Cell Differentiation *In Vitro*: Model Systems

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Secondary article

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Because conditions of culture *in vitro* cannot be identical to those in the living system, cell lines cultured *in vitro* are referred to as 'model systems'. Not all cell types are capable of differentiation *in vitro*. Procedures for studying differentiation in model systems depend on the type of culture that can be employed. Techniques employed include liquid suspension cultures, monolayer cultures and complex culture systems involving semisolid matrices or liquid overlay methods. Such *in vitro* culture techniques have applications to the study of differentiation in basic research and clinical applications.

Introduction

When mammalian tissues are dispersed into single cells or aggregates of cells and placed in a sterile environment that resembles the environment of the cells in the body, they are often able to proliferate for a considerable time. The conditions for growth can be manipulated to allow the proliferation of a single cell type. Thus, studies can be made on homogeneous, or nearly homogeneous, cell populations under conditions that limit the number of variables encountered by the cells when they are part of tissues, and thus simplify the analysis of the complex processes that constitute the life of the cell. Originally, tissue culture experiments were performed in glass bottles or plates and were said to be conducted '*in vitro*' from the Latin word for glass, *vitrum*. However, since these conditions are never exactly the same as those in the living animal, '*in vivo*' – from the Latin *vivere*, to live – they are referred to as 'model systems'. In most cases, however, particularly if the cells are not transformed into neoplastic (cancerous) variants, the cells cease to proliferate. This can either be because of the inherent limitation of their proliferative ability (i.e. senescence) or because of induction of a programme for cell death – apoptosis. More rarely, when the cells are in early developmental stages, they acquire characteristics of functioning, mature cells that mimic some stage in a more advanced normal development. This last process is designated as cell 'differentiation'.

Not all cell types are capable of differentiation *in vitro*. First, the cells have to be either in the cell division cycle (cycling cells) or capable of entering the cell cycle (G_0 cells) given an appropriate mitogenic stimulus. Second, the cells have to be capable of survival and proliferation *in vitro*, which in successful attempts is achieved by supplying the environment, nutrients and growth factors/cytokines necessary for specific cell types. In the case of G_0 cells this is achieved by removing growth restraints present *in vivo*. Third, conditions have to be provided conducive to the acquisition of phenotypic and functional characteristics approximating those of the more mature cells. These conditions are provided by hormones or chemical agents (differentiation inducers) or by alterations in culture medium or conditions, such as growth in monolayers or in suspension, that initiate the pathways necessary for the subsequent changes in gene expression.

Outline of Methods

Procedures for studying differentiation in model systems depend on the type of culture that can be employed. Some cells can be cultured in suspension and this is a convenient and cost-effective as well as labour-efficient way of producing large masses of cells for biochemical studies. Suspension cultures are routinely used for cultivation of leukaemia cells, but other cell types, e.g. HeLa cells derived from cancerous epithelium of uterine cervix, have been adapted to grow in suspension. Most mammalian cells, however, grow as monolayers on solid support surfaces, which nowadays are usually plastics. In addition, cell aggregates can be cultivated in more complex culture systems.

Liquid suspension cultures

Haematopoietic cells do not normally grow attached to solid surfaces, so they can be grown in suspension. Differentiation of leukaemic cells can be studied using primary cultures or established cell lines. The principal advantage of primary cultures is that the events are more likely to reflect the normal processes that take place *in vivo*. The disadvantages are that the success rate of maintaining sufficient number of cells in culture for extensive experiments is low, so that parameters of differentiation that can be studied are limited, and it may be difficult to repeat the findings. Conversely, established cell lines may have drifted genetically from their *in vivo* progenitors, which may skew

the results of differentiation induction, but there is no limit on cell numbers that can be obtained. Ideally, the conclusions derived from experiments in which established cell lines are used should be confirmed with primary cultures.

Primary cultures are in most cases obtained from human blood or bone marrow samples taken with the subject's consent. In essence, the procedure starts with the removal of patient's cells from the serum and from acidic heparin, which may inhibit cell growth, and separation of the cells into the principal blood components using a density gradient such as Ficoll-Hypaque. The buoyant cells at the gradient–specimen interface include lymphocytes, monocytes, neutrophil precursors from blast to myelocyte stage, proerythroblasts and erythroblasts, and leukaemic cells in cases of leukaemia. The cells in the pellet consist predominantly of erythrocytes, but granulocytes are also present. T lymphocytes can be separated from the other cells by forming rosettes with neuraminidase-treated sheep erythrocytes. To separate monocytes/macrophages from the other cells in the interface, the interface cells are washed, resuspended in 10% fetal calf serum (FCS) and placed horizontally in a tissue culture flask placed in a 37°C incubator for 1–3 hours. Removal of the supernatant fluid and unattached cells leaves the adherent monocytes and macrophages on the flask surface. The separated cells can be grown into colonies and attempts can be made to establish long-term cultivation if they are derived from immortalized cells such as leukaemic stem or progenitor cells. Alternatively, the cells can be used as differentiation models in primary suspension culture. It is usual to add antibacterial and antimycoplasmal agents during establishment of cell cultures. However, this does not guarantee mycoplasma-free cultures and testing is important to exclude an inapparent infection, as infected cultures produce misleading results in studies of differentiation.

The choice of cell type and the maturity of the cells depends on the objective of the differentiation study. Models are available that mimic different lineages and stages of haematopoietic cell differentiation. For instance, there is a great variety of human myeloid cell subtypes, such as HL60 cells, which are promyelocytic type cells that can differentiate to the granulocytic, monocytic or macrophage phenotype; U937 cells, which differentiate towards the monocytic phenotype; and K562 cells, which have the capacity to differentiate to erythroid or megakaryocytic phenotypes, depending on the nature of the differentiation inducer. Other available myeloid cells include HEL cells, KU 812, MEG01 and ML-1.

Induction of differentiation of haematopoietic cells is primarily accomplished by the addition of selected chemicals or biological signalling molecules to the culture system. For example, HL60 cells can be induced towards the granulocytic phenotype by exposure to appropriate concentrations of the solvent dimethyl sulfoxide or the morphogen retinoic acid, while the carcinogenic phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) produces a macrophage-like phenotype, and the hormonal form of vitamin D (1,25-dihydroxyvitamin D_3; 1,25D_3) or the nucleoside analogue arabinocytosine (AraC) produces a monocytic-like phenotype. The inducers are not phenotype-specific; in the K562 cell system TPA induces some features of monocytic differentiation, while AraC induces erythroid differentiation, and 1,25D_3 inhibits this action of AraC. Unlike HL60 cells, U937 cells exposed to retinoic acid differentiate into macrophage-resembling cells.

For an optimal differentiation system, the conditions must be carefully standardized. If established cell lines are used, it is possible to obtain well differentiating sublines that provide more homogeneous cell populations and facilitate performance of kinetic experiments. This can be achieved by subcloning cell populations and selecting appropriate colonies. Differentiation-resistant subclones are also useful as controls in diverse differentiation studies, and may also be identified in subcloning experiments, but cultivation in increasing concentrations of a differentiation agent is a more effective method for development of resistance. For example, HL60-G cells, subcloned from an early passage of HL60 cells, show signs of differentiation within 6 hours of addition of 1,25D_3 and all cells in the culture express CD14, an early marker of differentiation, within 48 hours (Zhang *et al.*, 1994). This is in contrast to reports from various laboratories that HL60 cells require 4–10 days for differentiation. Conversely, HL60-40AF cells show complete resistance to the differentiating actions of 1,25D_3.

Optimal conditions for cell differentiation also require attention to culture conditions. There is usually a range of cell concentrations at which the cells can be seeded and allowed to grow, without the necessity of subculturing, which disturbs culture equilibrium and complicates quantification in kinetic experiments. Concentrations less than $2 \times 10^4\, ml^{-1}$ may not allow HL60 cells to proliferate, and below $5 \times 10^4\, ml^{-1}$ differentiation is poor. When concentrations are too high, many cells die. Thus, in this differentiation system the optimal starting cell number is $3 \times 10^5 \pm 1 \times 10^5\, ml^{-1}$. The nature of the medium should be the same as the medium recommended for optimal growth, and supplementation with serum/growth factors is generally not a critical factor. In the author's laboratory the less expensive bovine calf serum supplementation was found to be superior to fetal calf serum for differentiation of HL60 cells, even though it is less rich in growth factors. Also, as mentioned above, inapparent infection with mycoplasma has led to erroneous conclusions in various differentiation systems. Such infections are more common when antibiotics are routinely used for culture maintenance, so frequent testing for mycoplasma is recommended. A simple test that can be performed in most laboratories has been published (Studzinski *et al.*, 1973), but if facilities are available this can be done by polymerase chain reaction (PCR) or microbiological techniques.

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Alteration of culture conditions can also be used to induce differentiation. An interesting example is cultivation of freshly isolated blood mononuclear (gradient interface) cells with monolayers of human umbilical vein endothelial cells grown on an endotoxin-free collagenous matrix (Randolph *et al.*, 1998). The monocytes penetrate into the subendothelial collagen within one hour, but in the following two days approximately half the CD14-positive monocytes reverse the migration back though the endothelium. Two forms of differentiation have been detected in this system; the cells remaining in the subendothelium mature to macrophages, while the reverse-transmigrated cells show morphological appearances and markers of mature dendritic cells (Randolph *et al.*, 1998).

Assessment of differentiation depends on comparing the appearance and the functional capabilities of the more mature cells against the undifferentiated or partially differentiated cells used to initiate the differentiation system. Reduction or cessation of proliferative activity, though sometimes included in the criteria for differentiation, is a separate (albeit related) process, and should only be considered as confirmatory of otherwise demonstrated differentiation. On the other hand, the numerous cell surface markers available and flow cytometric analysis have largely supplanted the more laborious cytochemical and immunological methods for assessing differentiation-related changes in the phenotype. Cell morphology can be suggestive of the lineage of differentiation, but is difficult to interpret and of little help to the nonexpert. This is because *in vitro* differentiation is a caricature rather than an accurate replica of *in vivo* differentiation, so the recognition of morphology is more an art than a science. Functional characteristics, such as phagocytosis by maturing myeloid cells, are easy to recognize but the process is excessively laborious. Demonstration of enzyme activities or proteins associated with differentiated cells is therefore a reasonable compromise. This is shown, for instance, by measuring the activity of an esterase ('NSE reaction') or by detecting reactive oxygen species generated during phagocytosis ('NBT reaction') in differentiated monocytic and myeloid cells. Similarly, the appearance of haemoglobin signifies erythroid lineage differentiation. Assessment of large numbers of cells is facilitated by the use of flow cytometry, and changes in the levels of expression of numerous proteins or their accessibility to antibodies provide markers of differentiation and lineage identification.

Monolayer cultures

A large number of mammalian cell differentiation systems have been established in monolayer culture. Most of the considerations and caveats discussed with reference to suspension cultures apply also to this system. It is especially important to pay attention to cell density in culture, since adherence to the surface of the culture vessel is critical for cell proliferation, which is limited by the availability of this surface if cell density is too high and the cells remain confluent for a significant period of time. Forced detachment often occurs and results in cell death by a process known as 'anoikis', but it may also result in differentiation. Advantage is taken of this in a model for keratinocyte differentiation. Here, mouse keratinocytes are detached from the substratum and are placed in a complex specialized medium containing 1.45% methylcellulose, which provides a semisolid support for the cells for initiation of the differentiation process.

Another differentiation system regulated by cell confluence is the model for terminal adipogenesis. Several established mouse proadipocyte cell lines, e.g. 3T3-L1 cells, are available and are believed to provide faithful models of adipocyte differentiation *in vivo*. The evidence for this includes the development of normal fat pads in animals injected subcutaneously with *in vitro* differentiated adipocytes. In this model system spontaneous conversion to the adipocyte phenotype can occur in a significant proportion of the cell population 2–4 weeks after they reach confluence. Thus, either detachment from substratum or forced confluence can result in differentiation, depending on the cell type.

Examples of other model differentiation systems in monolayer culture include human colon cancer and prostate cancer cells, breast cancer cells that can be induced to make milk proteins and fat droplets, muscle cells that can form contracting myotubes when changed from growth factor-rich FCS-supplemented medium to medium supplemented with a lower concentration of horse serum, osteoblast cell cultures that can form mineralized bone matrix, and a variety of neuronal differentiation systems. Also very frequently used are rat pheochromocytoma PC12 cells, which can be differentiated into mature neurons when cultured with nerve growth factor (NGF) and adenosine 3′,5′ cyclic monophosphate (cAMP). Mouse embryonic stem (ES) cells are multipotent cells and can be induced to differentiate to neuroectodermal cells (neurons) with retinoic acid, and to mesodermal cells with dimethyl sulfoxide. Other multipotential cell lines have been established from the neural crest and the neuroepithelium.

Complex culture systems

Cells grown in semisolid culture matrices such as soft agar or methylcellulose form multicellular colonies that often differentiate according to their inherent potential. Collagen can also provide a more physiological matrix. Induced differentiation of breast cancer cells in semisolid gel matrix can result in formation of duct-like structures. Three-dimensional growth can also be obtained by growing cells in 'liquid overlay', where cells are placed into medium above a nonadherent layer. When cell clusters

have grown to sufficient size they are placed into magnetically stirred suspension cultures, or into multiwell plates, where they can grow into 'multicell spheroids' (Durand, 1999). In some cases these spheroids have structures that resemble organizing tissues, such as formation of tubular and trabecular structures within the spheroids of renal carcinoma cells (Lieubeau-Teillet *et al.*, 1998). Structures resembling endometrium have also been described when endometrial cell lines are cultured in the presence of ovarian steroids on artificial basement membranes composed of Matrigel (Mukherjee *et al.*, 1993). Complex structures such as pancreatic acini can also be maintained in culture using the methods described in this section, but although phenotypic modulation of such structures can sometimes be observed, so far only limited information on differentiation processes has been obtained using these complex systems.

Applications

Basic research

Currently, one of the major interests is in elucidating the relationship between cell cycle control and differentiation. Several systems have proved to be particularly useful in this regard. Adipocyte differentiation illustrates an unexpected aspect of this relationship. Monolayers of 3T3-L1 adipoblasts can be maintained in active cell cycle, but when the cells reach confluence they are temporarily arrested in the G_1 phase of the cell cycle and begin to express early markers of adipocyte differentiation. These 'preadipocytes' re-enter the cell cycle and undergo mitotic clonal expansion when exposed to a medium containing 3-isobutyl-1-methylxanthine, dexamethasone and insulin. This shortens the time necessary to reach permanent G_1 arrest and accumulation of fat droplets from two weeks to three days (Rubin *et al.*, 1978), and shows that cell cycle traverse accelerates and may even be required for differentiation as shown by the ability of inhibitors of DNA replication to block differentiation in bone and erythroid cells. Another system in which differentiation precedes cell cycle arrest is exemplified by the various leukaemia cells induced to differentiate, in which a proliferative burst has been described in the very early stages of induction of differentiation. Further, the seeming irreversibility of 'terminal' differentiation appears in many cases to be illusory – re-entry into the cell cycle has been demonstrated for such terminally differentiated cells as the myotubules (Endo, 1992) and 1,25D_3-differentiated HL60 cells (Studzinski and Brelvi, 1987).

In vitro differentiation systems also provide excellent opportunities for studies of intracellular signalling pathways. Published studies include investigations of the Ras/MAPK pathway operating in differentiating PC12 and HL60 cells, as well as in keratinocyte cultures.

There appears to be no limit to the ways in which *in vitro* differentiation systems can be utilized for molecular and cell biology studies.

Clinical applications

The modalities that can be used for cancer therapy include differentiation therapy. *In vitro* systems provide a unique testing ground for this form of treatment of human cancer since human cells can be used to evaluate the effectiveness of agents proposed for clinical use.

Model differentiation systems are also useful in many other aspects of cancer research. In cancer diagnosis, recognition of the lineage of colonies differentiated in semisolid media may occasionally be helpful to determine the nature of poorly differentiated leukaemic cells and thus provide information regarding treatment modalities that are likely to be the most effective. Differentiation systems also provide guidance for optimizing procedures aimed at regenerating damaged human tissues such as skin, muscle or nerves.

Future Developments

Among the emerging advances in the field, two appear to have special potential for a major impact. From a pure technology standpoint, microarray hybridization and related sophisticated analysis tools provide a vast complement of genes whose expression can be determined by measuring the abundance of mRNA. *In vitro* differentiation model systems should prove to be exceptionally amenable to this form of analysis, since these systems generally consist of homogeneous cell populations that can be sampled incrementally during the progression of the differentiation programme. Of course, identification of differentiation genes will require a conceptual synthesis of networks through which these genes relate to one another, a formidable undertaking. This may become facilitated by the alliance of forty US laboratories being established to map cell signalling pathways.

Another new development in differentiation research relates to the recent finding that some stem cells have trans-differentiation potential. Surprisingly, neural stem cells (NSC) from mouse forebrain, embryonic or adult, systemically injected into irradiated mice of a different genetic strain were found to produce a variety of blood cell types including myeloid, lymphoid and early haematopoietic cells (Bjornson *et al.*, 1999). Since NSCs can be continuously expanded in culture, this system can provide a source for repopulation of bone marrow with unlimited numbers of haematopoietic cell precursors. Similarly, injection of immature nerve cells derived from mouse

embryonic stem cells into rats paralysed by blows to their spinal cords resulted in some recovery of movement in these animals. If these findings are applicable to human NSCs, there could be major implications for treatment of human diseases.

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