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SCANNING X-RAY MICROANALYSIS OF MICROCARRIER CULTURED ENDOTHELIAL CELLS: ELEMENTAL CHANGES DURING THE TRANSITION TO CONFLUENCY AND THE EFFECT OF IONOPHORE A23187

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Abstract

Porcine endothelial cells were grown on microcarrier beads and examined by scanning electron microscopy (SEM) at various times after initiation of culture. Total cell coverage on the bead surface varied from mean values of approximately 7% (3h) to 80% (96h). Beam penetration into the subcellular matrix presents a major problem with SEM X-ray microanalysis of microcarrier cultured cells and necessitates the use of an accelerating voltage not exceeding 10kV. At this voltage and below, X-ray contribution from elements present in the microcarrier bead has minimal effect on the determination of cell elemental levels. Washing the cells with 0.15M sucrose was the least perturbing of the rinsing techniques investigated. removing surface culture medium but not internal diffusible ions.

X-ray microanalysis revealed detectable levels of Na, P, S, Cl, K and Ca in the cells, with well-marked changes from initial attachment to confluency. The level of K decreased from approximately 1.0% at 3h to 0.4% at 24h, with a corresponding decrease in the K/Na ratio. This unexpectedly low level of K was invariably observed after 24h, and is a genuine feature of established microcarrier culture.

The effect of ionophore A23187 was determined at the 3h culture stage, and resulted in significant increases in the concentration of divalent cations (Mg^{2+}, Ca^{2+}), monovalent ions (Na^{+}, Cl^{-}) and a decrease in the level of K^{+}.

Key Words: Endothelial cells, Microcarrier cultures, Cations levels, K/Na balance.

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Introduction

Cultured cells provide a useful experimental model for the study of cell physiology and the possible biochemical effects of drug treatments. Such in vitro systems provide an alternative to the use of whole animals, and the observation of one cell type in a culture model allows for a more thorough understanding of that cell type in isolation from the influences of interactions with other cells which occur in vivo.

The growth of cells on bead surfaces (microcarrier cultures) cells can be particularly useful. Such cultures have been widely used for in vitro studies, since large numbers of cells can be grown in highly reproducible conditions for easy sampling and manipulation of cells in situ on the bead substrate. During microcarrier culture, the cells undergo marked physiological changes as they progress from an actively dividing to a quiescent state (James-Kracke et al.,1980). Microcarrier culture of endothelial cells is well-established and has been used in the past for virus production (Giard et al., 1977), as a model for investigating the microcirculation (Busch and Owen,1982; Gryglewski et al.,1986), blood cell aggregation (Brigham et al.,1984) and blood endothelial cell interactions (Brigham and Meyrick,1984).

In this study, microcarrier cultures of endothelial cells are used for the investigation of changes in elemental composition by X-ray microanalysis. In general, the use of X-ray microanalysis with cultured or isolated cells can involve a range of preparative techniques and experimental approaches (Zierold, 1991), and the application of this technique to single cells on the surface culture beads has both advantages and disadvantages. One of the major advantages of carrying out scanning electron microscope (SEM) X-ray microanalysis on microcarrier cultures lies in the ease and quality of specimen preparation. The

occurrence of cells as a single layer on the surface of microcarrier beads is particularly useful, since it enables rapid freezing, with optimal retention of cell constituents. Identification of cells on the bead surface is simple, and there is no interference from other cells. The major disadvantage of microcarrier cultures for SEM microanalysis lies in the danger of electron probe overpenetration into the bead matrix, and it is important to adopt operating conditions that minimise this.

Preliminary X-ray microanalytical studies of the endothelial/microcarrier system cells revealed a range of detectable elements, with unexpectedly low levels of potassium at a confluent stage of growth (Hall, 1990). The present work was carried out to provide a more detailed assessment of the applicability of using SEM X-ray microanalysis with the endothelial/microcarrier system. The specific objectives of the research were to determine optimal specimen preparation and microscopical parameters for X-ray microanalysis, determination of elemental changes during the progression to confluency and use of the microcarrier model as a test system to monitor the effect of compounds that perturb cellular ionic composition.

Materials and Methods

Cell culture

A pellet of porcine thoracic aortic endothelial cells in a small volume of Dulbecco's Modified Eagle's Medium (DMEM) (GIbCo, Paisley, UK) containing L-glutamine (1g/ml), 25mM HEPES, 20% foetal calf serum (Flow ICN, Rickmansworth, UK), penicillin/streptomycin and gentamycin solution (GIbCo), was added to a microcarrier bottle (Techne, Cambridge, UK) containing 200 mls of DMEM and 0.7g of 'Cytodex' microcarrier beads (Pharmacia Fine Chemicals, Uppsala, Sweden). These beads are 133-215 μm in diameter and have a surface layer of purified, sterilised, denatured pig-skin type I collagen, which provides a surface for cell adherence and active growth (Reid & Rojkind, 1979). The bead culture was incubated at 37 C for 4h, with stirring for 5min in every 30 min, to allow for cell attachment. After 4h, the microcarrier culture was continuously stirred at 40rpm and incubated at 37 °C, for 96h, with sampling at specified times.

Ionophore experiment The effect of ionophore A23187 (Sigma, UK) was tested by thoroughly mixing 1ml of ionophore solution (in DMEM medium) with 3ml of 3h bead culture to make a final ionophore concentration of 0.1μM. Cells were harvested after 5 min treatment and processed for X-ray microanalysis as described below. Control preparations, with the addition of 1ml of DMEM without ionophore, were processed and tested in parallel.

Preparation of samples

10ml aliquots of cell culture medium containing beads were taken at 3h, 24h, 48h, 72h and 96h after the initial incubation period, and samples processed for scanning microscopy either by chemical procedures (for fine structural observation) or cryopreparation (for X-ray microanalysis).

Chemical processing Samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (0.1M) at room temperature for 1h, washed in buffer then postfixed in 1% buffered osmium tetroxide (1h). Samples were then washed in buffer, dehydrated in an acetone series to 100% acetone, freeze-dried and gold coated.

Washing and cryopreparation Each sample was pipetted onto a 5μm nuclepore polycarbonate filter (Nuclepore Corporation Ltd.), rapidly washed and quench-frozen.

Washing procedures. Washing is essential to remove all traces of the growth medium surrounding the cells before freezing, otherwise this will remain on the cell surface and yield medium-derived peaks in the X-ray emission spectra which will confuse interpretation of those from the cultured cells.

Several washing procedures were investigated to find an optimum protocol for removal of growth medium with retention of internal ion levels. Each washing step was carried out at 37°C and took 4s. The washing solutions were pipetted over the filter membrane bearing the microcarrier preparations, which was then immediately quench-frozen and freeze-dried, as described below.

The washing solutions tested were:
(1) 1ml 0.1M $MgCl^2$ in 10mM HEPES/Tris buffer, pH 7.4. This wash was repeated and followed by a third consisting of 1ml 0.15M ammonium acetate. Tris buffer, pH 7.4 (Rabito et al.,1987).
(2) 1ml 0.1M $MgCl_2$ in 10mM HEPES/Tris buffer, pH 7.4, followed by 1ml 0.15M sucrose.
(3) 1ml 0.15M sucrose.
(4) Control: 1ml DMEM

Cryopreparation. Microcarrier prepar-ations on filters were quench-frozen in melting Freon at liquid nitrogen temperature, freeze-dried at -80°C in an Edwards 150 Pierce tissue-drier overnight then mounted and carbon-coated in preparation for SEM X-ray microanalysis.

Microscopy and quantitation

Assessment of bead coverage The area coverage of cells over bead surfaces was

determined at each stage of the 96h time-course from SEM micrographs that were taken randomly and at constant magnification. Perspective at the edge of the bead precluded simple area measurement from the complete bead image, therefore measurements of cell coverage were restricted to the central area of each bead circumscribed by 2/3 of the radius. Cell coverage was expressed as a proportion of full confluency (ie. % total bead area) and was determined as a mean value from 15 separate beads at each sample time.

X-ray microanalysis X-ray micro-analysis was carried out using the Cambridge Stereoscan 360 SEM fitted with a Link Analytical detector and AN 10,000 analyser (LINK ANALYTICAL Ltd., High Wycombe, UK). Analyses were performed with a range of accelerating voltages between 7.5kV to 25kV. Livetime was maintained at 100s and the working distance kept at 25mm. Individual areas of interest were probed in the spot mode, with the probe area covering a major part of an individual cell (including nucleus and cytoplasm), and count rate was kept at 1000 c.p.s. by adjustment of the probe current to 1-2nA.

For each sample, the mass fraction of individual elements was calculated as the mean value from at least 15 cells (on separate beads), provided that the elemental peak was significantly greater than the continuum in at least two thirds of all spectra. Differences between mean mass fractions were statistically analysed using Duncan's multiple range test at the 95% level of significance.

Quantitation. Quantitative determin-ation of elemental composition of both cultured cells and gelatin standards (see below) was carried out using the LINK SYSTEMS ZAF/PB correction program, with reference to stored inorganic standards for the elements of interest. Elemental profiles were stored on disk and from these, peak/continuum ratios were calculated within the characteristic peak areas. Energy calibration was made each time with reference to a cobalt standard.

Accuracy of the quantitation was confirmed by reference to secondary gelatin standards. These were prepared by separate addition of different inorganic salts (Na_2HPO_4, $MgSO_4$, KCl & $CaCl_2$) to dialysed 20% gelatin (porcine Type A, SIGMA). 100µl droplets of the mixture were deposited onto SEM stubs, followed by quench-freezing, freeze-drying and carbon-coating as previously. The final dry weight concentrations of the test elements varied from 3 - 13%, depending on the particular element concerned. Mean elemental mass fractions calculated from these standards were normally correct to within 2% of the known elemental levels.

Beam penetration. Penetration of the electron beam into the subcellular (bead) matrix is an important source of potential artefact when analysing monolayer cultures, since the cell layer is so thin (about 5µm). The effect of beam penetration was determined by:

(1) Comparison of changes in elemental ratios of microcarrier-cultured cells between 15 and 7.5kV. Beam penetration was examined by determination of specific elemental ratios, which were corrected for the differential effect of different kV settings by adjustment of the net integrals in reference to bulk agar/gelatin standards containing relatively high levels (about 20%) of the test elements.

(2) X-ray analysis of endothelial cells on a matrix containing a marker element. Specimens were prepared by allowing endothelial cells in suspension to attach to the surface of carbon-formvar nylon grids (under similar conditions to the initiation of microcarrier-bead cultures) followed by freezing, freeze-drying and carbon coating as previously. X-ray emission spectra taken from the endothelial cells at different kV settings were then compared with spectra taken directly from grid material to determine the presence or absence of extraneous marker elements.

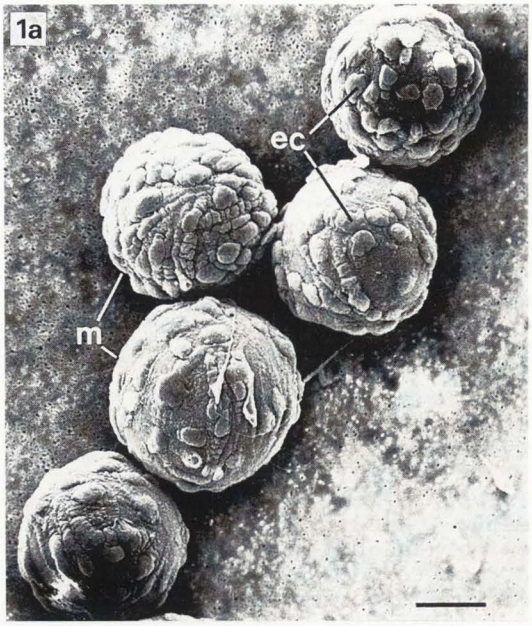
Results

SEM observations of endothelial cell growth during the culture period

The characteristic appearance of microcarrier preparations is shown in Fig.1 (chemically processed cells) and Fig.2 (freeze- dried cells). Differences in image definition between these two types of preparation can largely be accounted for by the use of carbon rather than gold coating in the freeze-dried material.

The appearance of individual microcarrier beads showed marked changes during the culture sequence. Three hours after the addition of endothelial cells to the microcarrier bottle, cell attachment is sparse, with areas of the microcarrier bead clearly exposed. By 48h, much of the surface of the bead is covered by endothelial cells, which have an elongated appearance with a smooth surface (Fig.1). After 96h, endothelial cells typically have a more complete coverage of the microcarrier beads and display the typical cobblestone morphology of a monolayer of cells in confluence (Fig.2).

Quantitative changes in the area cover over the four day sampling period are shown in Fig.3. Although the coverage of cells for beads within an individual sample was variable (see, for example,



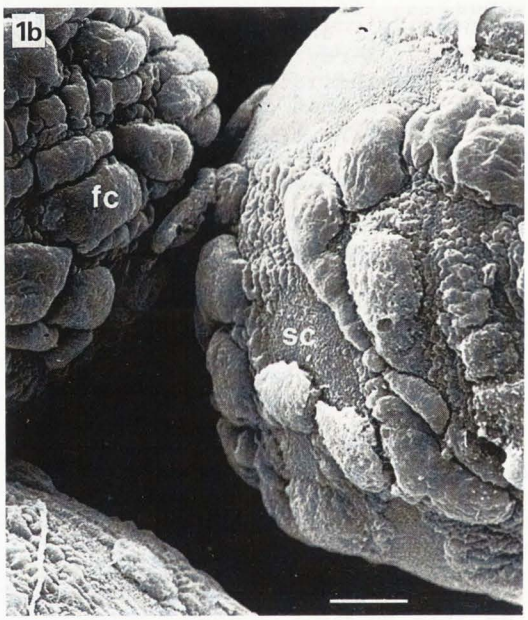


Fig.1 Scanning electron micrographs of cytodex beads with surface endothelial cells in a chemically-processed microcarrier preparation (48h sample).
(1a) Low power view of a group of beads deposited on a filter membrane. m - microcarrier beads, ec - endothelial cells.
(Bar = 40μm)
(1b) Detail from two adjacent beads, one of which has an area of cells in a fully-confluent (fc) state, while the other has sub-confluent cells (sc).
(Bar = 10μm).

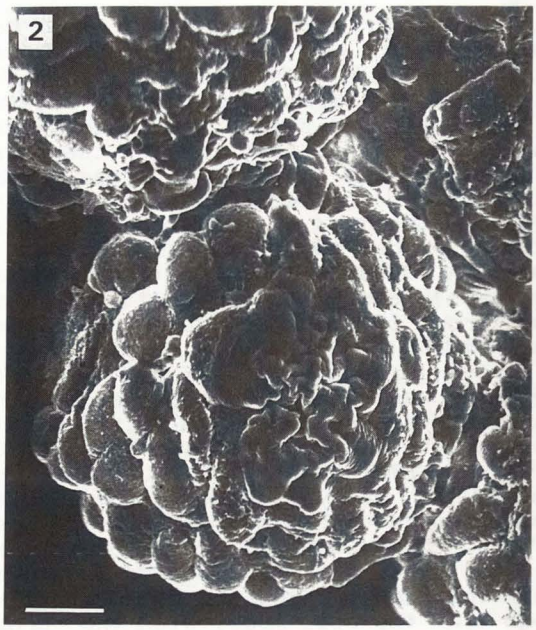


Fig.2 Freeze-dried preparation, showing fully confluent cells from a 96h sample.
(Bar = 10μm).

Fig.1b), mean values showed a clear and linear increase upto a mean value of approximately 80% cover at 96h.

X-ray microanalysis of microcarrier bead cultures: establishment of optimal preparation and operational parameters

Qualitative data Although the emission spectra obtained from the endothelial cells (Figs. 4 and 5) showed considerable variation in relation to specimen preparation, sample time and accelerating voltage of the probe, a number of elements were routinely detected - including Na, P, S, Cl, K and Ca. Emission spectra taken from the bead matrix (Fig.4c) invariably had a high Cl peak, plus a small peak of Na.

Quantitative data Accurate determination of elemental concentrations in the endothelial cells depends not only on the use of valid quantitation procedures, but also on the use of correct cell preparation techniques (particularly washing conditions) and the employment of appropriate microanalysis parameters (particularly probe voltage).

Optimal washing conditions. The elemental composition of 3h cells that have been washed in various media is shown in Table 1. This sampling point is particularly useful for the assessment of washing, since the cells have an optimal level of K at this stage (see later), and the differential removal of Na and Cl but not K during washing should be especially clear in this sample.

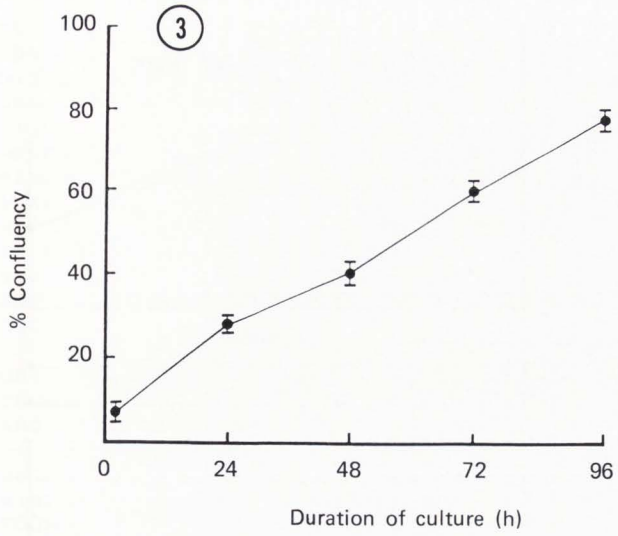


Fig.3 Changes in coverage of bead surface during the culture period.
Increasing cell confluency is expressed as percentage cell cover against sampling time.

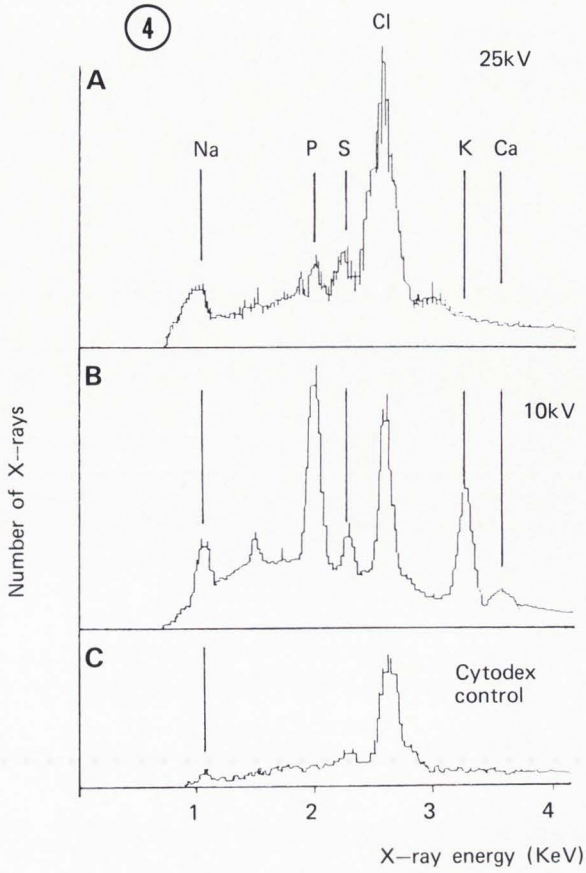


Fig.4 X-ray emission spectra obtained from unwashed endothelial cells on microcarrier beads, collected at 25kV. 10kV and from a region of exposed microcarrier bead at 10kV.

Table 1 Effect of different washing media on the elemental composition of endothelial cells

	Na	P	S	Cl	K	Ca
(1) Control	2.4 ±0.3	1.3 ±0.5	0.7	3.1 ±0.4	1.2 ±0.7	0.2
(2) $MgCl_2$/ Amm acet	2.3 ±0.2	0.9 ±0.2	0.7 ±0.1	3.3 ±0.6	0.2 ±0.1	0.1
(3) $MgCl_2$/ Sucrose	1.5 ±0.3	1.4 ±0.1	2.3 ±0.2	2.5 ±0.3	0.3 ±0.1	0.1
(4) Sucrose	0.5 ±0.1	1.6 ±0.2	0.5	1.4 ±0.4	1.0 ±0.1	0.1

Mean values are derived from 10 separate analyses, with 95% confidence limits.
Duncans Multiple Range Test Pairs of samples significantly different at the 95% probablity level were:
Na - 1(3,4): 2(3,4): 3(1,2,4): 4(1,2,3)
P - 2(3,4)
S - 1(3): 2(3): 3(1,2,4): 4(3)
Cl - 4(1,2,3)
K - 1(2,3): 2(1,4): 3(1,4): 4(2,3)
Ca - no significant differences
(Where 1 - 4 refer to the control plus three washing procedures)

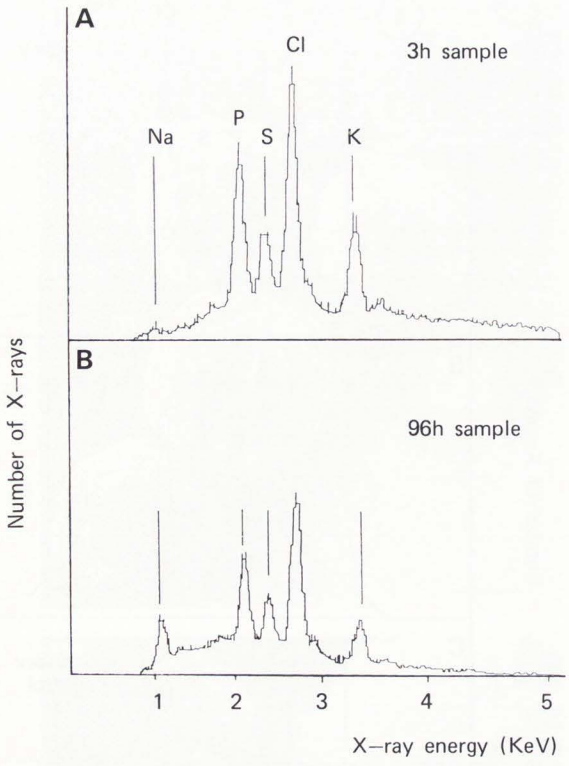


Fig.5 X-ray emission spectra from sucrose-washed microcarrier preparations sampled at 3h and 96h after initiation of culture. Accelerating voltage - 10kV.

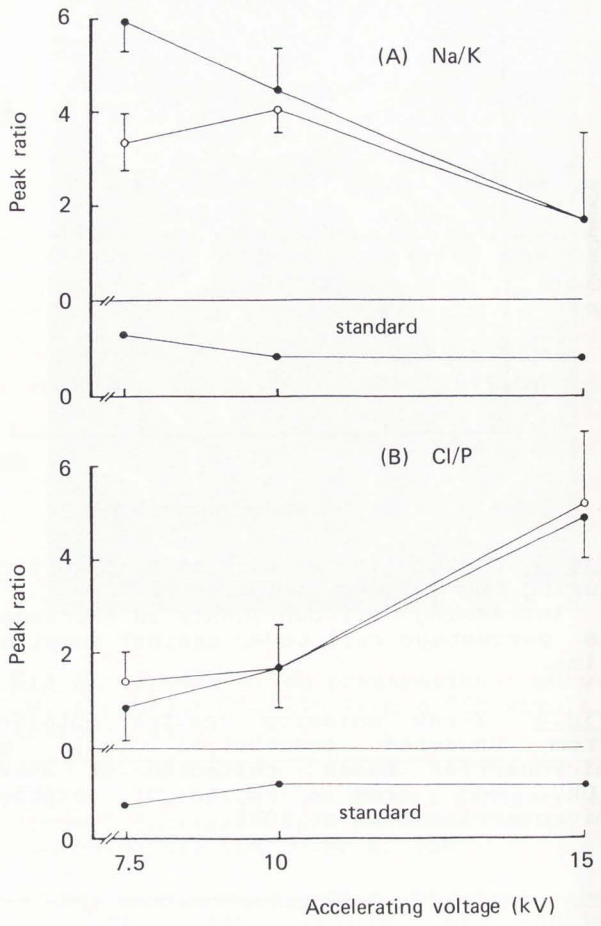


Fig.6 Effect of accelerating voltage on elemental ratios.
(6a) Shows the non-corrected (-●-●-●-) and corrected (-o-o-o-) Na/K ratio. The latter is derived by multiplying the direct values by ratios taken from an Na-K gelatin standard. There is no significant difference in the adjusted Na/K ratio as accelerating voltage is reduced from 15 to 7.5kV.
(6b) Changes in the Cl/P ratio are illustrated and derived as in 6a. The Cl/P ratio shows a significant fall from 15kV to 10kV, but levels off below 10kV.

All ratios are derived from integral means derived from samples of at least 10 spectra.

The results show that washing the cells with sucrose solution leads to a significant and substantial loss of elements that are present at high level in the growth medium (Na and Cl), but other elements (particularly P, S and K) that are present mainly in the cells are unaffected. Washing in sucrose thus has no significant affect on either the mass fractions or the relative proportions of these constituents (elemental ratios) within the endothelial cells. The loss of Na and retention of K during washing is shown clearly by comparing Figs. 4b and 5a. These ionic changes are consistent with the removal of excess medium from the surface of cells without perturbing the ionic integrity of the cell. In contrast to this, treating cells with $MgCl_2$ washes (combined with ammonium acetate or sucrose) causes major loss of K without removing surface medium.

Accelerating voltage and beam penetration. During bulk analysis, the degree of beam penetration is dependent on both the specimen density and the primary energy, with a decrease in penetration as the accelerating voltage is reduced (Zs-Nagy et al., 1977; Boekestein et al., 1980; Wroblewski et al., 1987). In the case of the endothelial cell monolayer, the low density of cells in the freeze-dried state and the shallow depth of the cells (approximate thickness, 5μm) both result in the likelihood of penetration of the beam through the endothelial cells into the bead matrix.

The importance of accelerating voltage in the X-ray microanalysis of microcarrier-cultured cells is illustrated in Fig.4, which shows the appearance of X-ray emission spectra from unwashed cells

at 25kV and 10kV. Similar results were obtained with washed preparations. At the higher voltage, overpenetration of the beam into the bead matrix results in a high Cl peak (largely derived from the matrix) and reduced peaks of cellular elements such as Na, P, S, K and Ca. At 10kV, where X-rays are generated mainly within the endothelial cells, the Cl peak is not so pronounced and cellular elements are much more defined. Clear peaks of K and Ca were normally observed at 10kV but not 25kV.

The importance of accelerating voltage on beam penetration was further investigated in terms of elemental ratios and use of a matrix with a marker element.

Elemental peak to peak ratios were measured for window integrals of the elements Na/K and Cl/P - where K and P are found exclusively in the cell, Na is present largely in the cell, and Cl is present at substantial levels in both cell and bead matrix. These ratios are shown in Fig.6 at different accelerating voltages, and are presented both as direct ratios and ratios that have been adjusted to take account of the differential effect of voltage change. This results in an increased peak size of low atomic number elements analysed at lower kV, and a reverse effect at high kV. Adjustment was achieved by normalising the elemental ratios in relation to bulk organic standards (Fig.6).

The corrected Na/K ratio does not show any significant change with alteration in kV (Fig.6a), which is consistent with the occurrence of these elements almost entirely within the cells. In contrast to this, the ratio of Cl/P shows a steep and significant decline from 25 to 10kV (Fig.6b), consistent with a reduced penetration at lower kV's into a Cl-rich bead matrix. The Cl/P ratio at 7.5kV did not differ significantly from the value at 10kV, suggesting that only minimal beam penetration into the bead occurs at these low kV levels, making 10kV an ideal setting for microcarrier cells.

X-ray analysis of endothelial cells on a marker-matrix involved the use of cells attached to the surface of nylon grids. These showed a close similarity in terms of cell size and general morphology to cells of microcarrier bead preparations. X-ray emission spectra from single cells lying over grid bars also showed a similar range of elements to microcarrier cells, with corresponding spectral differences in relation to the electron probe voltage. X-ray emission spectra taken from cells at 25kV, and directly from grid bars, invariably showed a clear peak of Ti, derived from the nylon matrix. No Ti peak was observed at voltages of 15kV or below, suggesting that overpenetration of the beam was minimal at these lower voltages.

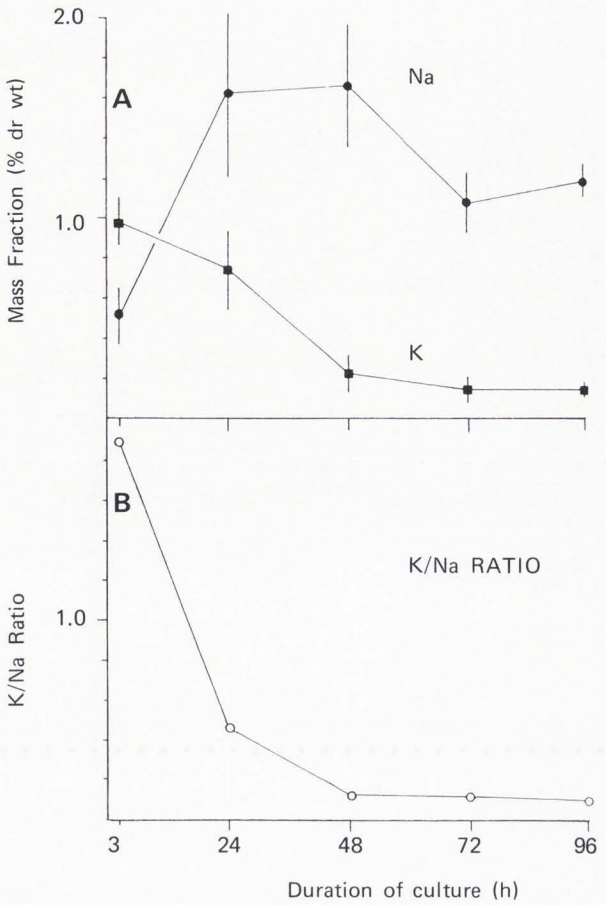


Fig.7 Changes in the level of Na and K during culture, and alteration in the K/Na ratio. Mean values are derived from 15 separate cells, and are given with 95% confidence limits. Sucrose-washed preparation.

Changes in the elemental composition of endothelial cells during the transition to confluency

The major cellular elements noted in the previous section were present at detectable levels throughout the culture sequence, as illustrated in Fig.5, which shows typical examples of X-ray emission spectra taken at 10kV from the 3h and 96h samples. Changes in mean elemental mass fractions during a typical culture period are shown in Figs. 7-9, and were as follows:

Sodium The mass fraction of Na varied between 0.5 and 1.7% dry weight (Fig.7a), with an initial rise between 3h and 24h before levelling off to a constant value.

Potassium The level of potassium fell significantly between 3-48h, from 1.0% to 0.4% (Fig.7a), with no significant variation after this point.

K/Na ratio Changes in the ratio of these major monovalent cations (Fig.7b)

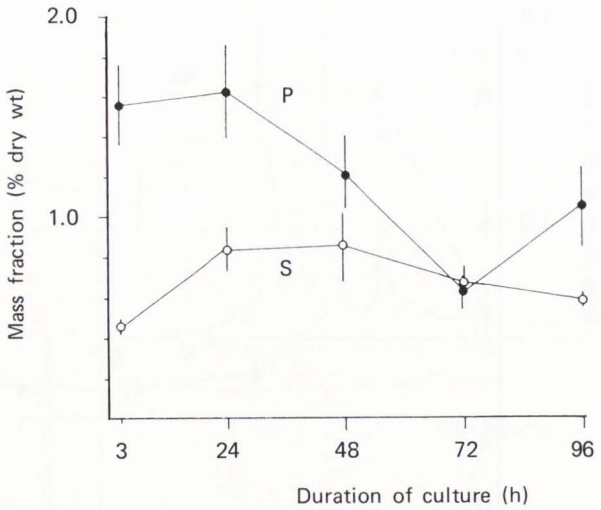


Fig.8 Changes in the level of P and S during culture. Legend as in Fig.7.

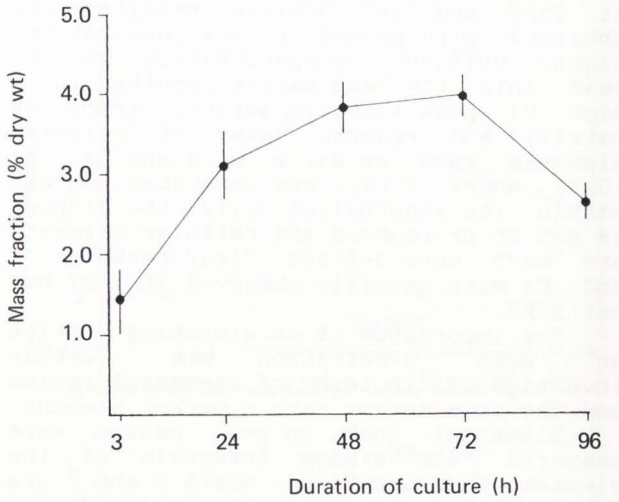


Fig.9 Changes in the level of Cl during culture. Legend as in Fig.7.

Table 2 Effect of ionophore A23187 on the ionic composition of 3h microcarrier-cultured cells

	Na	Mg	P	S	Cl	K	Ca
Control	0.6 (±0.1)	0.1	0.8 (±0.2)	0.5	2.2 (±0.3)	0.9 (±0.2)	0.1
A23187 treated	1.2* (±0.3)	0.3* (±0.1)	0.9 (±0.2)	0.6 (±0.1)	3.0* (±0.3)	0.1*	0.6* (±0.3)

* Significantly different from control cells at 95% probability level.

show a steep fall during the 3-48h period, in line with changes in the level of both elements during the early part of the sequence. The ratio remains low, with no significant change for the remainder of the period.

Phosphorus The mass fraction of P showed a significant fall between 24-72h, followed by a gradual rise during the rest of the sequence (Fig.8).

Sulphur The level of S varied over a small range (between 0.4 to 0.9%) with, with little change during the course of the experiment (Fig.8).

Chlorine The Cl mass fraction varied between 1.5% and 4.0%, rising to a peak value at 72h, with subsequent decline.

Microcarrier cultures as an experimental test system - the effect of ionophore A23187

The use of the microcarrier bead cultures to monitor the effect of agents that perturb the ionic balance of the cell was examined using ionophore A23187. Table 2 shows the effect of a 5 min treatment of this compound on a 3h cell culture compared to untreated (control) cells.

Ionophore treatment resulted in significantly increased levels of divalent cations (Mg^{2+}, Ca^{2+}), monovalent ions (Na^{+}, Cl^{+}) and a significant reduction in the concentration of K^{+}.

Discussion

In this study, microcarrier cell cultures have been used in conjunction with SEM X-ray microanalysis to develop an

in vitro system for monitoring changes in endothelial cells caused by pharmacological and cell-derived agents. The work provides information on the selection of optimal conditions for specimen preparation and X-ray microanalysis. Changes in elemental composition during culture and the effects of ionophore A23187 as a test compound were also examined.

Specimen preparation

Microcarrier samples can be removed from the culture vessel with minimal physical disturbance to the cells, and with no need to carry out any centrifugation steps. Great care must be taken, however, in the removal of culture medium during subsequent washing. During this process the medium around the cells must be removed without causing loss of internal diffusible elements. Of the washing solutions tested, only 0.15M sucrose met these criteria, removing substantial amounts of Na and Cl (present mainly in the growth medium) but causing no significant change in the level of K (present mainly in the cell). The successful use of sucrose as a washing agent for cultured mammalian cells has been previously reported for various cell types, including smooth muscle cells and chondrocytes (Wroblewski et al.,1983). These authors additionally report the damaging effect of ammonium acetate as a washing agent, causing loss of K from the cell - as also do James-Kracke et al.(1980) for cultured aortic smooth muscle cells.

Electron probe penetration

Beam penetration through the cell monolayer presents a major problem, since the generation of X-rays from the underlying bead matrix results in the combined production of extraneous (bead matrix) peaks and reduced detectability of cellular elements. The results of beam overpenetration are demonstrated very clearly in this study, which shows the need to reduce the accelerating voltage to 10kV or less. Reduction of the accelerating voltage to this value leads to a levelling of the Cl/P ratio, suggesting that probe penetration at 10kV is almost entirely contained within the cell. An accelerating voltage of 10kV also provides a sufficient overvoltage for detection of the elements K and Ca, whereas 7.5kV may not provide sufficient excitation to detect these and other biologically important elements (Zs-Nagy et al.,1977). The use of whole cells overlying a marker matrix (nylon) as part of the penetration-testing procedure follows the approach of Clay et al.(1991), who were examining overpenetration of single algal cells.

Changes in elemental composition during culture

The most conspicuous change in elemental composition during culture occurred during the early stages of bead colonisation, with a sharp fall in the level of K and a corresponding rise in the level of Na. The restriction of a high K/Na ratio to the earliest sampling times was unexpected, but was repeatedly demonstrated during successive experiments with both washed and unwashed preparations, and is a genuine feature of the endothelial cell/microcarrier system. The low level of K^+ (0.4% dry mass) over most of the sampling period, in particular, contrasts with the results obtained by other workers for animal cells (both *in vivo* and *in vitro*), where levels in the range 1.6-2.7% dry mass are normally obtained (Zierold, 1981; Warley et al.,1983; Wroblewski et al.,1983; Warley,1986). The increased level of Na^+ during establishment of the microcarrier culture is paralleled by an increase in the level of Cl^-, suggesting that these ions are balanced within the cell during the first 48h. Close correlation in the intracellular occurrence of these two ions has also been noted in other X-ray microanalytical studies on cultured mammalian cells (see, for example, Warley,1986) and on prokaryote cells (Sigee and Hodson, 1992), and may be of general significance. Fluctuations in other elements appear to be more variable between experiments, suggesting that more specific factors may determine their changes within individual cultures.

Elemental changes that occur during cell culture presumably reflect changes in cell stress and physiology during the sampling sequence. In early culture, the cells are actively growing and spreading, but once they have grown to confluency they enter a quiescent phase, during which cell function and viability may vary. Changes in the elemental composition of cultured cells during the transition to confluency have also been reported by Proll et al.(1979), who showed that confluent fibroblast cells had markedly lower levels of a range of cations (including K^+) after the active phase of mitosis and DNA synthesis that occurred during subconfluence.

Microcarrier cultures as experimental test models

In these endothelial microcarrier preparations, the earliest pre-confluent culture stages were considered the most suitable for metabolite testing. Only these cells had K and Na levels that were comparable to other animal cells, and only in these cells was the level of K sufficiently high for changes to be clearly monitored.

In accordance with this, testing of

ionophore A23187 was carried out on 3h-cultured cells, and demonstrated a clear influx of the cations Ca^{2+} and Mg^{2+}, as expected with this compound. Changes in the balance of Na/K balance also occurred, possibly reflecting some nonspecific cell membrane damage, and a rise in the level of Cl occurred in parallel to the sodium changes - in line with previous observations on correlation between these two ions.

In general, the studies reported in this paper suggest that microcarrier culture systems may have considerable potential in testing for compounds that perturb the ionic composition of cells. We have shown that elemental levels in the cultured endothelial cells vary considerably, and it is clearly important to be sure of the physiological status of the particular cells under test. Once the cell culture and microscopical parameters of microcarrier preparations in general are better understood, these should prove to be ideal models for pharmacological testing.

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Discussion with Reviewers

A. Warley : What exactly was the standard used for quantitation? If this was the Agar/gelatin/KCl/NaP standard referred to in Material and Methods, why were the particular quoted concentrations chosen, as they do not resemble the expected concentrations in animal cells?
Authors : The quantitation system is based on the use of primary inorganic standards, with verification of accuracy using secondary agar/gelatin standards of known (supplemented) ionic content. Elemental levels in these standards ranged from 3% (approximating to cellular levels) to 13%. The latter levels were intentionally high to provide the best statistical comparison of elemental ratios at different kV settings, where detector efficiency at different accelerating voltages was being determined.

K.-Zierold : How can you measure element contents in thin cells on a bulk substrate considering the different continuum portion of the substrate contributing to the X-ray spectrum ?
Authors : The authors agree that probe overpenetration of the biological specimen presents a major problem in any experimental system where cells are thinly spread over a solid substrate. In these experiments, probe overpenetration was minimised to a level at which characteristic peaks from the substrate were effectively eliminated, but there would still be a low level of substrate continuum contribution.

A. Warley : It seems to me that all the media used for washing the cells were hypotonic. Was this so, and would it not affect the distribution of elements across the cell membrane ?
Authors : On the assumption that the endothelial cells were in osmotic equilibrium with the growth medium, their osmolarity would be about 0.35M. The washing media used were thus hypotonic, but the washing process was very brief, and the data presented in Table 1 suggest that any transient effects on elemental distribution across the cell membrane do not lead to any differences on total cell concentration between the sucrose wash and the control.

G. Roomans : Do the authors know whether the Na and Cl content of the microcarrier beads is due to the bead material or to culture medium trapped in the bead ? If the latter is the case, possibly the entrapment of culture medium could vary with the extent to which the cells cover the beads.
Authors : High levels of Na and Cl are derived both from the bead matrix and from entrapped culture medium. The entry of culture medium into the bead probably occurs during the initial stages of culture, when there is minimal cell coverage of the bead. This would result in penetration of the entire bead, and entrapment of medium would thus not relate to the degree of cell coverage at later stages.

K. Zierold : Have you tried other substances in the washing solution, for example Ba or Sr to close K-channels ?
Authors : We have not tried the effects of Ba and Sr, but think this would be an excellent idea. Our experimental approach was to use as sinple a washing system as possible, removing excess medium from the cell surfaces, but having minimal metabolic effect on the cells.

A. Warley : Were any viability studies carried out ? The values for K obtained over most of the time course are low and coupled with a high value for Na and Ca would suggest cell death.
Authors : Viability studies, such as exclusion of indicator dyes, have not been carried out. All indications are, however, that the cells were viable, since cell division and colonisation were taking place right up to the 96h sample. Fine structural examination of the cells in ultrathin section did not reveal any morphological signs of cell death. It should also be noted that microcarrier systems (including the one used here) have been extensively used for physiological investigations, and appear to be metabolically intact and biochemically active.

K. Zierold : Is the decreasing K/Na ratio with culture time specific for the studied endothelial cells or for the used culture conditions?
Authors: We have so far shown a decreasing K/Na ratio only for endothelial cells in microcarrier systems. Changes in ionic composition with transition to confluency have been observed in other culture systems by other workers (see discussion) and it will be of considerable interest to see how general this phenomenon is.