

Review-Hypothesis

Primary culture, cellular stress and differentiated function

Alan P. Wolffe and Jamshed R. Tata

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, England

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Isolation of specialized cell types for the analysis of tissue-specific gene function often results in loss of the differentiated phenotype. Examples of this type of phenotypic change following tissue disaggregation are reviewed together with possible explanations. Close similarities between the effects of cell isolation with those of other cellular stresses such as heat or anoxia point to common biochemical mechanisms being involved. This suggests that the study of freshly isolated cells will contribute significantly to our understanding of the nature of cellular stress and its consequences for the maintenance of phenotype and induction of tissue specific gene expression.

Tissue disaggregation Cellular phenotype Hormonal responsiveness Heat shock protein Culture shock

1. INTRODUCTION

Primary culture of differentiated cells is now widely used to study the phenotypic expression of specialized tissue-specific functions. These studies are often vitiated by the loss of differentiated characteristics, either very shortly after tissue disaggregation and cell isolation or during the subsequent maintenance of the cells *in vitro* over periods of days and weeks. Although the loss of phenotype during prolonged maintenance of cells in culture has been extensively observed [1,2], little emphasis has been placed on the more immediate consequences of cell isolation. This review concerns the transient loss of specialized function usually encountered in freshly isolated cells for primary culture and emphasizes the strong similarities with the effects on cellular phenotype following induction and recovery from the well known response to heat shock and other cellular stresses [3].

2. CELL ISOLATION

As the loss of cellular phenotype often occurs

during or immediately following cell isolation, it is essential to outline the procedures involved. Primary cell cultures are commonly prepared either by allowing cells to migrate out of fragments of tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate. In general, the requirement for high yields of cells means that enzymatic digestion of the tissue biomatrix is the method of choice [4,5]. Crude trypsin is the enzyme most frequently used for embryonic tissue, but is widely recognized to damage membranes and alter normal cellular function [6,7]. The large amounts of fibrous connective tissue in adult organs has led to the use of collagenase in isolating terminally differentiated cells [4,8]. The concentration of enzyme, the method of tissue dispersal and the centrifugal force used for washing the cells all greatly influence the final yield of viable cells.

Liver parenchymal cells have been more extensively studied in primary cell culture than any other cell type. These cells are particularly susceptible to trauma, their isolation requiring the use of the

gentlest methods available [5,9]. Berry and Friend [10] established the basic protocol involving a two-step perfusion of the liver *in situ*, first with calcium-free medium, followed by a calcium-rich medium containing collagenase. Tissue disaggregation is then continued *in vitro* most commonly by finely chopping up the tissue and agitation in enzyme-containing medium.

In earlier studies, the perfusate was intensively oxygenated but Seglen [11] omitted oxygenation to facilitate sterility without any apparent deleterious effects [12]. The use of high concentrations of Hepes (20 mM) to buffer the perfusate maintains the yield and quality of cells, as judged by morphology, respiration and several hormonally responsive functions [13,14]. Cells isolated in the absence of supplementary oxygen show some discrete morphological alterations, such as retraction of the mitochondrial matrix and abnormal appearance of endoplasmic reticulum, similar to those seen in rat liver parenchymal following hypoxia *in vivo*, although these features are readily reversible [15].

Tissue disaggregation by the collagenase digestion procedure has also been used for preparation of cells from other epithelial [16–18], mesenchymal [19,20] and endocrine tissues [21]. The tissue-specific functions of these cells are then either analysed immediately or more commonly during the first few days in culture.

3. LOSS OF PHENOTYPE OF CELLS IN SUSPENSION OR SHORT TERM CULTURE

Investigation of cellular function during the first 24 h after isolation often involves the maintenance of isolated cells in suspension, during which time they are agitated to provide both oxygen and prevent aggregation [22,23]. Alternatively, they may be allowed to settle onto a substratum to which they attach rapidly and, in the case of liver parenchymal cells, reform tissue characteristic structures such as trabeculae and bile canaliculi by the second day in culture [24,26].

The viability of isolated cells has been assessed in various ways: vital dye exclusion, lactate dehydrogenase leakage [27], fine structural analysis [14], attachment to the substratum [28], respiration [11], gluconeogenesis [14] and the capacity to respond to various hormonal and other

stimuli (see below). Several laboratories have adopted the maintenance of normal levels of cytochrome P450 as a criterion for liver parenchymal cell viability [29,30]. Rat hepatocytes cultured for 24 h lose 70% of their cytochrome P450 [31,32], while in bovine adrenal zona glomerulosa cells the loss of capacity to synthesize aldosterone accompanies the decline in cytochrome P450 following isolation [33,34]. This decrease can be prevented by supplementing the culture medium with a variety of nutrients, growth factors and hormones [35,36], or by the incorporation of hyperphysiological concentrations of nicotinamide or pyridine derivatives (metapyrone) in the culture medium [37,38].

Almost all mouse liver-specific mRNAs decline following parenchymal cell isolation due to inhibition of their transcription [39]. Of all hepatic mRNAs the best studied gene product in primary cell cultures is serum albumin [26,40,41]. Fifty percent of total plasma protein synthesis in liver is devoted to albumin both immediately after cell isolation and *in vivo* [42–44]; its synthesis and secretion rapidly decline to negligible levels during the first 2–3 days in culture [45,46]. This reduction in synthesis is due to both a rapid decline in cellular albumin mRNA levels [26] and perhaps translational control as well [47]. Addition of insulin to the culture medium partially alleviates this decline in both mRNA levels and albumin synthesis [46–48], while serum itself appears ineffective [45]. The relatively enhanced synthesis of other adult type plasma proteins during maintenance of cells in culture (see below) indicates that the decline in albumin synthesis is selective and not due to a general loss of cellular viability as seen in rat hepatocyte suspensions [22,49].

Although the synthesis of cytochrome P450 and albumin represents the best documented example of loss of constitutively synthesized tissue specific proteins in short term culture, other examples are also known, including both secreted and intracellular proteins [50,51], where specific protein synthesis rapidly decreases following cell isolation.

It should be emphasized that the above losses occur in the first 2–3 days of cell culture and these short term studies should be distinguished from long term cultures. In the latter, phenotypic changes may be associated with selection of more actively proliferating cells under the limitations of

nutrient, hormone and substrate availability [1,8].

The isolation procedure removes the cell type of interest from its normal environment leading to the loss of identifiable plasma membrane domains [52]. Epithelial cells are found to be rounded and dispersed singly or in small clumps [24,26]. Maintenance of normal *in vivo* levels of constitutively synthesized tissue specific proteins is significantly enhanced by culturing these cells on or within specific biomatrices such as collagen gels [53,54], or matrices from other natural tissue glycoproteins such as fibronectin [50]. Complex biomatrices have also been employed, as for example, that secreted by bovine corneal epithelial cells [41], or isolated from rat liver [55]. However, liver parenchymal cells are able to synthesize their own collagen biomatrix in culture [56]. Albumin synthesis can be sustained in long term cultures (>20 days) by co-culturing liver parenchymal cells with another liver epithelial cell type [57,58], however in these studies it is clear that the rate of albumin synthesis increases during maintenance in culture to peak values at 10 days in the presence of fetal calf serum which may reflect hormonal induction, and also a partial recovery of rates of synthesis and secretion to those found *in vivo*.

4. HORMONAL RESPONSIVENESS IN SHORT TERM CULTURES

Perhaps the best indicator of the maintenance of differentiated function after isolation of cells is their ability to respond to specific hormones that rapidly modulate metabolic activity or promote their growth and development [59]. It is the rapid and transient metabolic actions of hormones such as glucagon and insulin which have been widely studied in isolated cells. However, hormonal responsiveness is often reduced or even lost as a consequence of a reduction [60], or even elimination [61], of the number of hormone receptors on the cell surface immediately following cell isolation. Following establishment of primary cell cultures hormone receptor numbers gradually increase, leading to the recovery of full hormonal responsiveness [60,61]. This recovery appears to be dependent on RNA and protein synthesis. Proteolysis alone appears inadequate to explain these decreases in cell surface receptors, since isolation of cell membranes without the use of collagenase

[62] also results in receptor loss. Hormone binding is also known to decline following surgical trauma such as partial hepatectomy [63].

The effects of addition to cultured cells of hormones that regulate metabolic activity (*i.e.*, insulin and glucagon) are usually rapid and often act via changes in cAMP metabolism [64] or metabolite transport processes at the plasma membrane [65,66]. Rapid and transient metabolic actions of hormones acting at the plasma membrane would appear therefore to be retained or rapidly recovered in cell suspension or primary cell culture, maximal hormonal responsiveness being achieved 48 h after cell isolation [67].

The actions of hormones with relatively slow growth and developmental actions has been more difficult to study in isolated cells, and hence less extensively investigated. One problem is the often rapid metabolism of the hormone in the target cell. For example, triiodothyronine [68] and steroid hormones [69,70] are rapidly metabolized in hepatocytes, thus accounting for the requirement for high doses of hormone in eliciting a full physiological response [24,71]. Frequent replenishment of the culture medium with the hormone may entirely alleviate variable or suboptimal responses [69,71] allowing the same quantitative hormonal effects as seen *in vivo* [72,73].

As is the case for immediate metabolic effects, hormonal effects on protein and RNA synthesis are also reduced following cell isolation, even if adequate hormone concentrations are maintained in primary culture [26,74-79]. In general, response to growth and developmental hormones recovers to maximum levels after 5-10 h in suspension culture [22,75,76] or after 2-3 days in primary cell culture on fixed substratum [24,26,45].

The levels of hormonally regulated tissue specific mRNAs and proteins may rapidly decline following cell isolation, *e.g.*, albumin [46] or α_2 -globulin [80]. Glucocorticoids, which are known to enhance albumin synthesis [81,82], help to maintain its rate when added to hepatocytes soon after their isolation [50,83]. In this respect the full induction of vitellogenin synthesis in primary cultures of amphibian or avian liver parenchymal cells offers many advantages, such as the competence of male liver to respond to estrogen, offering a 'zero' background of prior vitellogenin gene expression and being independent of cell division

or DNA synthesis [72,84,85]. The levels of certain fetal isozymes and α -fetoprotein are known to increase following cell isolation, a process often termed 'dedifferentiation' or 'retrodifferentiation' [86]. The acquisition of a fetal phenotype [8] is, however, an unlikely explanation for loss of specialized, adult cell functions. These fetal characteristics are associated with rapidly proliferating hepatocytes and the rate of DNA synthesis may determine their expression [87,88]. It is also important for this discussion to realise that 'dedifferentiative' changes in phenotype of this kind do not occur until 3–6 days after cell isolation.

There are many similarities between hormonal induction of gene expression and the induction by drugs of detoxicating enzyme systems [89,90]. For example, phenobarbitone is ineffective in increasing cytochrome P450 levels for the first 24 h after isolation of liver parenchymal cells from normal or hepatectomized adult rats [30,31]. The capacity of the cells to accumulate cytochrome P450 in response to phenobarbitone has been shown to increase to a maximum level only after 4 days of culture [91].

In addition to its rapid metabolic effects on isolated cells, insulin enhances the relatively slow lipogenesis in short term rat hepatocyte primary cultures [92,93], the action requiring continuing RNA synthesis following a 24-h lag period after cell isolation [92,94]. A similar latent period was observed with the stimulation of lipogenesis by triiodothyronine in primary cultures of chick embryo hepatocytes [25].

In summary, following cell isolation there is quite commonly an extended lag period during which the response of cell cultures to growth and developmental hormones is slow and suboptimal in comparison to the *in vivo* response. The specialized tissue-specific functions generally recover, albeit not fully in all cases, to give maximal hormonal responsiveness after usually 2–3 days in culture.

5. CULTURE SHOCK AND THE LOSS OF DIFFERENTIATED FUNCTION

Explanations offered for the loss of specialized tissue specific gene function following cell isolation and short term primary culture include: (i)

removal of tissue substratum [95]; (ii) nutrient and hormonal deprivation [2]; (iii) onset of DNA synthesis or cell division [87]. These suggestions, however, do not provide a biochemical mechanism. Furthermore, some of the above factors are not associated with the loss of phenotypic function, e.g., in *Xenopus* liver parenchymal cells which do not divide in culture [26] and the failure of cell–cell contact, cell substratum interactions and hormone or growth factor supplemented serum to prevent short term loss of phenotype in mouse hepatocytes [39].

An important factor influencing protein and nucleic acid metabolism is cell morphology [96,97]. Reattachment of mouse fibroblasts in suspension culture to a substratum results in a rapid recovery of overall protein synthesis within a few hours, although complete restoration of mRNA, rRNA and DNA synthesis require about 18 h of culture after reattachment. This recovery process is dependent on extensive cell spreading and appears to be shape dependent. In these fibroblasts, actin mRNA levels are specifically down-regulated following detachment from the substratum. Similar recoveries in protein and nucleic acid metabolism are seen following attachment of cells isolated for primary culture to the tissue culture dish surface [98,99].

Another important factor, overlooked until now, and relevant to cell structure, is the increase in stress protein synthesis in freshly isolated cells, recently characterized in our laboratory [26]. The synthesis of stress or heat shock-like protein declines during the first 2–3 days in culture concomitantly with the reformation of cell–cell contacts and substratum interactions. These proteins are known to associate with cytoskeletal elements [100–102] leading to alterations in cell morphology [26,103] that are reversible on return to the normal incubation temperature.

Stress proteins may be synthesized in embryonic tissue at normal temperatures and are generally thought to be constitutively synthesized at low levels in unstressed tissues [104–106]. Although the only established role of heat shock proteins is in thermotolerance [107], diverse forms of stress lead to the induction of their synthesis. Many of these stresses are produced during cell isolation: (i) mechanical trauma [108,109]; (ii) deprivation of glucose [110,111]; (iii) potential influx of Ca^{2+}

[112]; (iv) anoxia [113]. Anoxia (and recovery from it) is particularly relevant to the isolation of cells, since many tissues used for primary cell culture have a high requirement for oxygen, in particular liver. Highly differentiated cell types might be deprived of oxygen both during preparation or subsequent maintenance in culture [114]. Stevens [115] proposed that growth in primary cell culture was limited due to the high requirement for oxidative metabolism in isolated hepatocytes in comparison to most secondary cell lines or fibroblasts.

Most highly differentiated cells stop synthesizing normal or non-heat shock mRNAs or proteins upon trauma or stress [116–120]. Pre-existing mRNAs are either sequestered in a non-translatable form or degraded, while recovery of normal protein synthetic patterns usually requires 12–36 h after return to normal temperature [119,120]. There may also be a transient paralysis in hormonal responsiveness during this period [120].

There are therefore many similarities between the effects of heat shock and cell isolation, which may point to common biochemical mechanisms. For example, isolation of rat liver parenchymal cells results in estrogen receptor levels falling to 50% of the *in vivo* level, with a further 17% fall in the first hour of suspension culture [51]. In *Xenopus* liver parenchymal cells, heat shock leads to the disappearance of estrogen receptor, its synthesis explaining the 24–36-h lag period of estrogen responsiveness following return to normal temperature. Addition of estrogen prior to heat shock prevents this loss, therefore allowing an immediate hormonal response [120]. Similar protection by ligand is seen with the stabilization of cytochrome P450 by pyridine derivatives following hepatocyte isolation [38]. Heat shock leads to the dephosphorylation of ribosomal protein S6 [121,122], which is the same protein whose dephosphorylation following establishment of primary cultures selectively affects albumin synthesis [47].

If the inhibition of tissue-specific function following both cell isolation and heat shock are due to common mechanisms following cellular stress then monitoring cellular protein synthetic patterns will indicate the degree of stress. It may be presumed that maximal hormonal response or recovery of specialized function will not occur un-

til the cells have recovered from the stress which would be indicated by the decline in stress protein synthesis. The study of heat shock has concentrated on the mechanisms of induction of stress proteins, whereas cell isolation studies have focussed on the maintenance of normal differentiated function. If common biochemical mechanisms are involved in both then the study of isolated cells will contribute substantially to our understanding of cellular stress and its effects on normal differentiated function. At the same time, the recognition of cellular stress will allow the full exploitation of primary cell cultures in studying the regulation of phenotypic function.

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