Mammalian Cell Culture Methods

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Keywords

Cell Cloning
Generation of a colony from a single cell. Subculturing results in a cell strain.

Cell Cycle
Ordered sequences (G_1, S, G_2, and M) of cellular syntheses between two cell divisions.

Cell Line
Subcultured primary cultures. A cell line can be finite or continuous.

Cell Strain
Cell line that has been purified by physical separation, selection, or cell cloning.

Continuous Cell Line
Indefinite proliferation. This immortalization of a cell line may be induced by a viral gene transfer or was already acquired by some cancer cells before cultivation.

Primary Culture
Freshly isolated cells in culture until the first passage into a subculture.

Serum
Blood fluid without cells and clotting factors.

Suspension Culture
Cells proliferate isolated from each other when suspended in growth medium.

Tissue Culture
Accustomed term for the cultivation of animal cells. Originally: Fragments of tissues maintained *in vitro*.

Mammalian cell cultures originate from tissue explants or cell suspensions as primary cell cultures that can be subcultured with a limited life span. By transformation, these cells might lose some of their original properties and establish permanent growth. Many of these continuous cell lines are aneuploid and genetically unstable, nevertheless, our knowledge of molecular, physiological, biochemical, and biophysical properties of cells is notably based on investigations with such cell lines. Since the synthesis of various bioproducts such as vaccines, monoclonal antibodies, enzymes, and hormones is accomplished with cell cultures, many efforts were made to develop and improve cell culture technology. Besides this impetus, cancer research also stimulated the progress of cell culture methods as can be seen with the development of three-dimensionally growing cultures. These cultures provided a better understanding of tumor invasion and revealed the importance of the extracellular matrix for physiological regulations of cell–cell interactions that

cannot be observed with monolayer or suspension cultures. This knowledge helped to improve the cultivation of cells that are used in clinical treatments as is the case for wound healing with implantation of epidermis or for defect organs such as liver, which can be supported with bioartificial organs during a temporary extracorporal bypass.

1 Principles

1.1 Development of Cultured Cells

Animals are three-dimensionally organized complex multicellular creations. Their tissues and organs maintain specific internal milieus and are separated from each other and from their environment by specialized endothelial and epithelial cells. The adult human body consists of hundreds of cell types and altogether of approximately 50 to 100 $\times$ 10^{12} cells from which about 10^9 cells vanish within one hour. This sounds more alarming than it really is because most of these cells are replaced within the same time. The highest turnover is found for blood and epithelial cells; after wounding, organs and tissues might start proliferation albeit they are normally almost quiescent. Even when most cells have the potential for proliferation, it is not an easy task to cultivate them *in vitro*, that is, in Petri dishes, flasks, or bioreactors.

A stimulus to study isolated cells was given in 1858 when Rudolf Virchow postulated that pathological characteristics may be detected on a cellular level. First experiments to cultivate cells were performed with non-mammalian cells such as amphibians with their high capacity to regenerate lost limbs and with chicken embryos that are easily accessed and proliferate with a high activity. Successful attempts to maintain cells *in vitro* date back to 1885 when Wilhelm Roux kept isolated nerve fibers of chicken embryos alive in a warm saltbroth. Much effort was put in the establishment of an appropriate medium in which the cells could survive and even proliferate. In 1895, Paul Ehrlich succeeded to grow and propagate mouse tumor cells by intraperitoneal injection in mice where they grew as single-cell suspension in ascites. This *in vivo* cultivation revealed that the liquid of the peritoneum obviously contains all nutrients necessary for cell proliferation. Consequently, human ascites was used to keep pieces of human skin alive *in vitro*, as was demonstrated in 1898 by Ljunggren who grafted them back to the donors, including himself. A significant step forward was achieved in 1907 by Ross Harrison, who cultivated spinal cords of frogs in a hanging drop of coagulated frog's lymph and studied the outgrowth of nerve fibers during several weeks. In 1912, Carrel reported on the permanent life of tissues outside the organism when he cultivated embryonic chicken cells in an extract of chicken embryos. He introduced aseptic methods and cultivated the cells as monolayers in glass flasks, but that he has kept these cells for more than 30 years is certainly not true since *in vitro* proliferation of normal cells is limited to about 50 divisions, which became known

later by the investigations of Hayflick and Moorhead. Carrel and coworkers had regularly fed their cultures with insufficiently filtered extracts of chicken embryos, which contained fresh cells.

Different media were adopted for specific cells, which grew as monolayers on different substrata such as glass, polypropylene, ceramics, and so on. Earle and coworkers treated mouse fibroblasts with methylcholanthrene and cultured these transformed cells in medium with a bicarbonate/CO_2 buffered salt solution. These L-cells have been growing as a continuous cell line since 1943, and at present their use might only be outnumbered by the first continuous human cell line HeLa, which was derived from a human cervical carcinoma by Gey and coworkers in 1952 (Fig. 1). Modified medium formulations were used by Dulbecco to grow animal viruses in cultured cells and Eagle described in 1955 the minimum requirements of nutrients for appropriate synthetic media, which, however, still required the addition of serum from young animals. Another important step was made by Levi-Montalcini and colleagues who isolated of a protein, which stimulates the growth of certain nerve cells. Similarly, to this nerve growth factor, other growth factors were later isolated from serum (see Sect. 2.2). With better-defined media, more complex cells could be cultivated as was the case with the successful development of a hybridoma cell line for the production of monoclonal antibodies by Köhler and Milstein.

Even from the very beginning of tissue culture, three-dimensional explants were always used, and most experiments are still performed with monolayer and suspension cultures. This might change in the near future since many regulatory processes are better studied in three-dimensionally growing cells that perfectly match the *in vivo* conditions.

1.2 Selection of Cells

Most animal cells are cultivated as monolayers since the majority of cells is anchorage dependent and not very selective with regard to a substratum. This type of cell cultivation offers advantages for microscopic inspections during growth or during experiments and it allows micromanipulation of the cells with electrodes. Normal cells in a primary culture usually stop proliferation by a so-called contact inhibition when they come in close contact on a flat surface. By serum deprivation, the proliferation of these cells can also be inhibited and they remain quiescent in the G_1- or G_0-phase

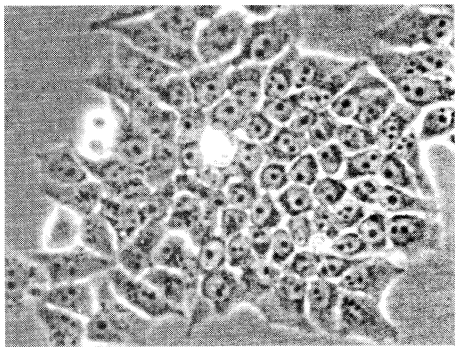

Fig. 1 Monolayer of HeLa cells during logarithmic growth. Diameter of a cell is about 15 µm. Light-microscope phase contrast picture.

of their cell cycle. After addition of serum to these cultures, the cells reenter the cell cycle and progress in a synchronized manner. Under these conditions, signals from individual cells are multiplied since effects occur synchronously in all cells as has been demonstrated for growth factor–induced channel openings in embryonic rat fibroblasts. In spite of the high variance of samples, cells of primary cultures are recommended when specialized functions should be investigated under controlled culture conditions.

Cultures with selected properties are available with transformed cells. They are not contact inhibited and continue to grow as long as the medium is not depleted of nutrients. Numerous investigations in cell biology as well as in cancer research were performed with these continuous cell lines and cell strains, and much of our knowledge on cellular regulation, synthesis, and proliferation is based on these cells. For the production of cellular material, cells were selected after transformation, hybridization, or transfection. In catalogs of the American Type Culture Collection (ATCC), of the European Collection of Cell Cultures (ECACC), or of other national culture collections, appropriate cells for many purposes can be found.

Since production is usually optimized for a high yield, cells were further selected for anchorage-independent growth, which qualifies them to proliferate and to synthesize the requested product in single-cell suspension in large volumes. However, as is evident from intact organisms, producer cells must not necessarily be in a proliferative status. This understanding finally led to cultivation methods that allow the cells to grow as three-dimensional cell aggregates. This might end in organ-like cultures where the cells stop to proliferate but still synthesize substances or function as bioartificial organs as is the case for extracorporal cultivated liver cells.

1.3 Cell Cycle and Growth Curves

Under optimal conditions in culture, cells may divide every 10 to 20 hours except when cells may be stressed by transferring from one culture vessel into the next or by exhausted medium. Between two cell divisions, a cell must synthesize all its materials such as DNA, RNA, proteins, lipids, and so on. These processes could occur continuously or in discrete phases. As has been demonstrated by Quastler and Sherman, DNA is replicated in a separate phase that starts a few hours after division and ends a few hours before the next division. This DNA synthesizing period is called *S-phase*, it is preceded by the G_1- and followed by the G_2-phase, whereas the M-phase covers the mitosis and the cell segregation between the two G-phases. Similarly, as in an adult organism, cells may stop proliferation and rest in the G_0-phase.

When cells are cultivated as suspension culture, they exist as isolated single cells and can easily be harvested without enzymatic treatment and transferred directly into a new culture vessel. Under these conditions, cells may immediately continue to proliferate at a high rate. During mitosis, one cell divides into two cells and, therefore, the number of cells increases to $N + 1$. Since one "old" cell disappears after its doubling time and two "young" cells are instead added to the culture, we must distinguish between growth rate $G = (dN/dt)$, which indicates how many cells are added to a culture during a certain time period, and the birth rate

$B = 2G$, which gives the number of cells that are "born" at a certain time point. A culture of continuously proliferating cells has, therefore, twice as much young cells as old cells and its age distribution declines exponentially.

Under these conditions, an exponential growth is observed, which is described by

$$N = N_0 \cdot e^{\mu \cdot t} \quad (1)$$

with N: number of cells; N_0: number of cells at the start of the culture; growth parameter $\mu = ln2/t_d$; t: time; t_d: doubling time of cells. Every cell has an individual cell age τ that starts after mitosis at $\tau = 0$ and ends at $\tau = t_d$ with the next division. Under optimal conditions, the individual doubling time of a cell could be identical with the population doubling time. However, during growth, the medium will gradually be exhausted and some cells may stop proliferating or are prolonged in their cell cycle because one or more restriction points cannot be passed. Furthermore, when monolayer cells are transferred from one vessel into the next by trypsinization and mechanical treatment, they might additionally be stressed by temperature shifts and their proliferation in the new vessel starts delayed. These delays result in characteristic growth curves as is shown in Fig. 2 for BICR/M1R$_k$ cells. For fitting these points with a curve, the growth parameter μ must be modified to $\mu = \mu_1 - \mu_2 N$ and leads to the Verhulst–Pearl equation:

$$N = \frac{\mu_1 \cdot N_0 \cdot e^{\mu_1 \cdot t}}{\mu_1 + \mu_2 \cdot N_0 (e^{\mu_1 \cdot t} - 1)} \quad (2)$$

This equation represents realistic growth conditions in which the proliferation also depends on the number of cells and the available amount of nutrients in a medium. A typical growth curve starts with a lag-phase in which the number of cells is rather constant before the cells start with exponential growth and enter the log-phase. For practical reasons, the ordinate of a growth curve is logarithmically divided and, therefore, the log-phase is characterized by a straight line. When the medium is deprived of nutrients, cells stop proliferating and for a while the number of cells is constant – the culture is in its stationary phase before cells start to die away.

1.4 Cell Profiling

Altogether, several ten thousands of cell lines may be kept worldwide; therefore, a reliable identification of the cells becomes mandatory. Besides microbial contamination and phenotypic drift, cross-contamination between cells occurs more

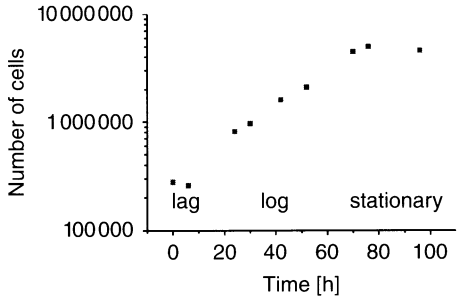

Fig. 2 Growth curve of BICR/M1R$_k$ cells with a typical lag-, log-, and stationary-phase. This continuous cell line originates from a mammary carcinoma of the Marshall rat.

often than it is detected and leads to severe misidentifications of cell cultures. Within 10 years of the start in culture of the human cancer cells HeLa, other human and animal cell lines were contaminated with these fast-growing HeLa cells as was detected by genetic markers. These alarming reports did not stop this carelessness and in 1976, Nelson-Rees and Flandermeyer reported more than 90 cell lines that were contaminated with HeLa cells. It is still estimated that up to one-third of all cell lines is of different origin or species to that claimed. These cross-contaminations result from careless handling as might be the case when several cell lines are transferred simultaneously and medium is added to all cultures with the same pipette. Aerosols and small droplets with cells might adhere to the outside of the pipette and are thus transferred into the stock medium from where they can contaminate other cultures. Cross-contamination could be detected by experienced molecular biology laboratories with several time-consuming and expensive methods, but none was suitable for use as an international reference standard. With the development of new PCR-based forensic techniques, an inexpensive method, the short tandem repeat (STR) profiling, has been applied to DNA from human cell lines. STR generates standard numerical codes for lengths of polymorphic DNA loci, which qualify as universal reference standard for human cell lines. Masters and colleagues recommend DNA profiling of cell lines as a normal practice and suggest this analysis as a prerequisite for publication. This DNA fingerprint will not only help to identify cell lines and greatly enhance the confidence in these studies but should also allow comparisons of published results on a more solid basis as momentarily possible.

1.5 Ethical Rules

Working with continuous cell cultures does not seem to cause severe ethical problems; however, when primary cells are acquired from humans and animals, several critical implications arise. For clinical treatments as well as for research, the use of human stem cells from embryos, fetuses, or adults is strictly regulated by national and international legislation. However, research with other human cellular material must also be justified and can only be approved by local research ethical committees when the demands of national guidelines are enforced. Similarly, the use of animal tissue requires ethical and legal approval, often by local animal welfare committees. Animal welfare aspects include the minimizing of pain, suffering, distress, and lasting harm as well as the restriction to the absolutely necessary number of animals. Experiments with cultures of permanently growing cells do not exclude animal welfare considerations since most cell lines are still maintained with purchased fetal bovine or newborn calf serum. Even when little or no influence can be taken on the collecting process, there are also technical arguments for considering a replacement of serum (see Sect. 2.2).

2 Techniques

2.1 Preparation of Primary and Continuous Cultures

A primary culture starts with biopsies from solid tissues or organs, preferentially from embryos, but tumor cells are

also often selected because of their high proliferation capacity. These firmly attached cells have to be disaggregated to single cells, a procedure that combines mechanical dissociation and enzymatic detachment. After chopping the tissue with fine surgical scalpels, proteolytic enzymes such as trypsin, collagenase, or pronase are applied to open proteinaceous bonds between cells, a process that might be accelerated by the appropriate temperature and thorough pipetting. This cell suspension will be diluted with medium plus serum; centrifuged and resuspended in medium for several times before the cells are transferred into Petri dishes or flasks. Most cells are anchorage dependent and will adhere to an appropriate substratum after a few hours. At that time point, the cultures must be washed free of all cellular debris and damaged cells, since only a small portion of cells may have survived the isolation procedure. Microscopic inspection will then reveal the yield of attached single cells, but also aggregates of cells will be found from which an outgrowth of cells can be observed after a couple of days. Usually, a mixture of cell types will be present in these primary cultures, the majority being fibroblasts that will proliferate better than other cell types. Since they are also motile, they can grow out from cell aggregates and spread over the substratum. Epithelial cells, on the other hand, are more or less immobile and tend to form patches on the substratum. Figure 3 shows a primary culture of embryonic mouse brain cells in which nerve cells have spread on top of other cells that are out of focus.

Adherent cells can be detached by trypsin, and enough cells might be harvested from these primary cultures to proceed with their propagation in multiple dishes or flasks. These subcultures can be used to multiply the selected cells and generate a new cell line that contains all the different cells of the primary culture. If these cells are selected from normal tissues, these cultures have a limited life span and must, therefore, be multiplied by several cultivation passages, collected, and stored in liquid nitrogen so that planned experiments can always rely on identical cell populations (see Sect. 2.5). Critical cells may not proliferate, especially when the density of transferred cells is too low. This problem can sometimes be solved either with conditioned medium or with feeder layers. The so-called conditioned medium is taken from a culture of logarithmically growing cells and added to freshly propagated cells. This medium contains cell specific factors that mimic the presence of numerous cells and stimulate the growth of critical cells. A similar effect can be obtained with irradiated feeder layer cells, which form a monolayer and serve as substratum for critical cells.

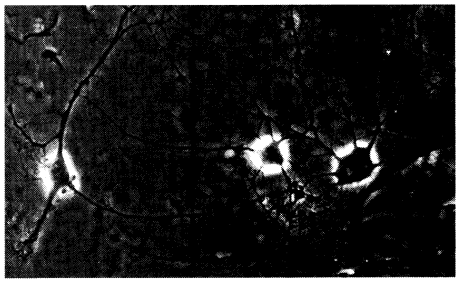

Fig. 3 Primary culture of embryonic mouse brain cells. Nerve cells have spread on top of other cells, which are out of focus. Light-microscope phase contrast picture.

By transforming and cloning cell lines, permanently growing cell strains with specific properties can be derived, and many of them are available from cell banks. A majority of experiments is, therefore, performed with these continuous cells.

2.1.1 Monolayer Cultures

Plasma membranes of animal cells carry fixed negative charges that allow cell–cell adhesion by divalent cations (Ca^{++}, Mg^{++}), but cells may also adhere to artificial surfaces that are either negatively or positively charged. Cells form a monolayer when they adhere not only to each other but also to the surface of culture vessels. Different cells form differently arranged monolayers: epithelial cells (Fig. 1) show a typical cobble stone appearance, whereas transformed fibroblasts (Fig. 4) may grow criss-cross and pile up when a high density is reached. The cells' morphology might indicate that the medium is exhausted and its replacement or a subculture is necessary. For propagating the cells, the medium is withdrawn and trypsin is added with a Ca^{++}/Mg^{++} free balanced salt solution. The time and temperature required for this treatment depend on the sensitivity of the cells, and other enzymes might also be required for a gentle disaggregation. In any case, the enzymatic treatment should be as short as possible to avoid unnecessary stress. Therefore, the cells must be washed with medium to block the activity of trypsin by its serum. The cells can then be detached from the surface by repetitive pipetting of medium over the monolayer, a procedure that also disperses floating cell aggregates. Portions of this resulting single-cell suspension are used for subcultures, and an aliquot might be used for determining the concentration of cells. The number of cells that are necessary for starting a new culture depends on the cell type, on the kind of planned experiments, and on the type of culture vessels. A few hours after starting the incubation of new cultures, they can be microscopically inspected to exclude any damage that might have occurred during the passage.

2.1.2 Suspension Cultures

Several monolayer cell lines have lost their anchorage dependence by transformation and are now also kept as suspension cultures. HeLa cells are amongst these cultures, which grow as monolayers and in suspension. Primary suspension cultures can be obtained from normal lymphocytes, which are anchorage independent. They have a limited life span, but lymphoblastoid cells with unlimited growth in culture are also available as are other ascites tumor cells that have lost their anchorage dependence already *in vivo*.

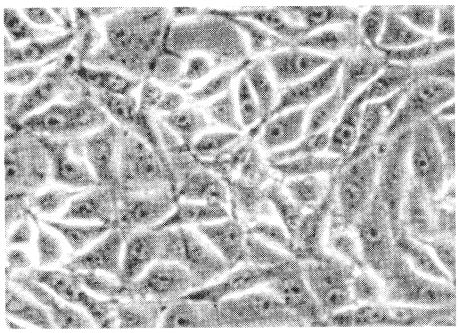

Fig. 4 Monolayer of BICR/M1R_k cells during logarithmic growth. Light-microscope phase contrast picture.

2.1.3 Three-dimensionally Growing Cultures

Not only may primary explants grow three-dimensionally but also transformed cells can be cultured as so-called multicellular spheroids in suspension. For a few anchorage-dependent cell lines, it is sufficient to start a culture of these cells in a stirrer flask for suspension cultures. When cells meet each other in this suspension, they will stay together and thus form larger spheroidal aggregates within a couple of weeks. Most other cell lines must first be kept in dishes with a nonadhesive surface to induce this three-dimensional growth. These Petri dishes are commercially available and are mainly used for cultivating bacteria on agar. After a few days of cultivation in these dishes, the cells have formed irregular aggregates, which are transferred into stirrer flasks in which they form multicellular spheroids and may be kept and treated for several weeks (Fig. 5). Spheroids can be harvested directly with pipettes, and medium is easily replaced since the spheroids precipitate quickly when they are no longer stirred. When such a spheroid is placed on a culture dish, the cells quickly adhere to this charged surface and within a day many cells will have migrated from the spheroid and proliferate again as monolayer cells.

When biopsies of organs are cultured in stirrer flasks, they will maintain morphological and physiological properties, and many cells remain fully differentiated and proliferate sparsely. Even when fresh explants are required for new experiments, these cultures offer some advantages for complex experiments that might, for instance, include immunological reactions as has been shown with lymphoid tissue and HIV infection.

Epithelium, which separates an organism from its environment or an organ from its surrounding fluid, is formed by layers of different cells. When cultured *in vitro*, epithelial cells often form flat monolayers of different cells but epidermal cells may be kept as organotypic flat multilayers in a liquid–gas interphase. This *in vitro* skin is another example for the many types of three-dimensionally growing cultures that are not only used to study tissue regeneration but are also applied in biomedical treatments.

2.2 Cell Culture Media

The basic components of cell culture media are inorganic salts, glucose, and organic substances, which are also present in blood plasma of animals. Their concentration varies with different compositions of

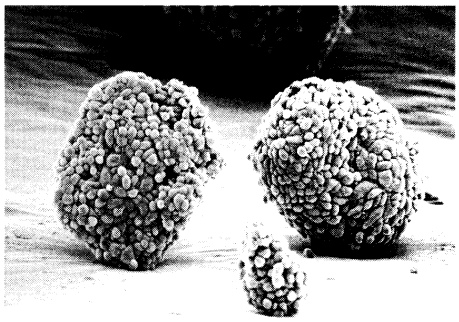

Fig. 5 Multicell spheroids of BICR/M1R$_k$ cells. The diameter of the larger spheroids is about 350 µm. Scanning electron microscope picture.

media that were designed for specific cells with special properties. Most of these media are commercially available as solutions and also as powder that has to be dissolved in water. The selection of water, however, is not trivial since it may contain organic and inorganic material, which will not be removed by simple distillation so that other purification processes must be included. Differences in cell activity may be due to different water qualities; therefore, the use of ultrapure water is recommended. In any case, powdered media require sterilization by filtration through a pore size of 0.22 μm, preferably during bottling and before storage in a refrigerator.

All basic salt broths of cell culture media contain NaCl, KCl, $CaCl_2$, and $MgCl_2$. The concentration of these inorganic salts is set to adjust the osmotic balance of the cells and maintain their membrane potential. When this solution is buffered to a pH value of 7.2 to 7.4 with the pH buffering phosphates NaH_2PO_4 and Na_2HPO_4, it can be used to keep the cells alive for a short while. A complete medium requires much more additives for the functioning of inorganic salts as mediators for cell attachment or as enzyme cofactors. Carbohydrates (glucose) are added as energy source, and amino acids serve as nitrogen sources, although glutamine can also provide a carbon source via transamination. Essential and nonessential amino acids and water-soluble vitamins are also necessary as nutrients and additional energy sources; their concentrations vary with different medium formulations.

For proliferating mammalian cells, the buffering capacity of the medium is increased by bicarbonate ($NaHCO_3$). In this case, the medium's CO_2/HCO_3 content must be balanced by gaseous CO_2, which requires gassed incubators in which an atmosphere of 5–10% CO_2 in air can be maintained. Another buffering system is given by the zwitterion HEPES, which is often used together with the "natural" bicarbonate buffering system. With this buffer, cells can be manipulated under atmospheric conditions without drastic pH changes. For a rapid and easy control of the medium's pH, phenol red is added as indicator.

Many laboratories supplement their medium with antibiotics such as streptomycin sulfate and penicillin G. This reduces the risk of bacterial contamination but increases the development of resistant organisms and encourages the use of inadequate aseptic techniques. Transfected cells, however, may have been selected as resistant to puromycin or geneticin, and the use of these antibiotics is recommended for maintaining a selection pressure.

A very important component of a cell culture medium is serum, and in most cases newborn calf or fetal bovine serum is added. Serum is a complex mixture that contains not only essential growth factors but also plasma proteins, hormones, metabolites, and growth inhibitors. Since serum components are affected by age, health, and nutrition of the donor animals, the quality of serum will change from batch to batch and, therefore, every new batch has to be tested for the required quality standards. When bovine spongiform encephalopathy (BSE) spread in cattle, guidelines became effective, which should minimize the risk of BSE transmission via medicinal products and the necessity for media without any material from animal origin has increased.

At the beginning, the so-called serum-free media contained factors that were isolated from serum and stimulated the

growth of certain cell types, which can be seen from their name: fibroblast growth factor (FGF), nerve growth factor (NGF), epithelial growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF). Other serum-isolated additives include interferons, hormones, and attachment factors. With these components, a serum-free medium was still an undefined medium since it contained components that were subject to variation. Effects to develop defined media where the chemical structure and the concentration of every component are known were successful. These media are consistent from batch to batch and are optimized for growth of specific cell types and product synthesis. Almost every cell line requires its own expensive serum-free medium, its use is therefore still limited to productions with expensive purification processes and to some biomedical treatments.

2.3 Equipment

2.3.1 Vessels

Monolayer cells need a substratum to adhere, and this material must be of good optical quality since microscopic inspection of the cells is mandatory for most experiments, but other material might be advantageous when it comes to production. Cells preferentially adhere to negatively charged surfaces and, therefore, the plastic vessels for cell culturing are charged during the manufacturing process. Many laboratories use transparent disposable multiwell plates, Petri dishes, and T-flasks made of polystyrene. Their volume varies between 0.1 and 250 mL medium and with the corresponding surface areas. However, other mechanisms are also effective in cell adhesion. When cells are cultivated on glass surfaces, they might not adhere well when the glass flasks are first in use. After several passages of cells, the adhesion is enhanced, indicating a conditioning of these glass surfaces. This is due to specific receptors for cellular adhesion, which are part of the extracellular matrix of plasma membranes. They partly remain adherent to the glass surfaces when the cells move or are harvested. Even after cleaning and sterilizing, material is firmly connected with the glass and thus facilitates the attachment of cells of sequential cultures. This finally led to the use of special mixtures of extracellular matrix glycoproteins and proteoglycans by which any substratum could be made available for the cultivation of critical cells. Continuous cell lines such as HeLa adhere to different substrata, for example, carbon-coated glass (Fig. 6) and polished stainless steel (Fig. 7).

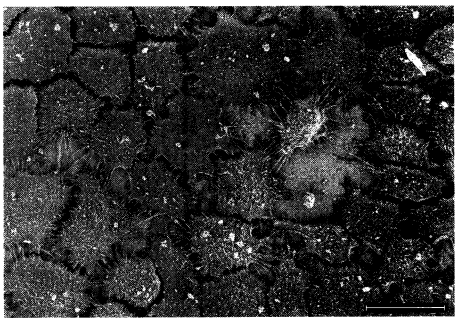

Fig. 6 Monolayer of HeLa cells during logarithmic growth on carbon- coated glass. Attached particles originate from serum and/or dead cells' debris. Scanning electron microscope picture, bar = 30 µm.

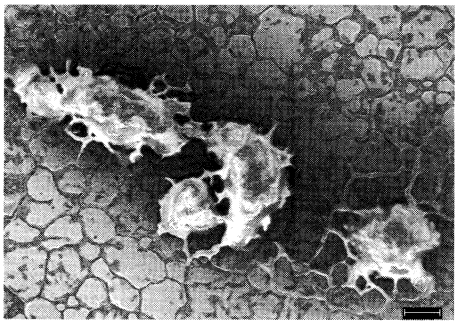

Fig. 7 HeLa cells grown on stainless steel. Note the amoeboid protrusions onto the nonpolished gaps. Scanning electron microscope picture, bar = 10 µm.

Many sophisticated systems have been developed to improve the yield of monolayer cells. Since the area of the substratum on which the cells can grow must be increased and the volume of the vessels must be expanded, risks of contamination and problems of regulation will also simultaneously rise. These tasks are solved with different strategies. A classical technique is the use of cylindrical bottles that are slowly rotated so that the total inner glass or plastic surface is available for cultivating monolayers with a small amount of medium. Many of these roller culture bottles can be rotated simultaneously on racks and thus increase the total amount of products. Other mass culture techniques make use of separated compartments in which the cells grow on permeable membranes and medium is provided in a compartment separate from the products, a strategy that reduces the cost of product purification. An example is the hollow fiber, which is not only used for adherent but also for suspended cells. The surface of substratum can also be increased when the cells are grown on microcarriers. These small beads with diameters of about 200 µm are fabricated from different materials, such as polystyrene, silica, glass, or dextran and may be coated with collagen or mixtures of cell adhesive substances. Cells can grow as monolayers on these beads, which are kept as suspension and thus mediate mass cultivation of monolayer cells.

Cells can also grow isolated in suspension cultures in which they must be kept floating with a minimum of shear stress and optimal conditions for growth and production. For small volumes, this is achieved with stirrer flasks or gyratory shakers, but cells may also be cultivated in air-lift bioreactors that may hold several ten thousand liters. In small stirrer flasks, the medium is kept in motion with magnetic stirrers and rotating paddles or pendulums, necessary controls are maintained by the incubator's devices. This is no longer possible when the volume of a stirrer flask exceeds one liter. Scaling-up in volume requires better controls of medium conditions; therefore, bioreactors are equipped with sensors for the regulation of gas, temperature, pH, and metabolites.

To prevent outgrowth of cells, multicellular spheroids and three-dimensionally growing biopsies are constantly kept in motion in stirrer flasks or gyratory shakers. For this cultivation in small volumes, an interesting alternative is given by rotating wall vessels that were designed for cell growth in low or zero gravity. Their rotation speed is so adjusted that the cells remain stationary, which not

only facilitates the formation of three-dimensional aggregates but also explants from organs and tissues can be maintained under these conditions.

2.3.2 Appliances

Essential equipments that are used in tissue culture laboratories are shortly mentioned here. A laminar flow hood protects not only cells from microbial infections but also the operator from hazardous materials (see Sect. 2.4). Incubators must allow regulation of temperature, CO_2, and humidity and provide good internal convection. An autoclave is necessary for sterilizing solutions, but medium must be filtered since many ingredients will not tolerate the high temperature in an autoclave. Wasted dishes and other disposable material that came in contact with pathogens or with cultures of primate or transfected cells must also be autoclaved before they are trashed. Glassware and glass pipettes can be sterilized in a dry heat at temperatures higher than 160 °C for at least one h. Besides refrigerators and freezers for storing substances and solutions, liquid nitrogen canisters should also be available for deep-freezing and storing cell lines. Ultrapure water might not always be necessary, but a cell culture laboratory should have access to a supply; in any case, deionized and distilled water must be available. Well-adjusted phase contrast microscopes, both upright and inverted, are important tools in a cell culture laboratory since microscopical inspection of the cells during growth and before an experiment can save time and costs when inappropriate cells are to be used. A balance should not be missing as well as centrifuges and many other helpful instruments such as pH meter, cell counter, dispensers, pipettors, and a glassware washing machine.

2.4 Safety and Biohazards

For any workplace, national regulations are effective that prevent operators from safety hazards of physical or chemical origin. Working in a tissue culture laboratory adds further sources of potential safety hazards, which originate from cell cultures contaminated with latent viruses or occult pathological organisms. A known risk comes from transfected cells for which not only the origin of the cell line but also the transfection vector outlines the risk. A low individual and community risk is given with microorganisms that are unlikely to cause disease in healthy operators or animals and with cell lines that are not of human or primate origin. These risk group I agents can be handled in a containment level I laboratory that matches a functionally designed standard microbiology laboratory. Aseptic techniques must be used and all liquid waste should be treated with bleach or similar detergents. Since during pipetting aerosols are formed that might carry cells and unidentified pathogens, it is good laboratory practice to protect operators with a laminar flow hood with an air barrier at the front opening and an exhaust filter.

Agents that are of moderate individual and limited community risk are classified as risk group II. This includes cultures of primate and human origin, recombinants, transfectants, and animal tumor cells. A separate containment level II laboratory with separate equipments is obligatory, and cells must be handled in a class II laminar-flow hood. Human cell lines that are virus-producing or are infected with pathogens have a high individual and a low community risk and are classified as risk group III. These cells can also be handled in a class II laminar-flow hood,

but any waste and material that came in contact with cells must be sterilized before leaving the separate containment level III laboratory. Only designated laboratory staff is allowed to work in this room.

The risk group IV is given when a high individual and a high community risk must be expected from the experiments. This includes human pathogens that produce very serious human or animal diseases that might be untreatable and readily transmitted. Biopsies and cell cultures carrying known human pathogens must be handled in a containment level IV laboratory. In addition to the requirements for a level III laboratory, this laboratory must be physically isolated and functionally independent of other areas and must have an air lock for entry and exit. Class III biological safety cabinets and/or positive pressure ventilated suits protect designed operators for whom a shower and change of clothes are obligatory when entering or leaving the laboratory. Authorization for handling transfected cells and human pathogens as well as the routine inspection of containment laboratories are regulated by national laws.

2.5 Storage

Primary cells have a limited life span and continuous cell lines may change properties by transformation, dedifferentiation, or because of genetic instability and infection. When production or research depend on certain properties, it is therefore essential to have a stock of cells with the original properties. Cell lines as well as small multicellular organisms and embryos can be preserved by freezing since much of the cellular volume is water that is essential for cellular metabolism. When water becomes to ice, cellular metabolism is stopped and cells can be kept for years under cryopreserved conditions when the temperature is deep enough.

Before freezing, cells should be maintained under routine conditions and must be inspected for contamination. Monolayer cells are harvested during exponential growth by trypsinization; suspension cultures are concentrated by centrifugation. Cells must be thoroughly washed and resuspended to a concentration of about 1 to 10 million cells per milliliter freezing medium. This freezing medium is composed of the appropriate growth medium plus a high concentration of serum (50% or more) plus dimethyl sulfoxide or glycerol. This cell suspension is transferred in plastic ampoules wherein cells will be frozen at a slow rate till they reach $-70\,^{\circ}C$ and will be kept overnight before they are transferred into liquid nitrogen and stored at $-196\,^{\circ}C$.

For thawing, an ampoule is placed in a $37\,^{\circ}C$ water bath and slowly agitated. Its content is transferred into a flask and diluted with medium. After 6 to 8 h, when viable cells are firmly attached, medium with dead cells and cellular debris should be aspirated and fresh medium is added.

3 Applications

Considerable knowledge of cellular structures and functions is the result of investigations with cells in culture. This includes intracellular synthesis processes for numerous macromolecules, such as nucleic acids and proteins, as well as for most other relevant cellular molecules. Intracellular energy and signal transfer, cytoskeletal architecture, transport through membranes, product formation, but also cell–cell interactions, malignant transformations, and

microbial infections are representatively mentioned for the numerous issues for which answers were found in cell cultures. A few examples for applications in research and production are given that concentrate on the importance of three-dimensionally grown cell aggregates, a method that might become the standard technique in the future.

3.1 Cellular Regulations

Complex cell interactions and epithelial differentiation were studied with organotypic cocultures of flat epidermal multilayers and revealed the normal regulation and balance of these cells. For this *in vitro* epidermalization, a collagen matrix at the basal side is required, whereas the keratinocytes are exposed to air. The collagen layer also supports dermal fibroblasts that synthesize the right extracellular matrix proteins. Together with the metabolites of the medium, which is separated from the collagen layer by a filter, all necessary components are available for proliferation and differentiation of the epidermal cells.

Intracellular regulations are not only dependent on the combination of cells but also on the culture conditions. This is trivial as long as necessary metabolites or messenger molecules are missing or are too low in concentration; however, with the same concentrations available in medium, cells may behave in a completely different manner when grown three-dimensionally as cell aggregates instead of as two-dimensional monolayers. An example is the so-called contact effect, which was observed for multicellular spheroids in which the tumor cells are more sensitive to irradiation than in two-dimensional growth. Later, it was detected that cells that were coupled to their neighbors via gap junction channels showed this effect more pronounced. Gap junction channels regulate an intercellular exchange of ions and molecules of up to 900 dalton and are normally found to be open in monolayer cells. However, in multicellular spheroids of several tumor cell lines, the permeability of these channels will be regulated, they were found open in two-day old spheroids and were closed two days later. It is still unknown what controls this channel closing, but channels may open again when the cells were allowed to grow as monolayer. Another growth-dependent regulation was demonstrated with β-galactosidase synthesizing L-cells. For this production, the cells had been transfected with a lacZ gene, which was under control of a β-actin promoter. This constitutive transfection led to a constant production when the cells were cultured as monolayer but β-galactosidase activity was downregulated in cells that were cultivated as spheroids. Since monolayer L-cells are motile, a permanent synthesis of cytoskeletal elements such as β-actin is required, but in spheroids the cells are rather immobile and the synthesis of β-actin is downregulated, which also effects the β-galactosidase activity. In contrast to the layered growth of epidermal cells, multicellular spheroids contain only one type of continuously growing cells, and these are exposed to gradients of metabolites, protons, and oxygen. Furthermore, spheroids establish an extracellular matrix, which is not found when the same cells are cultivated as monolayers.

3.2 Cancer Research

Malignant growth of cells can only be understood correctly when the modes for normal growth are known. Both cases can be

investigated under the same experimental conditions with cell cultures. A successful model for studying carcinogenesis *in vitro* is again the multilayered epidermis, which allowed examination of malignant transformation and tumor progression. A problem of metastatic behavior of tumor cells is invasion, it can be investigated *in vitro* with cocultures of three-dimensionally grown aggregates of normal and malignant cells. First, attempts were made with organ cultures from chick embryo or fragments of human endometrium, which were confronted with suspended tumor cells. When precultured embryonic chicken heart fragments were confronted with tumor cell spheroids, invasive behavior could be analyzed with immuno-histological techniques, and comparative studies revealed that this *in vitro* invasiveness matched the *in vivo* situation. Interestingly, it was shown that invasive tumor cells were coupled to the host via gap junction channels and that noncoupled HeLa cells became invasive after transfection with a connexin gene, which led to an expression of host-compatible gap junction channels. Several other prerequisites for invasion have been detected with similar cell culture models: Metalloproteases were found to be active during invasive processes of tumors from brain and the cell adhesion molecule e-cadherin plays a role when breast tumor cells invade the host. Weakening the cadherin-mediated cell–cell contacts facilitates detachment and migration of single tumor cells in a mesenchymal type of movement. Recent studies with new fluorescent probes allowed a time-resolved investigation of invasive processes in three-dimensional collagen matrices and revealed a supramolecular plasticity mechanism with a transition from a proteolytic mesenchymal toward a nonproteolytic amoeboid movement.

3.3 Production

Two modes of cellular production are possible: either the product remains within the cells or it is secreted into the medium. In any case, purification processes are necessary and, therefore, the culture conditions should be adjusted and optimized to increase the yield and avoid unnecessary costs. For biomedical treatments, the cells are often the requested product, which requires safe culture conditions. These should not only perfectly match the host's physiological conditions but must also be free of nonhuman additives that may be due to limited cleaning processes.

Monoclonal antibodies are produced by hybridoma cells in suspension culture. They are secreted into the medium and can be harvested with the culture supernatant. For mass production, hybridomas are often grown in hollow-fiber cultures that facilitates harvesting; the highest yield, however, might be achieved when hybridomas are grown as ascites culture in mice.

Amongst the first products of monolayer cultures were therapeutically important substances such as viral vaccines and tissue plasminogen activator, which were harvested from roller bottle cultures or from microcarriers in suspension cultures. With the availability of genetically manipulated cells, the production of therapeutically relevant proteins with animal cell cultures increased considerably. The list of these substances encloses insulin, interferon, interleukin, plasminogen activators, blood clotting factors, hemopoietic growth factors, hormones, vaccine, and so on. Many of these proteins have a complex tertiary structure and require posttranslational modifications that can only be synthesized in animal cells but not in bacteria. For mass production, producing cells

are often additionally modified so that they tolerate high shear stress when cultivated as suspension in large bioreactors.

An often-applied method in biomedical treatment is transplantation of skin. For this purpose, small pieces of skin will be taken from the patient, cut, and expanded to a mesh. They will then be cultivated, and keratinocytes will fill the interstice. These pieces can then be retransplanted onto the wounds of the patient. Another approach, which is still under development, is the temporary support of defect organs such as liver by bioartificial organs via an extracorporal bypass.

4 Perspectives

Biomedical treatment with primary or continuous cell cultures requires complex growth conditions, and some of the current approaches might lead to new therapeutic applications. Three-dimensionally growing cells do not completely represent the cells' behavior in human or animal, and monolayer cells are not necessarily representative for more complex culture systems. This does not argue against investigations with cell cultures as long as possible limitations are taken into account.

A great potential of research with monolayer cultures is given by certain cellular functions such as channel activities, intracellular transport, or protein synthesis. Examples are cells that were transfected with a fusion construct that leads to the synthesis of proteins that are labeled with a fluorescent protein. This labeling enables investigations of supramolecular dynamics by microscopic techniques such as internal reflection fluorescence or fluorescence correlation spectroscopy. Results obtained with these techniques offer insights into general cellular transport and diffusion processes.

Cellular production has been optimized with selected cell types that grow under adequate suspension- or monolayer-conditions and might still be expanded with other transfected cells and new recipes for synthetic media. A great potential for production can be expected from cell cultures of coelenterates and arthropods because pathogens are unlikely to transfer to human cells. Successful cultivation of three-dimensional aggregates of sponges has already been reported, and continuously growing insect cells are available for a longer time.

See also Antibody Molecules, Genetic Engineering of; Bacterial Cell Culture Methods; Immunology; Prokaryotic and Eukaryotic Cells in Biotech Production.

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