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Editorial

Pitfalls in Pit Measurement

There are two main approaches to assaying the resorptive behavior of osteoclasts *in vitro;* based on tissue culture and cell culture. The first is achieved by leaving the cells *in situ* on bone explants, and monitoring either the release of calcium or hydroxyproline into the medium [1–4] or examining histological sections to evaluate the loss of tissue or numbers of osteoclasts. Tissue culture is valuable because the cells are retained in their immediate milieu but removed from the influence of circulating hormones or other factors. Nevertheless, the complexity of the local environment is great, and the resorbable substrate is generally fetal or neonate tissue that differs in many characteristics from lamellar bone.

Cell culture has the advantage that the composition of the substratum may be chosen, and a flat reference plane proferred so that the measurement of resorption pits produced in the culture period is easier [5–7]. It suffers from the disadvantage that interactions between adjacent cells on and off the bone surface are disrupted, and the new relationships formed may not be representative of *in vivo* conditions. This method is commonly referred to as the isolated osteoclast resorption assay, but more properly may be considered as a bone cell culture resorption assay as osteoclasts are generally well outnumbered by other cells. Osteoclasts can be easily separated from bone by enzymatic digestion of the tissue. However, this alters their cell surface characteristics and may have a significant effect on their adhesion to a new substrate and their subsequent resorptive ability, as factors bound to the surface are lost. Gentle mechanical agitation of bone fragments also successfully releases bone cells which can then be seeded with receptors and substances bound to the cell coat left undisturbed.

The earliest work with the cell culture assay utilized scanning electron microscopy (SEM) stereophotogrammetry to measure the depth, volume, and other parameters of individual resorption lacunae [5–8]. Experience with the method showed a wide range of biological variability in single cell function in any one experiment [9–13]. Derivatives of this method have proved to be quite popular, especially ones from which data could be obtained more rapidly, if with reduced accuracy, and it is timely to review their purpose and validity.

Cell Counts: Attachment and Spreading

Information from the bone cell resorption assay may be of several types, each valuable if its meaning and limitations are understood. At its simplest, the number of osteoclasts compared with other cells adhering and spreading on a given substrate in unit time may be counted. The osteoclasts are usually represented either as the numbers of multinucleate cells (MuNC) per unit area, or compared with the number of mononucleate cells (MoNC), and expressed as the MuNC:MoNC ratio. If the cells are not overlapping, a simple stain such as toluidine blue suffices for such cell counts [14–16].

A further refinement is to count the number of MuNC staining for tartrate-resistant acid phosphatase (TRAP +ve cells). Although TRAP is not a specific marker for osteoclasts, it allows the distinction of osteoclasts from macrophage polykaryons which usually remain unstained, particularly in short-term cultures. This staining technique also has the advantage that osteoclasts can be visualized below mononuclear cells, even when multilayered, although not with ease below blood clots when an osteoclast is only lightly stained. A counting problem may arise where osteoclasts are contiguous because the cell boundaries cannot be seen.

Neither of these methods gives an exact count of osteoclasts, of course, and only the numbers of cells attached at the termination of the culture period are counted. Vital staining using, for example, neutral red [17] or a fluorescent DNA stain is required to monitor changes in the osteoclast numbers during culture.

Further information concerning the development of the typical attachment foci exhibited by osteoclasts under various experimental conditions may be gained by immunolabeling attachment proteins and examining them by fluorescence microscopy [18–20]. The attachment sites and cytoskeletal organization of osteoclasts are entirely different from other cells in the bone-derived cell population and can be seen on dentine or bone, as well as glass or plastic. An alternative method is to use interference reflection microscopy, or reflection imaging of the contact zone with confocal microscopy.

It is often valuable to discover simply whether putative osteoclasts are able to resorb a calcified tissue, or to find which tisues or other substrates can be resorbed by known osteoclasts. In this case, it is only necessary to record whether typical resorption lacunae are present. The use of a suitable substratum other than bone, such as dentine, is particularly apposite for the first experimental situation to remove doubt as to whether the resorption observed could have occurred *in vivo*.

Pit Counts: Resorptive Episodes

The simplest assay of resorption is a count of the numbers of discrete pits in the substratum after the removal of the cells. The pits may be identified using reflected light microscopy or, after staining with toluidine blue, by transmitted light microscopy. This is easy and quick, but counting number (or

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measuring the area resorbed) may reflect osteoclastic spreading and attachment rather than resorptive function [21, 22]. For this reason, the pit:osteoclast ratio is a much more useful measure of osteoclastic resorptive activity [23]. It complements but does not replace volume measurements of pits.

The pit count may also indicate the pattern of movement and resorption rather than the quantity of resorption. For example, one osteoclast under certain conditions may make several individual small pits, or under different conditions one large multilocular pit, but the volume of tissue destroyed—the work done—might be the same or it might differ. It is also necessary to define carefully what constitutes a separate pit: a clear rim of unchanged original surface between neighboring excavations should in theory be the deciding point, but try to get 2 observers to agree!

Areas: Adhesion, Spreading, and Resorption

A second analysis that has been widely used for its simplicity, and because of an assumed difficulty in measuring depths and/or volumes, is to measure the plan area of resorption per substrate slice or per unit area [24–34]. Together with a count of MuNC, a value for the mean area per pit is obtained. This method combines information about the adhesion and spreading of the cells, and the total area over which secretory ruffled borders of osteoclasts have been active at the level of the original surface of the substratum.

This is a relatively rapid technique for assaying some aspects of resorption and requires no unusual technology—reflection light microscopic (LM) and toluidine blue staining being the simplest methods, followed by SEM, with the specimen normal to the beam to eliminate foreshortening. One limitation is that the total area affected may not be realized if SEM using backscattered electron imaging [35–38] is not employed: demineralization is sometimes slight. Toluidine blue stains such areas, but a skilled eye is needed to detect the minimal staining. The method does not measure the amount (volume) of tissue resorbed and may be misleading because an increase in pitted area may occur with a decrease in pit depth and volume [11, 12, 39]. In this case, the cell may have spread more and/or have had a more extensive ruffled border zone, yet have done less work. A refinement is to record the area of each pit, using image analysis or stereology, and show the distribution of pit areas.

The measurement of area and number can be automated if a sufficiently contrasty image is available: toluidine blue or silver stained pits on the surface of dentine slices are sufficiently well imaged [9]. In the case of bone, however, natural internal features are present in the same size and contrast range, and distinguishing between these features requires the intervention of a human operator. For area measurement, approaches using a cursor on a TV monitor or cutting out the area of the image from a photograph have been used. The much simpler approach of measuring the width of pits—which gives equally useful information when averaged out over a large number of pits—has not been used until recently, although it represents a classical stereological approach [40].

The numbers of pits reported in many papers is very low and the proportion of the area of the substrates occupied by pits is also low. If the substrates are cut even from large mammal bones, the real areas of the substrates are low. Under these circumstances it is not surprising that counts of pit number and measurements of total area resorbed may be influenced as much by chance as by any real experimental or biological parameter [21, 22, 24–34).

Some workers have taken succour from early reports of a good correlation between area and depth, and area and volume, and concluded that it may be justifiable to dispense with the 3D measurement [41]. Such assumptions are, however, really unjustified because the correlations between Z and XY are not the same in all cases, and are not the same at different times during the development of a pit because of the lateral movement of the cell during resorption.

Cell Size

Pit size depends partly on osteoclast size: larger osteoclasts may make larger and/or more pits [37]. The resorptive activity of osteoclasts may also be increased by cell fusion, so that one with six nuclei, for example, can destroy more tissue than two cells with three nuclei. Ideally, the volume of each osteoclast should be known in relation to the volume of pits it has made in a known time. As the size of an osteoclast is related to the number of nuclei the cell possesses, some measure of this may be made by recording the numbers of nuclei of the osteoclast associated with each pit at the end of the culture period. The volume of tissue removed per osteoclast nucleus can then be found. For this to be anything more than a minimum value, a much more sophisticated procedure is required. Shorter culture times improve the closeness of the minimum to the real value, in so far as each cell may have only made one pit with which it is readily identified, but do not account for the varied lag time between settling and starting to resorb.

The volume of each cell (and its associated pit) may also be measured by SEM stereophotogrammetry [37]. Corrections have to be made concerning the volume shrinkage of the cell. Most methods require the identification of the same pit following removal of the cell; this is time consuming, but could be much improved by automatic methods for relocating positions on a specimen surface. Alternatively, values for the volume of the (wet, fixed) cell and pit may be obtained by automated, confocal, light microscopic imaging.

It is unlikely that this amount of information would be necessary for routine assaying of osteoclastic function in bone cell cultures unless cell size changes occur (by fusion or recruitment of new osteoclasts) as a result of the culture conditions. Knowing the volume resorbed per osteoclast nucleus might mean that fewer pits would need to be measured because it should reduce the variability due to the wide range of osteoclast sizes.

Depths of Pits

Extra information is obtained if the depths of the pits are measured either as the sole measurement or in conjunction with area measurements. The maximum depth is sometimes chosen [8], but experience shows that this is likely to be unrepresentative of the average depth and hence give a poorer indication of volume. Reflection LM or SEM have been used to make direct measurements (of dubious value [42]), or depth has been determined using SEM stereophotogrammetry [43, 44]. Regarding LM, the location of the maximum depth may also be difficult to find and its value unreliable unless reflection confocal microscopy is employed. As the depth of many pits is small, the error in measurement may be huge.

The depth of a pit changes with the nature of the substratum proferred, in particular with the mineral component composition and density [35, 38]. For this reason, a more

homogenous material may give better results. Certainly, this is one reason why comparison between experiments must be made with care. The depth for a given pit area is a useful indicator, but the area:depth ratio is not dimensionless and is therefore not a shape factor.

Shape factors supplement volume measurement, which give the quantity of work accomplished, by providing extra information on cell-substratum interactions as well as intercellular communications. The molecular nature of the substrate and the release from it of ions and organic molecules modifies the pattern of activity of the osteoclast, and this in turn is apparent in the shapes of the lacunae made.

Volumes of Pits: Amount of Resorption Accomplished

The work done by an osteoclast in making a pit is best estimated by measuring the volume of tissue destroyed. Estimations of volume from formulae using area and the depth at the deepest point in a pit are unlikely to be accurate [8, 45, 46]. Volumes and mean depths are better determined by SEM stereophotogrammetry or automated reflection confocal imaging and analysis. The confocal method can be speeded up and simplified to generate only profiles, from which depths and widths can be obtained [40]. Extrapolation from these values to volumes will be better than no attempt, but obviously imprecise compared with the other two methods.

Measuring the volume of an individual pit gives the work done by one cell in one resorptive incident. In longer cultures, several cells may contribute to the making of a complex resorption patch [37]; for this reason, culture periods are generally kept short. It also is possible for the volumes of pits produced in a culture to be unchanged in an experimental situation, but for the numbers of pits to be dramatically altered. Combining pit:osteoclast ratios with volume measurements, therefore, gives a more comprehensive view of the resorptive activity of the total bone cell population.

To examine the changing resorptive function of individual osteoclasts over time, individual pits may be followed using video recording with low intensity, oblique red light [13]. After fixation, the cells may be stained to count the numbers of nuclei, and their volumes measured either by SEM stereophotogrammetry or by automated confocal imaging and analysis. The cell is removed, and the pit's shape and volume is determined using the same methods. The rate of growth of the pit can then be found in terms of increase in surface area, and volume of each pit, or loculus in a multilocular pit.

Technical Problems

A 1985 review [9] of quantitative and qualitative microscopic methods which could be used in connection with this aspect of the study of the function of isolated osteoclasts, dealt with methods that had been used up to that date and considered the technical issues relevant to this work, including choice of substrates—dentine versus bone; substrate surface finish versus difficulties in detecting and measuring small pits; LM methods; vital microscopy; time lapse and video filming; normal transmitted LM with bright field illumination for stained specimens; interference LM—the Mach-Sehnder interferometer; phase contrast; transmitted and reflected dark field; vital staining (including fluorescence probe methods, neutral red to selectively stain osteoclasts, DASPMI for mitochondria, bisbenzimid for nuclei, methylene blue staining for nonosteoclastic cells); selective staining of the resorbed area for routine light microscopy (toluidine blue or silver staining and conversion of silver stain to silver sulphide); confocal LM of wet, dry, and glycerine and oil-immersed specimens; SEM specimen preparation (CPD versus air drying and freeze drying, whether or not to remove the demineralized collagen fringe with any alkali or oxidizing agent, e.g., NaOCl, NaOH, KOH, H_2O_2; metal coating for SEM and the use of such specimens in reflected light microscopy and the advantages of different metal coatings in terms of their later removability, for example, to be able to undertake a further treatment to remove the collagen fringe). Regarding the selection of microscopical method to detect image and measure resorption pits, this review drew attention to what could be achieved with reflection LM, in particular, dark field reflected light, as against SEM, and paid particular attention to the advantages of confocal microscopic methods that were at that time both new and hardly available.

Since that date, most work in the bone resorption field has neglected the need for appropriate methods of measuring the third dimension of resorption lacunae. A field that started with methods of measuring volumes has drifted into one where measuring areas and counting pits is seen to suffice.

Only two sorts of method have so far been validated for the measurement of volume (though others have been used): these are based either on the measurement of parallaxes in stereo-pair images from microscope systems with a large depth of field, or from determining the position of best focus using a microscope with a very shallow depth of field. It is worth looking at relevant aspects of this microscopical 3-D surveying.

Scanning Electron Microscopy and Stereophotogrammetry

Heights or depths can be determined from stereopairs of scanning electron micrographs [43–51]. XY co-ordinates are determined by direct measurements in the image plane. It is an important problem that there may be severe, unrecognized, barrel or pincushion and/or less regular distortions of the raster scanned by the electron beam on the sample in the SEM as well as of the raster scanned by the beam in the record CRT. The magnification across a typical SEM picture is not uniform [47]. These problems can be, but rarely are, overcome, for example, by imaging a regular grid which is used to calibrate the magnification in all parts of the field of view. Most users merely accept the magnification calibration given by the manufacturer's service engineer and do not know of the global and local errors involved.

The simplest formula for relating parallax to height difference assumes that co-ordinates are measured perpendicular to the plane of an image which would have been taken with the specimen half way between the two positions in which the two stereo images were actually recorded. Assuming parallel projection optics, which is a close approximation to the truth at the magnifications above 500 × and up to 2,500 × commonly used in measuring resorption lacunae, a height difference perpendicular to this plane is designated as $Z_c = (X_L - X_R)/(2\sin(G/2))$, where X_L is a distance between two features in the X (interocular, perpendicular to the tilt axis) direction, and X_R is the distance between the same two features in the X direction in the projection given in the right image. X_L-X_R is the parallax, and G is the tilt angle difference through which the specimen was tilted or the electron beam was tilted with respect to the specimen. Thus, the

precision of the determination of the height component is proportional to the precision with which the tilt angle difference, the parallax, and the magnification can be determined. Most commercial SEM specimen stages are arranged to indicate the tilt angle difference to no better than 1°. They may also suffer from mechanical backlash (hysteresis) and other nonlinearity problems. It is therefore frequently the case that the tilt angle difference can be determined to no better than 10% [48]. SEM stereo height difference measurements have been quoted with an implied precision of 0.1–0.01% (volumes of thousands of cubic microns quoted to decimal cubic micron precision); such extrapolations from depth measurements may have generated volume errors of 33% or more [46]. The measurement of the depths of a few pits has even been used to estimate volumes for many; the methodology (precision) of the depth measurement procedure is not described. It can never be valid to record the depths of a small proportion of the pits to compute the volume of tissue resorbed per slice; the range in the depths measurements is too great.

Technical reviewers of work submitted for publication should ask for important details such as how and how accurately and reproducibly the tilt angle difference could be determined, how the magnification and its possible local variations within the field of view were calibrated, and how and how accurately the parallax was measured. At the very least, it must be indicated how it was determined that all the errors involved were identical between stereoscopic pairs.

With digital SEMs, it is possible to tilt the sample automatically through a precisely determined angular range and then automatically recognize corresponding features in two digital images stored in computer memory. Algorithms are available for depth and volumetric measurements via this route. At the present time, however, the procedure is no faster than manual acquisition and measurement of SEM stereopairs, which allows subtle morphological features to be seen and interpreted from the 3-D image.

Slopes that are nearly parallel with the optic axis present a problem for volume measurement. Every point to be measured (and this must include the side slopes of the pits in true volume measurement) must be well represented in both members of the stereoscopic pair. As the sample has to be tilted to generate the pair, it is evident that some slopes may be eclipsed. The human eye-brain complexed to a stereocomparator interpolates the missing data points, reliably filling in the missing sides of the pits. Automatic image analysis cannot do this, and automated SEM stereophotogrammetry is weakest on this point.

User-oriented, precise equipment for parallax measurement [49–51] is not widely available in SEM and biologically oriented laboratories. One economic alternative is the use of a special pair of overlay grids, the stereoscopically fused grid giving a number of intersections in depth. The number of XYZ intersections in space that lie within the osteoclastic lacuna is counted to give a stereological volume measurement [52].

SEM stereo vision can be used as an aid to making precise measurements of depth. A real-time stereoscopic display is generated by deflecting the electron beam in the SEM such that it subtends at contrasting mean angles of incidence to the specimen surface. Depths are measured by moving the sample with appropriate X, Y, and Z stage controls, bringing one point after another into register with a cursor which marks the same position in 3-D space [53, 54].

The suggestion that Z co-ordinates could be measured by determining the height of the specimen at best focus in the SEM [55–57] is seriously flawed. The depth of field in an SEM (obviously depending on the final aperture) is typically many microns, yet height differences of 0.1 μm are required. Determining focus by measuring the final condensor lens excitation current (which is changed to change focal length) gives rise to the additional problem of hysteresis in the magnetic behavior of the lens.

Optical Microscopy: Conventional and Confocal

For good depth discrimination in optical microscopy, a high aperture lens should approach the sample surface closely. High aperture lenses are immersion lenses, usually designed for use with 170 μm coverslips. A coverslip unnecessarily increases the stand-off distance and reduces the possible numerical aperture for the objective lens. The large refractive index difference which allows for good reflection at the interface between air and a dry substrate is diminished by the presence of an immersion medium; frequently this leaves stronger reflections at internal features such as osteocyte lacunae and canaliculi or dentine tubules, making automation of measurement more difficult. The strength of the surface reflection can be restored by metal coating the sample as for SEM, which will also make it possible, using a 1.4NA immersion lens, to examine the steep sides of deeper pits which often reflect no light back into even maximum aperture (0.95) dry lenses.

The convenience of using dry samples for LM measurement is without doubt, but, as just noted, this will lead to undefined areas in the edges of pits which will affect volume measurements. This difficulty has been overcome in a program for measuring pits with automated real time confocal (tandem scanning) microscopy by making a linear interpolation between good data at the reference surface of the substrate and the bottom of the pit [60]. Straightening out the sides of the pits in this way results in a marginal reduction in the calculated volume.

The lateral resolution of the conventional optical microscope is most often given as 0.61 lambda/NA. The maximum NA for dry objectives is 0.95; the retina is most sensitive at lambda = 550 nm, giving a resolution of 350 nm. Regarding the vertical dimension, optical microscopy is clearly deficient in that even with the highest aperture lenses there is too large a depth of field and too small signal response with change of focus. Ordinary light microscopic methods will give a range for a single Z reading of ± 1 μm.

When dealing with the measurement of the depth and volume of shallow resorption pits, it is necessary to consider the surface roughness caused by cutting the substrate. Conventional LM does not have the depth resolution to make this determination, except where the expected depth of the pits is in excess of 5 μm; in this case, it might be posible to determine the real depth of the pits to ± 20% of 5 μm. Conventional reflection LM also does not provide the discriminatory power (given by SEM) to allow the recognition of preexisting resorption pits in bone wafers. Even with SEM resolution, critical evaluation must be exercized when using bone as an experimental substrate.

The depth response in automated, digital, confocal reflection LM is good enough to characterize the substrate surface roughness and to measure the depth of shallow pits [58–62]. Confocal microscopes work by illuminating features only in one plane and accepting the light only from the same features in the same plane, thus showing a sharp peak in signal brightness at focus [58, 59]. If a fine enough mechanical focusing mechanism is employed, the depth of a feature can be determined reproducibly to better than 0.1 μm with a high NA

objective. The steps between the focusing planes may provide one limit to resolution: fundamental limits may also apply. The essence of the procedure is to focus the microscope in very small increments, storing each image plane as digital data, which can then be compared with the data obtained from the previous plane. It is thus possible to build an image that records the depth at which focus was found at each pixel, when it is a simple matter to determine volumes [60].

Depths alone can also be obtained by a manual through focusing method: with an observer viewing a real-time confocal image, it is possible to determine a focus level at which the surface surrounding the resorption pit reaches its brightest value and to subtract this from the depth at which the bottom of the pit becomes brightest. Depths as such can also be derived from the (longitudinal chromatic aberration) color coding for depth which is found in the real-time, direct-view (tandem scanning) confocal microscopes illuminated with white light [58, 59]: focusing to set the surrounding original surface to a constant hue, the color of the base of the pit is observed.

At least one commercially available video-rate confocal laser LM system is available, complete with software suitable for measuring the depths and the widths (but not areas or volumes) of pits. Such measurements are made from single line profiles using cursors [40, 61, 62]. This technology is rapid, reliable, and reproducible—even if not yet cheap.

Interactions

Although much has been clarified concerning the cellular biology of mineralized tissue resorption, many fundamental biological questions remain. These are unlikely to be answered by the simplified versions of the bone cell culture resorption assay, but require an analysis that takes into account the cellular environment of each osteoclast and relates this to its performance. The general biochemical environment is, of course, of great importance. Nevertheless, of equal importance is the local biochemical environment of a particular cell—its contact with or proximity to cells of other lineages and with other osteoclasts or progenitor cells.

It is evident from comparing SEM pictures of surfaces resorbed *in vivo* and *in vitro* that the collagen fringe is more evident under most culture conditions than it is *in vivo* [10, 36]. Demineralization and destruction of the organic matrix may be differently affected *in vitro*. However, the depth of the fringe has generally remained unmeasured, although this is now technically possible and is a most important parameter for understanding the resorptive process. A development of this is the identification of conditions *in vitro* that will mimic the resorption-repair coupling process occurring *in vivo*, and stimulate the bone formation activity of osteoblasts.

We would urge that adequate descriptions of methods and reporting of data (including values found, rather than test:control ratios) are always presented so that the validity of conclusions may be properly assessed. The type of information sought and conclusions drawn must match the type of measurement made. The potential of the bone cell culture-resorbable substrate system is great for increasing our understanding of many aspects of the osteoclastic performance, from interactions with other cells through attachment, spreading, resorption, motility, and detachment.

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