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Viability Evaluation of Engineered Tissues

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Abstract

Biohybrid artificial organs encompass all devices capable of substituting for an organ or tissue function and are fabricated from both synthetic materials and living cells. The viability of engineered tissue could be related to the viability of implanted cells. The system of viability assay for mammalian cell culture can be applied to the determination of cell viability for engineered tissue. This review explores various methods of cell viability assay which can be applied to the viability evaluation of engineered tissue. The major criteria employed in viability assays include survival and growth in tissue culture, functional assay, metabolite incorporation, structural altercation, and membrane integrity. Each viability assay method is based on different definitions of cell viability, and has inherent advantages and disadvantages. In order to be able to assess the viability of cells with one assay method, it is desirable to compare the viability measurements from various assays derived from different criteria.

Key Words: Cell viability, membrane integrity assay, functional assay, fluorescent probes assay, morphological assay, reproductive assay, engineered tissue

Over the past few decades, the development of several tissue substitutes has been attempted with both synthetic and organic materials. Synthetic materials have been tried previously in experimental and clinical settings.[1,2] Some of the above attempts have failed to gain clinical acceptance due to either functional or biocompatibility problems. Recent technological advances resulting in improved maintenance of cell viability and function in culture and bioreactor designs have led to the development of a number of bioartificial devices. In tissue engineering, cell viability and function has been considered to be a crucial factor in determining the fate of artificial organs.

The term "cell viability" and the diverse assays used to for its determination can strongly influence the interpretation of experimental results. The determination of cell viability is often required in biological studies. In an effort to determine cell viability, a number of assays that correlate with either reproductive or functional capabilities have been developed. The major criteria employed in viability assays include the assessment of survival and growth in tissue culture, functional assays, metabolite incorporation, structural alteration, and membrane integrity (Table 1).

Occasionally, viability measurements derived from these different criteria do not correlate well with each another. For instance, a viability test based on membrane integrity judges cells with functionally undamaged membranes to be viable even if they have lost the ability to proliferate. It is the purpose of this review to examine the characteristics of the major groups of currently available assays as they are applied to engineered tissue. In addition, it is our intention to show that while some assays may be clearly superior in measuring viability, technical and practical considerations may limit their usefulness.

MEMBRANE INTEGRITY ASSAYS

Features distinguishing live from dead cells include the loss of transport function across plasma membrane which results from loss of membrane integrity. By far the most convenient viability assays involve the determination of membrane integrity via dye exclusion from live cells. Because membranes are so easily observed, the development of viability assays based on their integrity was natural. To relate viability with membrane integrity, it is assumed that a damaged

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Table 1. The Major Criteria Employed in Viability Assays

Category of viability assay	Assays	Principles
Membrane integrity assay	Exclusion dyes Fluorescent dyes LDH release Rhodamin ester (membrane potential)	The determination of membrane integrity via dye exclusion from live cells
Functional assay	ATP cell viability MTT, XTT assay DNA synthesis Protein synthesis	Examining metabolic components that are necessary for cell growth
Fluorescent probes assay	Fluorescent conjugates	Simultaneous cell selection and viability assay
Morphological assay	Microscopic observation	Determination of morphological change
Reproductive assay	Colony formation assay	Determination of growth rate

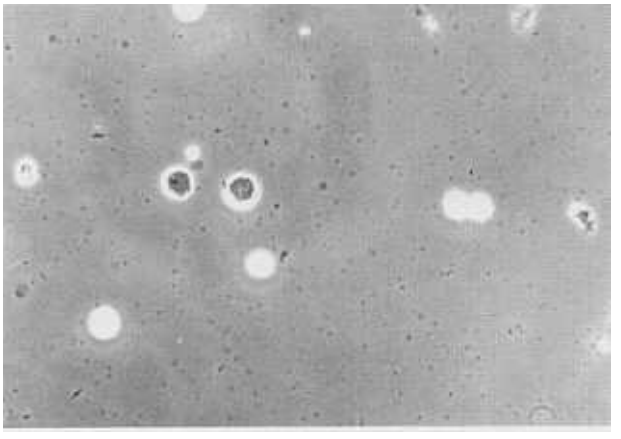

Fig. 1. Trypan blue exclusion assay with balb 3T3 fibroblasts. Lightly blue stained cells indicate dead cells; Translucent cells indicate living cells.

membrane will allow large, charged molecules, which are normally excluded by cells, unimpeded access to the cytoplasm. If the molecules inserted have special light-absorbing or fluorescent properties, damaged cells can be readily identified by microscopy. A variety of such dyes have been effectively employed, including trypan blue,[3-5] eosin,[3] Congo red,[6] and erythrosin B.[7,8] The classical example is the trypan blue exclusion assay. Under bright field microscopy, membrane damaged cells stain light purple-violet, whereas undamaged cells appear translucent (Fig. 1). Trypan blue has proved to be by far the most popular vital exclusion dye for mammalian cells.

However, there are several problems that limit the usefulness of such dyes. Cells must be counted within 3−5 min because the number of blue-staining cells increases with time after addition of the dye.[9] Such dyes are not recommended for use on monolayer cultures but rather they are intended for cells in suspension; thus monolayer cells must be trypsinized.[4,5] Where large numbers of samples have to be counted, it may be inconvenient to perform all the tests on the same day by counting one cell suspension at a time before staining the next sample. In tissue engineering, tissues can be engineered *in vitro* and *in vivo* for reconstruction using selective cell transplantation in combination with acellular matrices. This technology involves an interdisciplinary approach combining techniques of cell biology and materials sciences leading towards the development of functional tissues or organs.[10] In engineered tissues, because of the affinity of the dye for protein, trypan blue exclusion cannot be used to assess cell viability and proliferation in matrices in which collagen, fibronectin, or any other protein materials are used for cell adhesion.

Other variations include fluorescent dyes. Certain fluorescent dyes are more reliable indicators of cell viability than the more traditional colored dyes.[11,12] The intercalating dyes ethidium bromide (EtBr) and propidium iodide (PI) are known to be able to pass only through the membranes of dead or dying cells.[11,13,14] PI binds to nucleic acids upon membrane damage and becomes highly fluorescent. Because flow cytometric techniques depend on fluorescence, propidium iodide is ideally suitable for the rapid evalua-

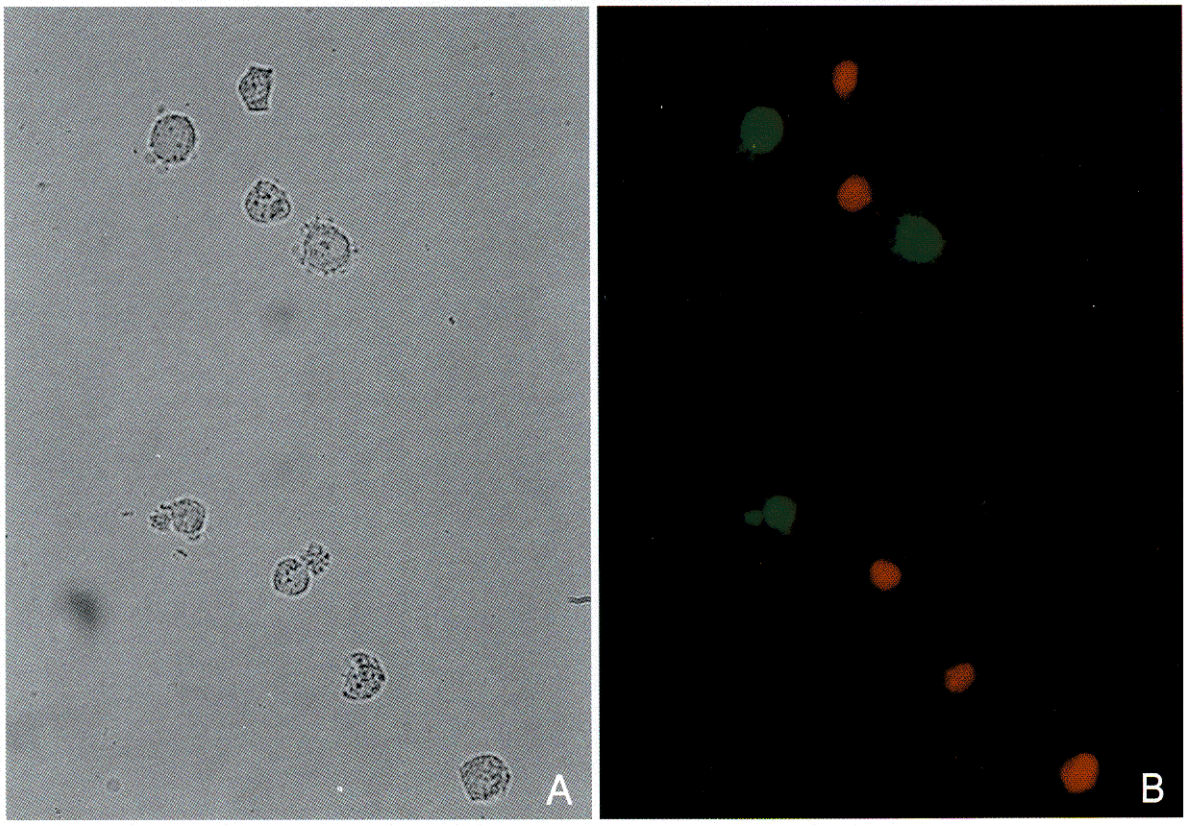

Fig. 2. FDA/PI double staining assay with HEL cells. (A) Cells before staining; (B) Cells after staining (green stained cells indicate living cells stained by FDA; red stained cells indicate dead cells stained by PI).

tion of the permeability properties of large numbers of cells while maintaining good statistical accuracy.[13,14] Double staining with acridine orange (AO) and PI is commonly performed on cell suspensions and can be evaluated by flow cytometry or fluorescence microscopy.[15] AO is a membrane-permeable, monovalent, cationic dye which binds to nucleic acids. A low concentration of AO causes a green fluorescence, while a high concentration causes a red fluorescence. PI is impermeable to intact plasma membranes, but easily penetrates the plasma membrane of dead or dying cells and intercalates with DNA or RNA forming a bright red fluorescent complex.[16]

An alternative approach uses inclusion dyes to test the integrity of the membrane. Several fluorometric dyes have been tested as indicators of membrane integrity. Fluorescein diacetate (FDA) has been used as a fluorometric assay of cell viability. FDA is a nonpolar ester which passes through plasma membranes and is hydrolyzed by intracellular esterases to produce free fluorescein. The polar fluorescein is confined within cells which have an intact plasma membrane and can be observed under appropriate excitation conditions. Undamaged cells retain the highly fluorescent fluorescein dye, whereas cells with damaged membranes are unable to do so and fluoresce only weakly. The live cells thus fluoresce a brightly greenish-yellow when illuminated at 450–480 nm.[17,18] FDA and PI are used in a simultaneous double staining procedure that can be performed in merely 5 min.[19] (Fig. 2) However, the staining of cells with FDA results in high background fluorescence. This fluorescence is presumably due to extracellular hydrolysis of the FDA or fluorescein that leaked through damaged membranes.

Cytometry refers to the measurement of physical or chemical characteristics of cells or other biological elements. Flow cytometry is a process in which such measurements are made while the cells or particles pass through the measuring apparatus in a fluid stream. Flow cytometry has important advantages over conventional techniques. It is rapid, highly sen-

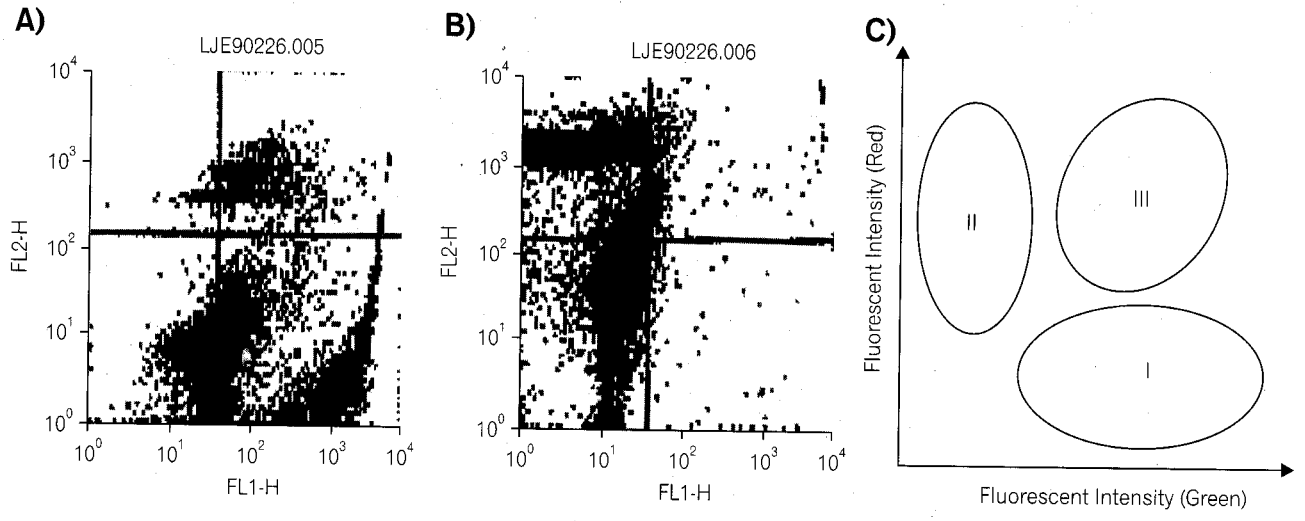

Fig. 3. Histogram of flow cytometry using FDA/PI double staining: (A) Histogram of live cells; (B) Histogram of dead cells; (C) Schematic diagram of histogram (Fluorescence intensity by fluorescein from live cells is distributed in the region (I); Fluorescence intensity by PI from dead cells is distributed in the region (II); Cells in the region (III) are double stained with fluorescein and PI, which may be dying cells).

sitive and allows multi-parametric analysis and cell sorting. The technique can be used to determine cell viability, intracellular calcium and pH levels, membrane potential, enzyme activity, membrane fluidity and endocytosis.[20] In the evaluation of cell viability, flow cytometry allows the simultaneous determination of the number of live cells and dead cells. For example, in the case of the FDA/PI double staining assay, the fluorescence intensity of fluorescein from live cells is distributed in the lower-right region in the histogram (Fig. 3A), and that of PI from dead cells is distributed in the upper-left region in the histogram (Fig. 3B). Both the above fluorescence intensities can be calculated to determine the number of cells separately.

In cell viability assay by membrane integrity, membrane potential can be determined in individual cells from the distribution of cationic dyes.[21] The equilibrium of intracellular ions in damaged cells may be lost due to the altered permeability to sodium and potassium resulting from the change of membrane potential. The live or dead status of the cell can be assessed by the determination of such change of membrane potential. The distribution of a select group of cationic fluorescent dyes can be used to measure membrane potential of individual cells with a microfluorometer. The essential attributes of these dyes include membrane permeability, low membrane binding and spectral properties which are insensitive to the environment. For example, rhodamine esters are nontoxic, highly fluorescent dyes which do not form aggregates or display binding-dependent changes in fluorescence efficiency. Thus, their reverse accumulation is quantitatively related to the contrast between intracellular and extracellular fluorescence and allows membrane potentials in individual cells to be continuously monitored.[21]

Lactate dehydrogenase release is a permeability assay that differs from those discussed above. It is a noninvasive means of identifying membrane damage.[22] Dehydrogenase activity is measured in cell or tissue supernatant fluids by observing the conversion of exogenously added lactate to pyruvate and measuring the increase in absorbance at 340 nm resulting from the conversion of added NAD to NADH. Alternatively, when increased sensitivity is desired, NADH can be measured fluorometrically.[23,24]

FUNCTIONAL ASSAYS

The overall failure of strategies attempting tissue replacement in the past led to the application of the principles of cell transplantation materials science and engineering towards the development of a biological substitute that would restore and maintain normal cell function. Cell transplantation has been proposed for the replacement of a variety of tissues. Implanted cells orient themselves spatially along the matrix surface.[10] In cell viability assays, functional assays for

implanted or seeded cells can be important predictor parameters related to the success of engineered tissues.

Functional assays typically evaluate viability by examining the metabolic components that are necessary for cell growth, on the premise that cellular damage will inevitably result in the loss of ability to maintain and provide energy for metabolic function and growth. Cellular energy is usually assessed by determining total ATP levels, although the energy charge of cells ([ATP]+0.5 [ADP]/([ATP]+[ADP]+[AMP])) has been suggested as a more appropriate indicator of cellular energy status.[25] The ATP cell viability assay measures light production as it interacts with the luciferin-luciferase complex. Quantification of the light produced has been shown to directly correspond with the number of viable cells.[26] This assay (the luciferin-luciferase system) has several advantages, including being highly quantitative due to the measurement of cellular response by cellular ATP instead of by counting colonies or cells, being reproducible and reliable, and furthermore it has been shown to have a successful assay rate.[27] This assay method is extremely sensitive, enough to detect the existence of even 50 cells,[25] but unless additional enzymes are added, only total ATP, not ADP or AMP, is measured. To overcome the above limitation, ^{31}P Nuclear Magnetic Resonance (NMR) has been applied to cell viability assay and has the distinct advantage of simultaneously measuring the multiple phosphate intermediates: ATP, ADP, and AMP. The major advantage of NMR is that it is a noninvasive technique such whereby cells and tissues can be probed under a variety of conditions.[28]

Functional assays one step removed from direct ATP measurements examine the activity of enzymes involved in the production of ATP. The most widely used method is the measurement of succinate dehydrogenase with a tetrazolium dye. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, which relies on cellular metabolism, is an easy and sensitive colorimetric assay of cell viability, activation and proliferation based on the use of the tetrazolium salt MTT.[29] Tetrazolium dyes serve as hydrogen acceptors and are reduced to strongly absorbing formazen product. Tetrazolium salt undergoes a color change caused by the activity of various dehydrogenase enzymes. The tetrazolium ring is cleaved in active mitochondria, and so the reaction

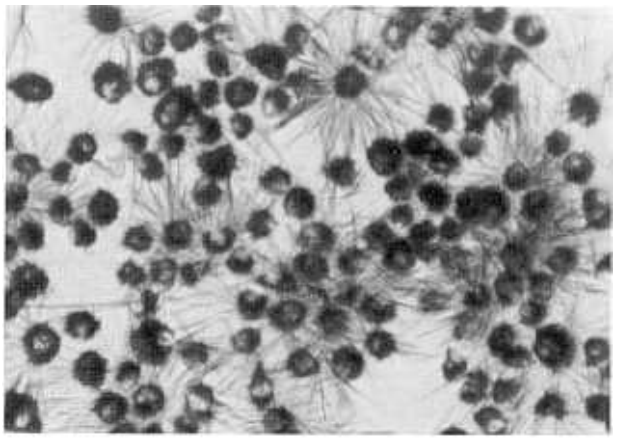

Fig. 4. MTT colorimetric assay with primary cultured rat calvaria osteoblasts.

proceeds only in living cells.[30] The quantity of formazen produced is measured at 565 nm. Cells that have lost viability do not reduce and absorb. The distinct advantage of the MTT assay is that many samples can be processed and analyzed semi-automatically within a reasonable time period.[29] One weakness inherent in the use of MTT is that the resultant colored product is insoluble, precluding direct spectrophotometric absorbance measurements without first dissolving the crystals (Fig. 4). However, a new tetrazolium salt, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT), has been synthesized by Paull and colleagues[31] and its bioreduction yields a highly colored formazen product which is water soluble.

Another set of frequently used functional assays measures viability by quantifying rates of DNA or protein synthesis.[32-34] DNA synthesis can be studied by measuring [^{3}H] thymidine incorporation. Liquid scintillation counting of the acid-precipitative fraction of ruptured cells or auto-radiography of individual cells is readily carried out. Because thymidine uptake depends on DNA synthesis, viability can be measured only in reproductively dividing populations. For non-dividing, metabolically active cell populations, measurement of the radio-labeled amino acid incorporation into proteins can offer an alternative means of assessing cell viability. Tissue remodeling and specific gene expression may be considered important processes for the implantation of biomaterials. The tissue remodeling process is accompanied by a fun-

damental reprogramming of fibroblast morphology and metabolism.[35-37] The homeostasis of fibroblast collagen metabolism is regulated in a complex manner by an interplay of various different mechanisms which include hormones, cytokines, and cell-matrix interactions.[38] In terms of tissue remodeling in implantation, specific protein synthesis, such as collagen, by cells may be an important factor for assessing the cellular function related to cell viability.[39]

FLUORESCENT PROBES ASSAY

A new generation of reagents, called fluorescent biosensors, that report on specific molecular events in living cells, has evolved from *in vitro* fluorescence spectroscopy and fluorescent analogue cytochemistry. Creative designs of fluorescent protein biosensors measuring the molecular dynamics of macromolecules, metabolites, and ions in single cells have emerged from the integrative use of contemporary synthetic organic chemistry, biochemistry, and molecular biology. The activity of intracellular proteins encompasses many sorts of chemical interactions that include the binding and release of ligands, assembly and disassembly of macromolecular structures, the interaction with membranes or organelles, and the catalytic conversion of specific substrates into metabolic or macromolecular products. The activity of a protein is defined by the environmental changes that occur either internally or on its surface.[40,41] Based on the fluorescent biosensor, a strategy has been developed to label specific sites in living cells with a wide selection of fluorescent or other kinds of probes. The incorporation of extrinsic fluorescent probes at specific locations in a protein allows for site selection as well as spectral selection. A wide range of reactive fluorescent dyes is now available that can be used to target specific sites, exhibit environmental sensitivities, and fit into specific spectral regions.[42]

In some cases of engineered tissue, simultaneous cell selection and viability assays are necessary to evaluate the target cell viability. A method for marking cells which allows highly sensitive detection of the cells in mixed populations has been previously described by the author.[43,44] Vascular endothelial cells (ECs) play an important role in physiologic hemostasis and blood vessel permeability, express immune-related functions in monocytes and macrophages, and

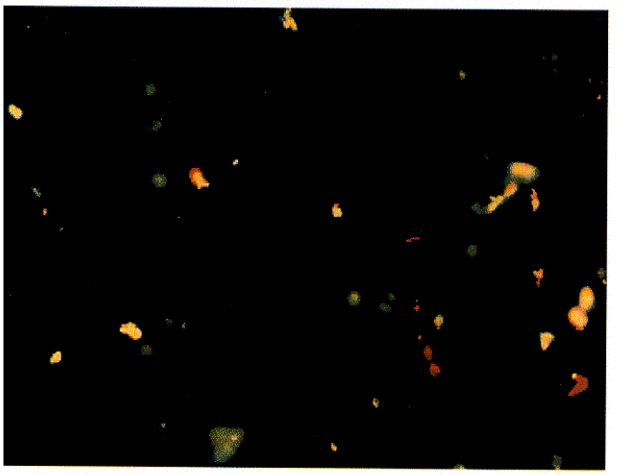

Fig. 5. Each part of quadrant statistics was observed under fluorescence microscopy. Live ECs preferentially expressed the green color of GS1. Dead ECs are double stained by the green color of GS1-FITC and the red color of PI, which results in yellow. Dead cells except dead ECs are identified by only the red color of PI.

the viability of ECs is important in predicting the post-operative function and durability of cryopreserved vessels for implantation.[45] Recent studies suggest that certain cells in a given species exhibit distinct cell surface glycosylation properties differing from other cells, and ECs appear to express certain a-D-galactosyl residues. It has been reported that the tetrameric *Griffonia simplicifolia agglutins* (GS1) shows prominent binding only to the a-D-galactosyl residue of blood vessel ECs.[46] Staining with fluorescein isothiocyanate (FITC) conjugated with GS1 differentiates ECs from the other cells in flow cytometry. PI intercalates DNA double strands in dead cells without regard to cell types, as their membranes lose integrity. Hence, flow cytometry analysis of the GS1-FITC and PI double staining is presumed to immediately determine the differential viability of ECs from whole cells. The use of fluorescent probes enables the rapid determination of the viability of ECs and whole cells from the same tissue without separating ECs from whole cells, and of the viability of each step in the cryopreservation process (Fig. 5).

MORPHOLOGICAL ASSAY

A number of large-scale, morphological changes that occur at the cell surface, or in the cytoskeleton, can be followed and related to cell viability.[47-51]

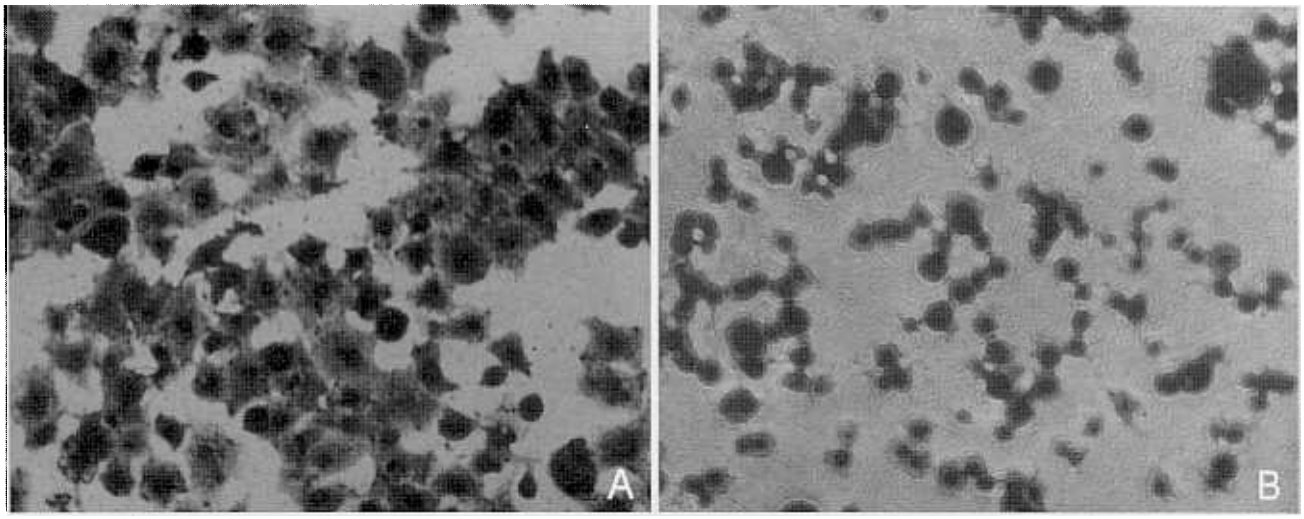

Fig. 6. Morphological assay: morphological characterization of osteoblast-like cells (MC 3T3-E1) cultured on surfaces. (A) Morphology of cells cultured on tissue culture plate; (B) Morphology of cells cultured on PLLA membrane.

Measurements of such changes are usually limited to specific situations and, therefore, have less utility than the membrane integrity or functional assays. Because energy is expended to regulate the concentration of intracellular water, irreversible volume changes can be used to indicate cell death. Damage can be identified by large decreases in volume secondary to losses in protein and intracellular ions or due to altered permeability to sodium or potassium.[52] The cytoskeleton change may be related to cell functions such as differentiation.[49-51]

In tissue engineering, the cell morphology is comparatively affected by its surrounding milieu, i.e., extracellacellular matrix (ECM) in engineered tissue, and may be related to cellular functions such as the secretion of ECM component. For instance, the ability of bone cells to migrate into the biomaterial and the relationships they establish between their membrane and the biomaterial surface may directly influence their activity. As is shown in Fig. 6, osteoblast-like cells display a spindle shape and orientation on the tissue culture plate (Fig. 6A), but cells on the poly L-lactic acid (PLLA) show rounded shapes (Fig. 6B). Environment might appear to influence not only cell orientation, but also the orientation of the collagen fibers deposited by cells.[53] Cytoskeletal change can involve either the breakdown of microtubular structures or the rearrangement of filamentous actin.[27]

REPRODUCTIVE ASSAY

Determination of growth rate also depends on assessment of cellular reproductive potential. The growth properties of cell cultures are determined by measuring the absolute increase in cell number over an extended period of time, generally by recording the increase in the number of cells at certain time intervals. The importance of cell proliferation and turnover for the maintenance of tissues, organs, and tumors is self-evident. Therefore, for an investigation of the response of proliferating cells in culture to engineered tissues, an assay that determines the productive potential, i.e., ability to proliferate, would seem to be the ideal choice. When used properly, colony formation assays are simple, highly sensitive and are commonly employed for measuring mammalian cell proliferation.[54-56] Colony formation assays involve obtaining a single cell suspension of the cell type desired. Enzymatic (trypsin or other protease) or mechanical techniques are commonly used to detach cells from the surface of the petri dish for single cell preparations. Once the cell suspension is counted and the cell density calculated, known cell numbers are plated onto petri dishes at low cell to surface area ratios for growth and division. Each viable cell will divide and eventually give rise to a colony or cluster of daughter cells. Statistically, for low cell to surface area ratios, each colony counted must have risen from a single cell. However, a number of considerations limit the usefulness of *in vitro*

colony formation assays. Cell survival, as defined by this technique, is dependent on the sustained proliferation of the single cells that are plated. Sterile conditions are necessary. Single cell suspensions are not always obtainable and clumps may form, with the consequence of survival being either under- or overestimated.

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