthetic RGD sites (one of the active sites of many attachment factors) may be included in SFM

to promote attachment (Yelian et al. 1993). Where growth factors are required, there is a variety of recombinant versions such as EGF, PDGF, FGF and NGF.

Trypsin is used regularly to dissociate anchorage-dependent cells. However, the most commonly used trypsin is porcine-derived. Trypsin can be replaced by non animal-derived proteases such as No-zyme (JRL) or rProtease (InVitrogen) from yeast (*P. pastoris*) or bacteria (*E. Coli*) (Hohenblum et al. 2000, Merten 1999a). Recombinant alternatives described as being animal-free components are available including rTrypsin (Cascades Biologics Inc.) or Trypzean (Sigma Aldrich).

Outlook

The industrial push for serum-free media that are chemically defined and either animal-free or animal-reduced, has led to the development of media that have low animal-derived material content supplemented with hydrolysates, recombinant proteins and plant-derived alternatives. Future developments may involve the replacement of hydrolysates with defined components and the continued use of recombinant proteins.

These formulations need to allow for increased productivity and ease of product purification and yet be reasonably cost effective for the manufacturer. Given the increasing demand for therapeutics (especially antibodies), the doses required for treatment and the production capacity, supply of demand will be problematic (Werner 2004). Not only will this require improvements in cellular production, increased scaling of reactor design but also decreasing cost and increased productivity from SFM are necessary. Other ongoing challenges in process control including removal of waste products (ammonia, glutamine and carbon dioxide) will need to be optimised (Chu and Robinson 2001). Attention to all these factors will help to meet future demands for therapeutics that are effective and free from adventitious agents.

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