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CELL ADHESION VARIATION DURING THE CELL CYCLE MEASURED USING IMMUNOLABELLING AND AUTORADIOGRAPHY TECHNIQUES

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INTRODUCTION: In the case of orthopaedic implants, one indicator of implant material compatibility is the adherence of soft tissue to the material in question. An *in vitro* investigation, initiated previously, focused on cell adhesion as a determination of 'implant surface-soft tissue' compatibility (Richards *et al*, 1997). Adherent cells (in this instance – fibroblasts) attach to substrates using unique sites known as focal adhesions. The aim of deriving a material compatibility status from quantifying the amount of focal adhesions expressed by a cell could not be met. This was thought to be due to practical shortcomings of the identification method used.

Osmium tetroxide was used to stain the focal adhesions. It primarily stains proteins; focal adhesions become distinct due to their high protein content. The staining produced inconsistent shading, thus distinguishing regions of interest using similar image analysis threshold levels could not be performed for each sample. A more specific method of immunolabelling a constituent protein of the focal adhesion was introduced. The protein chosen for labelling was vinculin, an integral component protein of the focal adhesion. The vinculin, as an antigen, is known to be highly resistant to both permeablisation and fixation methods utilised in immunolabelling. For electron microscopy purposes a gold label provides an easily identifiable marker, attached indirectly to the vinculin antigen during the labelling procedure. For image analysis reproducibility, the gold has a distinct greylevel making it easily identifiable at the 'thresholding' stage.

In the study by Richards *et al* (1997) cell adhesion patterns varied considerably on the same substrate, making the quantification of the cells' adhesion area unreliable. Observations have been made connecting adhesion variation to cell morphology and to cell cycle position. Hunter *et al* (1995) found cells with the greatest number and area of focal adhesions were well spread and flattened whilst those with the least number of focal adhesions were more rounded and less spread. Cross & ap Gwynn (1987) showed that for cell-cell adhesion, cells within the S-phase of the cell-cycle were most adhesive. Associated to what is known about the cells' morphology (grown on a substrate) during the cell cycle, it can be proposed that the cells' adhesion area will change radically during the cycle – primarily a marked increase in adhesion area density of cells in S-phase.

The goal of this study was to continue the development of the *in vitro* cell adhesion quantification test to remove variability from staining methods and the influence of the cell cycle. In addition, S and non S-phase cell adhesion was investigated using the developed immunogold labelling methods.

MATERIALS & METHODS: Swiss Balb c/3t3 fibroblasts were cultured on Thermanox (Polyethylene tetrapthalate) discs in DMEM media supplemented with 10% foetal calf serum at 37°C for 2 days. S-phase cells were labelled by a pulse of ^{3}H thymidine (2nCi/ml) in the culture medium for 30min. Cells were cultured for a further 2 hours in normal media before being processed for immunogold labelling of vinculin. For scanning electron microscope (SEM) visualisation of the adhesion sites on the whole cell, the gold label was enlarged with gold enhancement. Postfixation and staining was performed with osmium tetroxide. Samples were dehydrated through a graded ethanol series and critically point dried. For autoradiography (Owen *et al*, 2000) the discs were coated with 4nm of carbon by evaporation, and then covered with a thin layer of photographic emulsion in a dark room. They were left in a light tight box at 4°C for 7 days before developing the emulsion. Samples were embedded in LR White resin and the substrate was removed. The undersides of cells were imaged using backscattered electron (BSE) imaging. A general view of the cells at a high accelerating voltage (kV) visually differentiated S phase from non S-phase cells (the S-phase cells had distinctly labelled nucleuses). A lower kV was used to visualise the immunolabelled focal adhesions of the identified cells.

RESULTS & DISCUSSION: The focal adhesion sites were clearly marked with the gold label and the higher density of gold distinguishes it from any other element or stain (*e.g.* osmium) present within the cell (see *Figure 1*). An increased precision of

the quantification is attained as the probe only labels one constituent of the focal adhesion, unlike the osmium stain, which marked all the proteins within the focal adhesion. The labelling revealed that vinculin is also found in locations other than the focal adhesion, such as near the nucleus deeper within the cell body. At high kV the cells embedded in resin showed labelling throughout (see *Figure 2 (a)*), while the low kV of the same sample revealed labelling from only the area where the cell was in contact with the substrate (see *Figure 3 (b)*).

Image analysis results were calculated to assess cell adhesion density. The value was a percentage calculation of the adhesion area quantified in relation to the actual cell/substrate contact area. The results showed that the S-phase cells had significantly less adhesion density than non S-phase cells (1.3 ± 0.1 vs. 2.3 ± 0.2, mean $\pm$ S.E, $p=0.002$). It was also observed that the adhesion density varied significantly less for S-phase than non S-phase cells.

For *in vitro* compatibility tests comparing cellular adhesion on varying surfaces and materials, it should be considered to only quantify cells identified to be in the S-phase, due to the narrower distribution of results. Currently, we are investigating various biomaterials using this immunolabelling method.

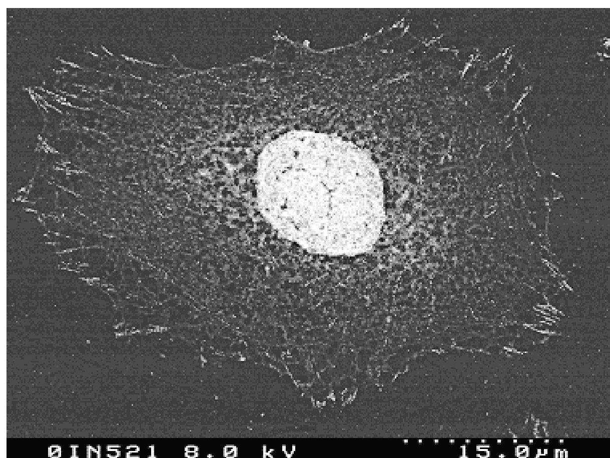
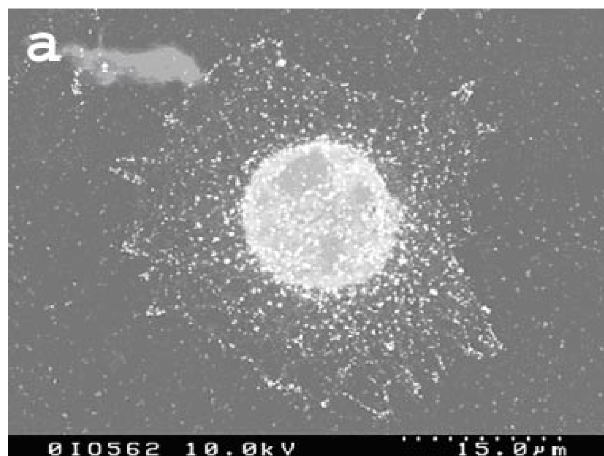


Figure 1. Adhesion sites of a fibroblast visualised by immunogold labelling of the vinculin. In this spread cell the label is mainly observed at the periphery.



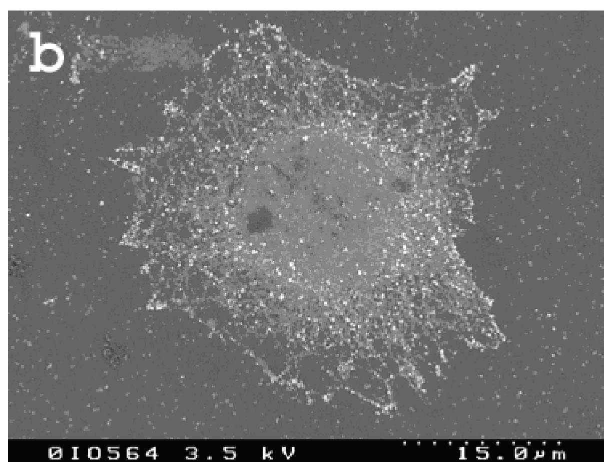


Figure 2(a) A fibroblast, immunolabelled with gold markers at the adhesion sites, can be identified as being in the S-phase by the silver deposit situated directly above the nucleus. The cell was imaged at high kV, so both the immunolabel and autoradiography label are visible. (b) The same cell imaged at a lower kV, in this case only the gold label is visible on the ventral surface of the cell.

REFERENCES:

Cross & ap Gwynn (1987) Cytobios 50, 41-62.

Hunter *et al* (1995) Biomaterials. 16, 287-295.

Richards *et al* (1997). Cells and Materials 7,15-30.

Owen *et al* (2000) European Orthopaedic Research Society, 10,135.