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Lactate Dehydrogenase Activity as a Rapid and Sensitive Test for the Quantification of Cell Numbers *in vitro*

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Abstract: Lactate dehydrogenase (LDH) has been used extensively as a marker for cell death both *in vitro* and *in vivo*. The release of LDH into tissue culture medium accurately reflects cell viability *in vitro*. We have investigated the relationship between cell concentration and total LDH activity in samples of cell lysate. Although there are differences in the amount of LDH present in different cell types, the total enzyme activity in a sample of cell lysate is directly proportional to the concentration of cells in the sample. The measurement of LDH activity *in vitro* provides a sensitive, accurate and cost-effective alternative to the use of either radioisotopic or dye-based assays for the determination of cell numbers.

INTRODUCTION

Tissue culture studies have greatly enhanced our understanding of the complex interactions which exist between biomaterials and cells *in vivo*. Investigations into the effects of test materials on cell morphology, survival, proliferation and function are now considered to be important elements of the preclinical testing of candidate biomaterials. Many of these in-vitro studies can be time-consuming and expensive to perform. As a result, attempts have been made to identify a preliminary screening procedure which makes it possible to identify potentially toxic materials at an early stage, thereby eliminating the need to conduct exhaustive testing on biomaterials which ultimately prove to have no clinical value. We have found cytotoxicity tests to be the most useful preliminary screening system and over the last 15 years have developed the lactate dehydrogenase (LDH) assay as a standard test for biocompatibility *in vitro*.

Lactate dehydrogenase (E.C. 1.1.1.27) is present within the cytoplasm of all mammalian cells. The normal cell membrane is impermeable to LDH and therefore the enzyme is released only if the plasma membrane is damaged. The release of LDH from cells has been used extensively as an accurate marker for cell death and is beginning to replace the more traditional radioactive ^{51}Cr release assay as a test for cell-mediated cytotoxicity.[1,2] In addition, LDH assays are used routinely in human medicine as diagnostic tests for myocardial injury[3] and neoplasia.[4] LDH assays have also been used in preclinical investigations into the biocompatibility of materials for use as surgical implants. Results from our laboratory and from other research groups have confirmed the reliability of the assay as a test for cytotoxicity *in vitro*.[5,6,7]

Our investigations into the effects of biomaterials on cell proliferation have focused on the use of growth curves generated by direct counting of cells in a haemocytometer. Although direct cell counting remains the most popular technique for measuring cellular proliferation over time, it can be problematic. The routine use of haemocytomers and, to a lesser extent, electronic counting devices is time-consuming and, in addition, the sensitivity of

each technique is limited. The haemocytometer, for example, is inaccurate at cell concentrations below 1×10^5 cells/ml[8] while electronic counters are unable to differentiate between live and dead cells and may overestimate the number of viable cells in a sample. Indirect methods of counting cells are based upon use of biochemical assays for quantifying either cellular DNA or cellular metabolism. ^{3}H-thymidine and ethidium bromide have been used extensively as markers for cellular DNA synthesis; since the amount of cellular DNA is almost constant for cells of the same type, the incorporation of these agents into DNA is directly proportional to the number of cells in the sample. A number of non-radioactive, dye-based cellular proliferation assays have also been used, of which the MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide) assay has been the most popular. This assay, first developed by Mosmann[9] is based upon the conversion of a colourless tetrazolium compound into a coloured formazan product. Only live cells are capable of metabolising the dye and the concentration of the formazan product, measured with a spectrophotometer, is therefore directly proportional to the number of live cells. Direct comparisons between the results from MTT assays and ^{3}H-thymidine assays have shown that there is no significant difference between the two techniques.[9,10] We have found the MTT assay to be unsuitable for our studies on particulate biomaterials; at high concentrations the particles accumulate in the bottom of the well and interfere with the measurement of optical density.

In an attempt to overcome these problems, we decided to investigate the possibility that data from LDH assays could be used to determine cell proliferation as well as cell survival. It is unlikely that all cells contain the same amount of LDH; the cytoplasmic distribution of the enzyme and its function as an important element in oxidative metabolism suggest that the amount of LDH present within the cell will be determined by the size of the cell and by its oxidative activity. However, the amount of LDH in cells of the same lineage is likely to be very similar, if not identical. We hypothesised that if these assumptions were correct, there would be a linear relationship between the number of cells in a sample and the LDH activity present in a cell lysate prepared from this sample. This linear relationship, in the form of a standard curve for enzyme activity versus cell number, could then be used as an extremely reliable test for the quantification of cell numbers.

This paper presents the results from our investigations into the validity of using LDH activity as a marker for cell proliferation.

MATERIALS AND METHODS

Cell cultures

The murine macrophage cell line, IC-21, was obtained from ECACC (Porton Down, UK). Cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 1% penicillin–streptomycin and L-glutamine. Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every 3 days and cells split at 80% confluency.

Primary cultures of human synovial fibroblasts (HSFs) were prepared from fresh samples of normal synovium as described by Rae.[11] The synovium was washed in sterile phosphate-buffered saline (PBS) and dissected into 1 mm^3 pieces which were then seeded out onto 6-well polystyrene tissue culture plates. Cells growing out from these primary explants were removed with trypsin–EDTA and subcultured in small (25 cm^2) tissue culture flasks. Cell monolayers were grown in Dulbecco's modification of Eagle's medium (DMEM) containing 10% foetal calf serum, 1% penicillin–streptomycin and L-glutamine and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cultures were fed every 3 days and split at 80% confluency.

The human osteoblast-like cell line, SaOS-2, was obtained from ECACC (Porton Down, UK). This cell line has been studied extensively and has been shown to possess the phenotypic features of normal osteoblasts.[12] Cells were grown in McCoys 5a medium supplemented with 10% foetal calf serum, 1% penicillin–streptomycin and L-glutamine. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every 3 days and cells split at 80–90% confluency.

Determination of cell numbers and preparation of cell lysates

Medium was aspirated from 150 cm^3 tissue culture flasks and the cell monolayer washed with 10 ml sterile PBS. Cells were disaggregated by the addition of 2 ml of either trypsin–EDTA (HSFs and SaOS-2) or cell dissociation solution (IC-21) and

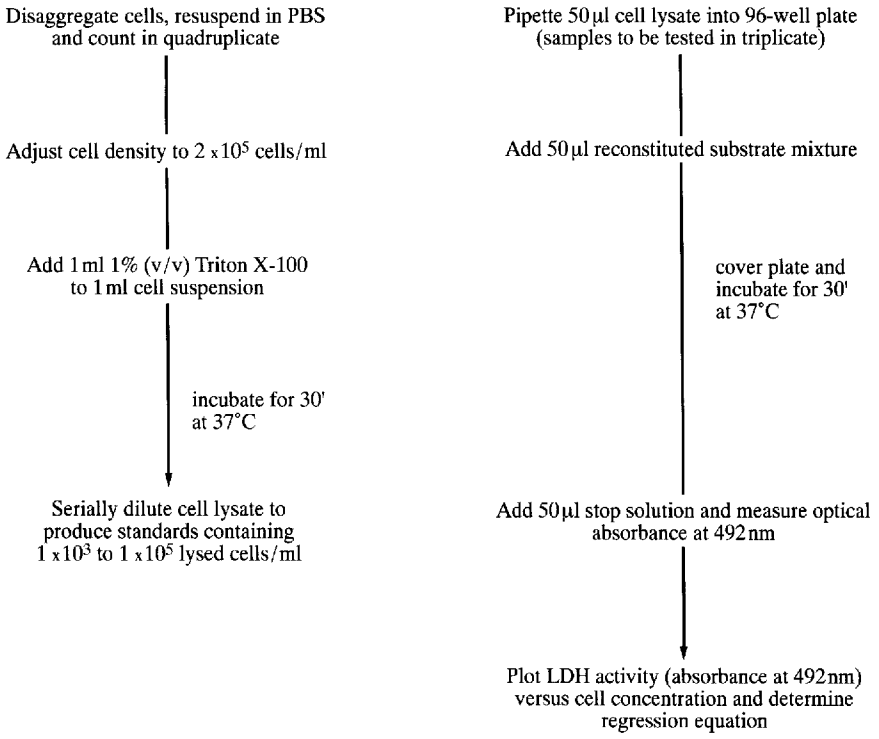

Fig. 1. Protocol for generating standard curves for LDH activity versus cell concentration.

were then resuspended in fresh PBS. Samples of these cell suspensions were stained with 0·5% trypan blue and examined in a modified Neubauer counting chamber. Viable and non-viable cells were differentiated on the basis of dye exclusion and the total number of live cells recorded in quadruplicate. The cell density was adjusted to 2×10^5 cells/ml by dilution with PBS and 1 ml of this cell suspension was added to an equal volume of 1% Triton X-100® in PBS. After incubation at 37 °C for 30 min, this cell lysate was serially diluted to give a range of standards containing from 1×10^5 to 1×10^3 lysed cells/ml.

A flow-chart for the preparation of LDH standard curves is shown in Fig. 1.

Measurement of LDH activity

The levels of LDH in samples of cell lysate were measured with a commercial LDH assay kit (CytoTox96®; Promega Corporation, Madison, WI). The basis of this kit is a coupled enzymatic reaction in which LDH present within the sample catalyses the conversion of lactate into pyruvate with the concomitant formation of NADH from NAD^+. The NADH is then used as a co-factor in the conversion of the tetrazolium salt, 2-*p*-iodophenyl-3-*p*-nitrophenyl tetrazolium chloride (INT), into a red formazan product; this second reaction is catalysed by the enzyme diaphorase, which is present within the assay substrate mixture. The absorbance of the formazan product is measured at 492 nm. Formazan concentrations are directly proportional to the concentration of LDH in the sample.[2]

Correlation between LDH activity and cell numbers

The levels of LDH in each cell lysate, expressed as absorbance values at 492 nm, were plotted against cell concentration.

Stability of LDH activity in cell lysates

Lactate dehydrogenase activity was measured in three samples of cell lysate at time zero. Lysates were then stored at 4 °C for periods of up to 1 week and LDH activities measured at intervals. Absorbance values on days 1, 4 and 7 were expressed as percentages of the control (day 0) value and results plotted against time.

Statistical analysis

Regression equations and coefficients were generated from standard curve data for LDH activity (absorbance at 492 nm) versus cell concentration.

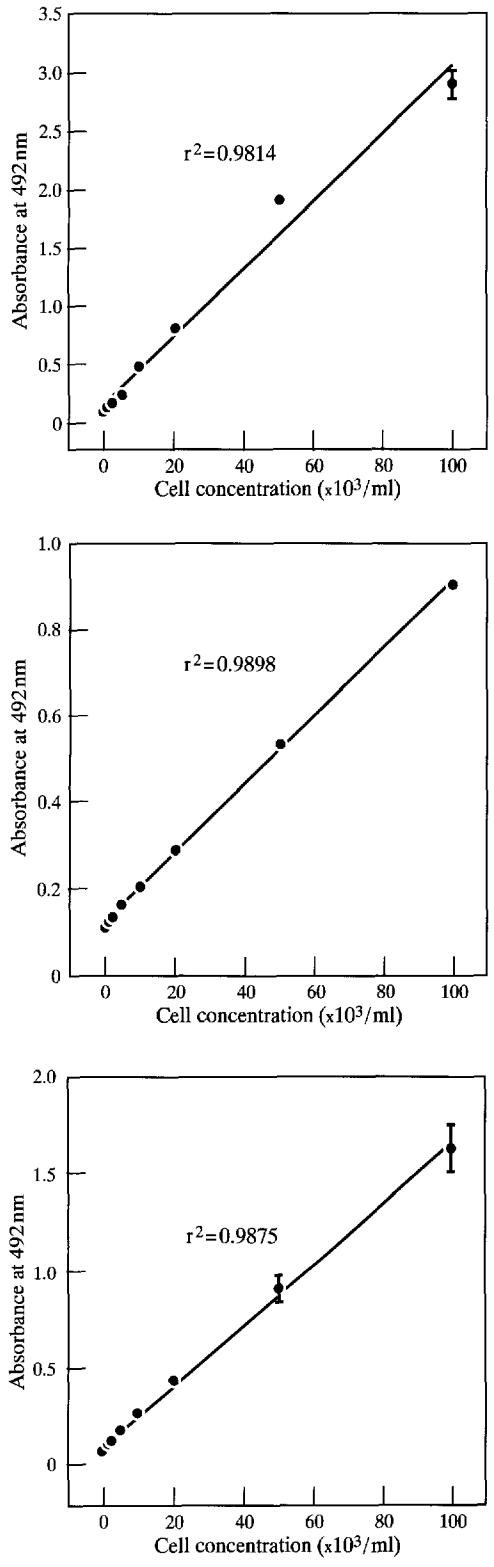

Fig. 2. The relationship between cell concentration and LDH activity (absorbance at 492 nm) in cell lysates. There is a linear relationship between the two variables for: (a) human synovial fibroblasts; (b) murine macrophages; and (c) human osteoblast-like cells. Data represent mean and standard errors of the mean for nine replicates.

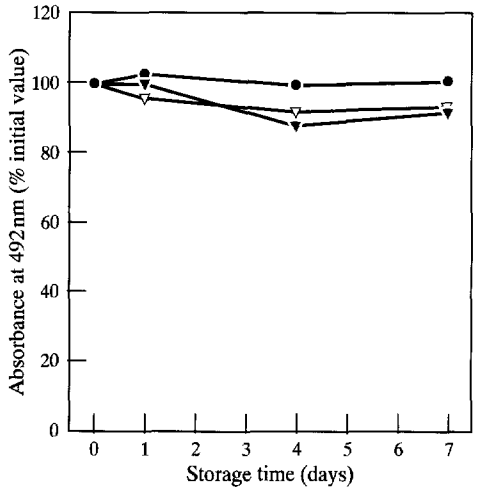

Fig. 3. The effects of storage at +4 °C on LDH activity in three samples of cell lysate. Data represent mean and standard deviations for duplicates at each time point.

RESULTS

Correlation between LDH activity and cell numbers

Linear relationships existed between LDH activity (expressed as units of absorbance at 492 nm) and cell concentration (up to 1×10^5 cells/ml) for each of the three cell types (Fig. 2(a)–(c)). Regression analysis confirmed the excellent correlation between the two variables ($r^2 = 0{\cdot}9814$, 0·9875 and 0·9898 for HSFs, SaOS-2s and IC-21s, respectively).

The maximal absorbance values and the slopes of the three curves were specific for each cell type. The levels of intracellular LDH increased in the order IC-21 < SaOS-2 < HSF.

Enzyme stability

LDH activity dropped rapidly after freezing at −20 °C (data not shown). LDH activity was stable at +4 °C for periods of up to 7 days (Fig. 3).

DISCUSSION

Accurate cell quantification is an important element of our research into biocompatibility. Since the existing techniques for cell counting are known to have limitations, we decided to investigate alternative approaches to cell quantification.

Lactate dehydrogenase assays are used routinely in our laboratory for the determination of biocompatibility and cytotoxicity *in vitro*. The commercial kit that we use for this purpose was originally marketed as a non-radioactive alternative to the ^{51}Cr

release assay for measuring cellular cytotoxicity. The results that we obtained with the assay demonstrated that the amount of LDH present in cell monolayers increased over time. When we studied the relationship between total LDH activity and cell concentration, we found a linear relationship for cell concentrations in the range 1×10^3–1×10^5 cells/ml. This relationship was valid for all cell types tested and in fact our own results with macrophages, fibroblasts and osteoblast-like cells are similar to those seen with peripheral blood lymphocytes (Moravec, personal communication). The amount of LDH present in a cell seems to be determined by cell size, with large cells having the highest amounts of enzyme. The sensitivity of the assay is such that cell concentrations as low as 1000 cells/ml (equivalent to 50 cells/well in a 96-well plate) may be detected; this compares favourably with the MTT assay, which has a detection threshold of 200 cells/well (Mosmann, 1983).

In order to use the LDH assay as a proliferation assay, a standard curve of LDH activity against cell concentration must be generated for each cell type being studied. We have found that the highest cell concentration that can be detected with the kit is 1×10^5 cells/ml. At cell concentrations above 1×10^5 cells/ml the concentration of LDH is so high that the substrate becomes rate-limiting. In addition, at higher absorbance values the accuracy of most spectrophotometers decreases, leading to potentially misleading results. If cell concentrations above 1×10^5 cells/ml are to be measured, the assay protocol can be changed in one of two ways. The easiest modification is to change the ratio of substrate to enzyme by reducing the volume of cell lysate added to the 96-well plate (use 25 μl instead of 50 μl). An alternative is to dilute the cell lysate before measuring LDH activity. Whichever technique is used, the substrate will once more be in excess, the reaction will proceed at the maximal rate and the concentration of formazan product will be proportional to the amount of LDH in the cell lysate.

Once a standard curve has been generated, it can then be used for all subsequent experiments with that batch of cells. New standard curves should be generated whenever cells are thawed, since it is theoretically possible that the levels of enzyme may be affected by prolonged storage.

Ito *et al.*[13] have also used LDH activity to monitor cell proliferation. Although the technique they describe is similar to the protocol that we have developed, it suffers from two major disadvantages. First, the spectrophotometric assay of Wroblewski and La Due[14] is time-consuming to perform, especially if a large number of samples are to be tested. In addition, the results from Ito's technique are only useful for predicting relative growth rates in test and control cultures of the same cell types; they do not produce absolute values for cell numbers. Since we express many of our test results (e.g. alkaline phosphatase activity) in terms of units per cell, a system in which absolute cell numbers are determined is clearly a prerequisite.

Lactate dehydrogenase assays can be used to determine cell viability and cell proliferation in the same experiment, without any modifications to the standard experimental design. The sole limitation of the technique is that it is only applicable to culture systems containing a single cell type. The technique is not valid for co-culture experiments in which mixed cell populations are used because each cell type is likely to contain different amounts of LDH, making the interpretation of results difficult.

In summary, this technique for measuring LDH activity provides a simple, quick and accurate method for determining cell proliferation and is ideally suited to in-vitro research on biomaterial biocompatibility.

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REFERENCES

1. Korzeniewski, C. & Callawaert, D. M., An enzyme-release assay for natural cytotoxicity. *J. Immunol. Methods*, **64** (1988) 313–20.
2. Decker, T. & Lohmann-Matthes, M.-L., A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular toxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Methods*, **15** (1988) 61–9.
3. Loughlin, J. F., Krijnen, P. M. W., Jablonsky, G., Leung, F. Y. & Henderson, A. R., Diagnostic efficiency of four lactate dehydrogenase isoenzyme-1 ratios in serum after myocardial infarction. *Clin. Chem.*, **34** (1988) 1960–5.
4. Rotenberg, Z., Weinberger, I., Fuchs, Y., Erdberg, A., Davidson, E. & Agmon, J., Elevation of serum lactic dehydrogenase levels as an early marker of occult malignant lymphoma. *Cancer*, **54** (1984) 1379–81.
5. Rae, T., The biological response to titanium and titanium–aluminium–vanadium alloy particles. *Biomaterials*, **7** (1986) 30–6.

6. Thomson, L. A., Law, F. C., James, K. H. & Rushton, N., Biocompatibility of particles of Gore-Tex cruciate ligament prosthesis: an investigation both *in vitro* and *in vivo*. *Biomaterials*, **12** (1991) 781–5.
7. Evans, E. J. & Clarke-Smith, M. H., Studies on the mechanisms of cell damage by finely ground hydroxyapatite particles *in vitro*. *Clin. Mater.*, **7** (1991) 241–5.
8. Freshney, I. R., Quantitation and experimental design. In *Culture of Animal Cells: a Manual of Basic Technique*, ed. I. R. Freshney. Alan R. Liss, NY, 1987, p. 227.
9. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65** (1983) 55–63.
10. Tada, H., Shiho, O., Kuroshima, K., Koyama, M. & Tsukamoto, K., An improved colorimetric assay for interleukin-2. *J. Immunol. Methods*, **93** (1986) 157–65.
11. Rae, T., The toxicity of metals used in orthopaedic prostheses. An experimental study using cultured human synovial fibroblasts. *J. Bone Joint Surg.*, (Br) **63-B** (1981) 435–40.
12. Murray, E., Provvedini, D., Curran, D., Catherwood, B., Sussman, H. & Manolagas, S., Characterisation of a human osteoblastic osteosarcoma cell line (SaOS-2) with high bone alkaline phosphatase activity. *J. Bone Miner. Res.*, **2** (1987) 231–8.
13. Ito, Y., Sisido, M. & Imanishi, Y., Attachment and proliferation of fibroblast cells on polyetherurethaneurea derivatives. *Biomaterials*, **8** (1987) 464–72.
14. Wroblewski, F. & La Due, J. S., Lactic dehydrogenase activity in blood. *Proc. Soc. Exp. Biol. Med.*, **90** (1955) 210–3.