Cell Culture Media

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This chapter is dedicated to my former Ph.D. student, Dietrich (Dieter) Möbest who brought with him much basic knowledge and a high interest in cell culture media. Due to a severe illness, Dieter could not further pursue his career after his postdoc time. His solid knowledge in biotechnology and specifically cell culture media and his constant optimism stimulated my own interest in the field and formed the basis of several joint studies investigating the role of culture medium in determining the fate of hematopoietic cells.

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What You Will Learn in This Chapter

You will learn the basic constituents of culture media for mammalian cells and their derivation. The historic development of constituents and additives to culture media such as amino acids, protein, vitamins for individual cell types from different tissues will be covered, with a focus on stem and progenitor cells. You will read on approaches towards the derivation of defined media, and the supplementation of serum additives. The relevance of physical parameters such as osmolarity and buffer systems will be approached. You shall gain an understanding on why essential components of cell culture media, of which several are in general use worldwide, have been added and assess the relevance of their presence in given cell culture settings.

3.1 Purpose of Cell Culture Media

The ability to preserve and amplify different cell types from animal and human tissues has been a key prerequisite for a multitude of discoveries in modern cell and molecular biology, including the identification of chromosomal aberrations in cancer and the production of monoclonal antibodies.

Cell culture media are the major constituent providing an environment securing the survival, further continuous propagation, and/or differentiation for cells that have been either freshly explanted from an organism or have been transformed from other primary cultures. Cell culture media shall in the first line provide the energy sources, confer oxygen to cells, contain a salt composition and pH which are beneficial to the cultured cells, and take up metabolites and debris of cultured cells. In addition, culture media are often used to provide signals for the survival, growth, and/or differentiation of the cultured cells. Thus, cell culture media have to replace the natural environment of the cultured cells in fluid form.

Experiments to isolate cells in culture date back in the early twentieth century, trying to establish expanded cells in plasma clots or fibrinogen clots and allowed for the preservation and isolation of different cell types such as neural tissue or bone marrow [23, 24]. First efforts toward the use of chemically defined media and continuing multiplication on serial subculture included studies by Fisher et al. [15] who supplemented a defined medium with dialyzed chicken plasma, serum, and embryo extract [15]. A multitude of different ingredients derived from tissue, blood, or organ extracts had been tried and established to isolate mammalian cells, including human cells. Among these were plasma clot culture, fibrinogen clots, embryonic cell extracts, and supernatants from explanted cells or tissues. ◘ Table 3.1 lists important purposes of cell culture media and typical basal ingredients mediating these functions.

3.2 Minimum Requirements for Culture Media for Mammalian and Human Cells

In 1955, Harry Eagle published pivotal work on the way to defined cell culture medium. He described a minimum of 27 different factors which needed to be present in "essential" medium (termed therefore, Eagle's Minimum Essential Medium) [13]. The selection of these ingredients, which included inorganic salts, glucose, amino acids, and vitamins, was justified by data omitting the respective reagent. The definition of these minimal ingredients laid the basis for the continuous and stable propagation of murine (L fibroblasts) and

Table 3.1 Main purposes of cell culture media and ingredients

Purpose	Typical ingredient
Energy source	Glucose
Confer oxygen	(free diffusion)
Provide ionic strength	NaCl, KCl, CaCl, $MgCl_2$
Maintain physiological pH	$NaH_2PO_4 * H_2O$, $NaHCO_3$
Take up metabolites and debris of cultured cells	(free diffusion/suspension) added serum or albumin (in part)
Provide survival/growth differentiation signals	(dialyzed) human, horse, or calf serum

Table 3.2 Essential constituents of basal media for cultivation of the HeLa cell and mouse fibroblast

Inorganic salts	Amino acids	Vitamins
NaCl (100 mM)	Arginine (0.1 mM)	Biotin (10-3 M)
KCl (5 mM)	Cysteine (0.05 mM)	Choline (10-3 M)
$NaH_2PO_4 * H_2O$ (1 mM)	Glutamine (2.0 mM)	Folic acid (10-3 M)
$NaHCO_3$ (20 mM)	Histidine (0.05 mM)	Nicotinamide (10-3 M)
$CaCl_2$ (1 mM)	Isoleucine (0.2 mM)	Pantothenic acid (10-3 M)
$MgCl_2$ (0.5 mM)	Tyrosine (0.2 mM)	Thiamin (10-3 M)
Carbohydrate	Leucine (0.2 mM)	Pyridoxal (10-3 M)
Glucose (5 mM)	Methionine (0.05 mM)	Riboflavin (10-4 M)
Other	Phenylalanine (0.1 mM)	
Serum (horse, fetal calf; 1–10%)	Threonine (0.2 mM)	
	Tryptophan (0.02 mM)	
Penicillin, streptomycin	Tyrosine (0.1 mM)	
Phenol red	Valine (0.2 mM)	

Adapted from Eagle [13]

human (HeLa uterine carcinoma) cell lines previously described by Sanford et al. [24] and Scherer et al. [41], allowing their growth also on glass culture surfaces. The essential constituents consisted of salts (NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, and NaH_2PO_4), glucose, 13 essential amino acids, and a number of vitamins of the B complex. Table 3.2 lists the ingredients of Minimum Essential Medium according to Eagle [13].

Careful experimentation by omitting single constituents demonstrated that each constituent is essential. A number of different carbohydrates could however substitute for

glucose: Galactose, mannose, and maltose were almost as active as glucose; some other sugars turned out to be only slightly less active. In the vitamin section, flavin adenine dinucleotide (FAD) could substitute for riboflavin, diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) for nicotinamide, coenzyme A for pantothenic acid, and cocarboxylase for thiamine. Phenol red was included to visualize changes in pH into the acid or alkaline direction which would lead to nonphysiological conditions. Especially, alkaline reactions would be encountered if CO_2 concentrations in the incubators fell below efficient levels.

As to serum, either horse or fetal calf serum was added, which usually had to be pretested on a batch basis. Serum concentrations between 1% and 5% were recommended for survival/experimentation, whereas 5–10% were considered to be useful for propagation of cultures. Alcohol-salt precipitation experiments proved that part of the serum constituents were inert, while others were only weakly active. When serum was exhaustively dialyzed, it was also inactive. However, serum fractions obtained by simple salting out with $(NH_4)_2SO_4$ followed by a 24-h dialysis were all active, to similar degrees.

Further experiments by Eagle demonstrated that cellular functions other than proliferation were less sensitive to the composition of the medium and the presence of the described "essential" components [14]. For example, the amount of poliomyelitis virus released into the medium by the HeLa cell line was quantitatively not affected by the omission of either serum protein, amino acids, or vitamins from the medium shown in ◘ Table 3.2. However, omission of either glucose or glutamine from the medium resulted in a marked decrease in virus production.

3.3 Natural Sources of Culture Media Ingredients

The results of the groundbreaking experiments in the 1950s by Eagle which defined 27 essential media components are still valid today in mammalian and also human cell culture. However, important factors could at that time not be substituted and remained unknown or poorly defined. For some constituents, this is still the case today.

Before the definition of essential medium components, such sources had been human- or animal-derived plasma, serum, lymph, extracts from adult tissues or embryos, or amniotic fluid. A main limitation of the natural media sources was and continues to be the variability between different collections (batches) and different organisms. Other limitations of serum as additive in culture medium are that they are non-defined and can contain, in addition to soluble ingredients, microparticles or exosomes which are difficult to remove. Animal-derived serum is also principally not suitable to culture cells or tissues which are used for transplantation in humans for more than one application, due to the formation of immune responses against animal proteins in patients after transplantation of cells which had been expanded in the presence of, e.g., calf serum [19].

Thus, in serum-supplemented culture, important stimuli continued to be provided by addition of little defined, natural sources. The variety of stimuli provided by serum is summarized in ◘ Table 3.3. Further functions of natural ingredients in cell culture media include activities to "detoxify" the culture, due to binding and inactivation of waste products, to provide (colloid) osmotic pressure for cultured cells, to increase the viscosity of medium and protect cells from mechanical damage, and to buffer against unphysiological pH.

■ **Table 3.3** Substances and activities contained in serum preparations used as additives in cell culture media

Amino acids	Vitamins	Carbohydrates	Trace elements
Lipids	Hormones	Growth factors	Minerals (e.g., Na+, K+, Zn2+, Fe2+)
Binding and transport proteins (e.g., albumin, transferrin)	Attachment and spreading factors (e.g., fibronectin)	Protease inhibitors (protect cells from proteolysis)	Proteases

3.4 Amino Acids

As described above, Eagle [13, 14] worked out and published a list of essential amino acids which allow for the continuous growth of several established cell lines in 2D culture systems. These essential amino acids are also used for the synthesis of nonessential amino acids and other metabolic intermediates such as phospho-l-tyrosine, S-sulfo-l-cysteine, or branched amino acids [17, 40]. L-serine and glycine are involved in the metabolism of nucleic acid precursors through the tetrahydrofolate cycle. Depletion of L-serine may be overcome by additional supplementation with glycine, which leads to L-serine production, avoiding slowing of the tetrahydrofolate cycle and one-carbon metabolism, and inhibition of cell proliferation [11, 22].

Amino acids have been found to be of limited stability in solution. Therefore, for every culture medium preparation, half-life studies have to be performed to determine the intactness of amino acids when storing medium at 4 °C. Alternatively, powdered media formulations have been developed which allow longer pre-use storage. In particular, L-glutamine is instable in aqueous solutions and decomposes to form cyclic pyrolidonecarboxylic acid with the release of ammonia [8, 35]. L-glutamine can be replaced with the dipeptide L-alanyl-L-glutamine (Glutamax), and L-cysteine replaced by *N*-acetyl-L-cysteine or *S*-sulfo-L- cysteine [32, 44]. Addition of more L-glutamine than necessary results in the buildup of ammonia which can be deleterious to some cell lines. Use of phospho-L-tyrosine in place of L-tyrosine has increased solubility of this more lipophilic amino acid [40, 47].

In addition, amino acids are capable of forming mixed crystals, especially amino acids with similar side chains, and cations can form coordination modes through interactions with the nitrogen in the amino group, the hydroxyl oxygen in the carboxyl group, and the carbonyl oxygen in the carboxyl group [21]. Although the composition of amino acids are generally fixed in the established culture media, for fed batch and perfusion cultures, determination of optimal concentrations of amino acids is often pursued, and depends on the metabolic requirements of the cells used [39, 45].

3.5 Proteins, Peptides, and Lipids: The Key to Development of Serum-Free Media

When using classical cell culture media which are described below in more detail, usually sufficient amounts of plasma proteins are supplemented through the addition of serum. In comparison with adult calf serum and horse serum, fetal serum is a relatively richer

source of growth factors and is appropriate for cell cloning and for the growth of fastidious cells [2]. Adult calf serum, on the other hand, is used in contact-inhibition studies because of its lower growth-promoting properties. For primary cell cultures, such as the culture of epidermis equivalents for skin explants, the substitution of single individual factors may suffice [26].

In cases where xenogeneic components are unwanted such as the ex vivo expansion of human cells for therapies, human additives, e.g., human serum, may be used since allosensitization against animal proteins needs to be avoided. One example is the ex vivo expansion of human hematopoietic stem and progenitor cells. Another example, human mesenchymal stromal cells (MSCs) which are used as cellular therapies in a variety of clinical diseases (for review, see [7]), human platelet lysate (HPL) has been established as an efficient additive to replace serum. HPL is at the same time a rich source of human growth factors which are released from platelets during the preparation process and proved to be an efficient replacement for animal or human serum in culture expansion for MSCs [3, 6].

The ultimate aim for the in vitro growth of human hematopoietic stem and progenitor cells has been a defined medium, without addition of serum or human plasma. Thus, the challenge has been the complete omission of serum. The development of serum-free media took a decisive turn when it was possible to provide three main constituents of serum in recombinant or purified form:

1. Albumin
2. Transferrin
3. Cholesterol (or other types of a lipid source)

The most commonly supplemented proteins and peptides in cell culture media are albumin, transferrin, and fibronectin. Albumin is the main protein in blood acting to bind water, salts, free fatty acids, hormones, and vitamins and transport them between tissues and cells. Albumin is also a key component securing the binding of added growth factors such as recombinant cytokines into serum-free media. Both transferrin (expressed in rice) and cholesterol are available in synthetic form as media supplements.

Today, a large number of serum-free cell culture media are on the market and in routine use, including culture media designed for propagation and/or differentiation of hematopoietic cells. By substituting of key functions of serum by the addition of albumin, transferrin, and a lipid source instead of serum [38], serum-free cultures could be established for cell lines and a number of primary hematopoietic and other cell types Arora et al. [2]. For example, serum-free medium for ex vivo expansion of hematopoieitic cells can be manufactured using as a basis an established basal medium such as Iscove's Modified Dulbecco's Medium (IMDM; explained below) [30]. Generally, recipes for such media are proprietal. A rather comprehensive list of FBS-free media for use in mammalian and human cell culture is given by the FRAME Initiative [16].

3.6 Vitamins

As shown above, the water-soluble B-group vitamins are present in the essential culture medium and are needed for cell growth stimulation. In contrast, lipid-soluble vitamins such as Vitamins A, D, E, and K are classically added to media through serum. The seven vitamins which have to date proved essential for the survival and multiplication of mouse

L cells and human HeLa carcinoma cells are choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamin. Omission experiments showed that if cells lacked these vitamins for 5–15 days, the deficiency became apparent by the cessation of cell replication and the development of specific cytopathogenic effects. These changes could be reversed by adding the missing vitamin. The maximally effective concentrations were elaborated for L cells and HeLa cells (Eagle et al. 1955a, b).

The stability of vitamins in culture media is however variable. Kurano et al. [25] found a relative instability of ascorbic acid and thiamine from alpha-modified Eagle's Minimum Essential Medium (MEM-alpha) when it was supplemented with 10% fetal calf serum. Vitamin B_{12} is present in sera at variable concentrations depending on species and due to its chemical instability is vulnerable to storage and handling. A wide range of Vitamin B_{12} is present in formulations of classic media. Basal media often contain no vitamin B12, RPMI-1640 and Iscove's Modified Dulbecco's Medium (IMDM) contain low levels (3–10 nM), and alpha-MEM contains 100 nM vitamin B_{12}, whereas H-Y Medium and McCoy's 5A Modified Medium contain 923 nM and 1.48 μM of vitamin B_{12}, respectively [40]. In general, hematopoietic stem and progenitor cell cultures contain primary, freshly isolated cells and have a limited ability to self-renew or even transform [12, 27]. The limited life span can explain a relatively low need for vitamin supplementation during their culture expansion. Vitamins, particularly lipid-like Vitamins A, D, E, and K, are transported by and bound to albumin. Importantly, the source and the purification grade of the albumin preparation added, especially to serum-free cell culture medium, determine the content of vitamins.

3.7 Buffering Systems

The gaseous CO_2 balances with the CO_3/HCO_3 content of culture medium. Cultures with a natural buffering system need to be maintained in an air atmosphere with supplementation of 5–10% CO_2 is usually maintained by an CO_2 incubator. This natural buffering system is practical, economical, and nontoxic [20, 37] and remains the major method to control pH in cell culture systems today.

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) is a chemical buffer using a zwitterion. It shows a superior buffering capacity in the pH range 7.2–7.4. A controlled gaseous atmosphere is no longer required [42]. In contrast to the bicarbonate system, HEPES is relatively expensive. For some cell types, it may be at higher concentrations. HEPES has been found to enhance the sensitivity of media to phototoxic effects induced by exposure to fluorescent light [46].

pH Indicators Most of the commercially available culture media include phenol red as a pH indicator, which allows constant monitoring of pH [36]. When cells grow and accumulate acid metabolites, a color change reflecting lowering of the culture pH is taking place toward orange and later yellow. At higher pH levels, the color turns purple. Media are bright red at pH 7.4, the optimum pH value for most cell cultures. Disadvantages of using phenol red include the ability of phenol red to stimulate steroid hormone receptors, particularly estrogen [4]. Thus, media without phenol red should not be used with estrogen-sensitive cells, e.g., from mammary tissue. Phenol red can also, when used in some serum-free formulations, interfere with the sodium-potassium homeostasis [18].

3.8 Basal Medium Formulations

In the following, the main characteristics of some basic cell culture media are described. Many of them are available as powder or in liquid form. Powdered media often do not contain sodium bicarbonate, because it tends to gas off and require the addition of three sodium bicarbonate upon dissolving it in water (3.7 g/L).

3.8.1 Eagle's Minimum Essential Medium (EMEM)

EMEM is one of the first widely used standardized media, formulated on his description of media, and was formulated from his basal medium described above [13, 14]. EMEM contains bicarbonate buffer, salts corresponding to the extracellular milieu (i.e., blood plasma), a number of essential amino acids, and sodium pyruvate. EMEM is usually supplemented with additional components and serum. It has been found suitable for a wide range of mammalian cells.

3.8.2 Dulbecco's Modified Eagle's Medium (DMEM)

Compared to EMEM, DMEM is richer, containing almost twice the concentration of amino acids and four times the amount of vitamins. Moreover, ferric nitrate, sodium pyruvate, and additional amino acids are included. Originally, it contained glucose at 1000 mg/L, but a variation with 4500 mg/L glucose has been proved to be better to culture a number of cells. As is EMEM, DMEM is a basal medium and does not contain proteins or growth-promoting agents and requires addition of fetal calf serum (FBS). DMEM contains sodium bicarbonate buffer system (3.7 g/L) and required the presence of CO_2 to maintain the required pH. DMEM is applied widely for culturing primary mouse, chicken, and human cells including fibroblasts. It is used as a basal medium also for embryonic stem cells.

3.8.3 Roswell Park Memorial Institute (RPMI)-1640

RPMI-1640 is a medium applied for many mammalian cells; it has been developed especially for hematopoietic cells and peripheral blood lymphocytes [31]. RPMI-1640 also uses bicarbonate buffering system, but differs from the most mammalian cell culture media by its ability to maintain a pH of 8.

3.8.4 Iscove's Modified Dulbecco's Medium (IMDM)

IMDM is a modification of Dulbecco's Modified Eagle Medium. Includes selenium as well as additional amino acids and vitamins. It lacks iron, with potassium nitrate replacing ferric nitrate. IMDM contains no proteins, lipids, or growth factors and required addition of serum. However, serum-free formulations have been derived from IMDM [30].

3.9 Co-culture with Nurturing Cells and Conditioned Media

Especially in the case of hematopoietic cells, cocultivation with other cells has been a major step leading to the successful growth of lymphocytes and immature hematopoietic cells in culture. Miller et al. [28] published the use of a leukemic cell line in the establishment of lymphocytes. Later, it was found that Epstein-Barr virus (EBV) was the transforming agent for the primary cells [29]. Also in the 1970s, the group of T.M. Dexter in Manchester together with colleagues found that bone marrow, when it was successfully established in culture, i.e., produced mature progeny from immature hematopoietic precursor cells, always contained a stromal cell layer containing adventitial reticular fibroblasts, endothelial cells, and macrophages which formed contact-dependent support of proliferating stem and progenitor cells [10]. Interestingly, hyperosmolaric conditions were beneficial in these culture systems. Moreover, spleen cells from other species could take over the support function for human hematopoietic stem/progenitor cells [1].

In cultures of hematopoietic cells, more recently it has been shown that removal of accumulating growth-inhibiting soluble cytokines can result in improved culture conditions. One relevant cytokine which inhibits hematopoiesis is transforming growth factor (TGF)-β1. By maintaining the concentrations of TGF-β1 below an upper threshold throughout hematopoietic cell culture, ex vivo expansion of hematopoietic progenitor cells could be enhanced and established over longer culture periods [9]. For other factors secreted into the medium, differential effects were shown. Bone morphogenetic protein 4 (BMP-4) at 10 ng/mL, but not at lower concentrations, was permissive for the emergence of cells capable to form not only hematopoiesis but also endothelial cells [34]. Early addition of vascular endothelial growth factor (VEGF)-2 positively influenced this development, whereas later addition did not. This example illustrates that addition and removal of growth factors during culture from the medium bears potential to design the outcome.

Finally, stem cells themselves may be a source of growth and differentiation inducing activity which can be used in culture media. Thus, conditioning of media for pluripotent stem cells by other stem cell types has recently entered the field of stem cell research and may widen the options of stem cell culture, as well as its use in diagnostics, research, and the development of therapies from cultured cells [5, 33, 43].

Take-Home Messages

- Cell culture medium requires essential salts, amino acids, vitamins, and energy sources. Most standardized media require addition of animal or human serum.
- Metabolites that are not further processed or toxic need to be taken up.
- Medium may be designed to provide specific biological signals to cultured cells, relevant for their survival, proliferation, and differentiation.
- Replacement of serum needs imposes a major challenge to medium development and may be accomplished through addition of albumin, transferrin, and a lipid source.

3

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