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SCANNING ELECTRON MICROSCOPY OF THE OSTEOCLAST-BONE INTERFACE IN VIVO

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Abstract

Rat femoral bones were studied by scanning electron microscopy to demonstrate the morphology of osteoclast-bone matrix interfacial relationships. Two general morphological types of actively resorbing osteoclasts were observed. One cell type was approximately ovoid with highly fimbriated borders, fully attached and closely adapted to the resorption surface. The ruffled border of such cells was composed of a number of individual filopodia which were of uniform, regular shape and approximately 2-3 µm in length and 150 nm in diameter. They were found to penetrate the bone matrix to a depth of 1 µm and interdigitated with the surrounding bone. The other cell type was elongated, covered smaller lining cells, and attached to the bone surface by pseudopodia. The bone-contacting face of pseudopodia formed a network of irregular membranous branches apposed closely to the underlying bone matrix. The results revealed the three dimensional ultrastructure of in situ relationship of osteoclast to bone matrix and necessitate a reinterpretation of the geometry of the secondary lysosome of these cells.

Key Words: Bone, rat, osteoclast, ruffled border, resorption, in vivo, morphology, light microscopy, scanning electron microscopy.

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Introduction

While osteoclasts were discovered and distinguished from megakaryocytes by Robin (1864) who coined the terms "plaques a noyaux multiples" and "myeloplaxes", he did not suggest any function for these cells. The peculiar notched, pitted, and eroded appearances of bone resorption surfaces were first observed by Howship (1871) who was not aware of the presence of osteoclasts. The relationship between osteoclasts and the resorption lacunae was established by Kolliker (1873) who suggested that the function of the cells was to resorb bone and named the cells osteoclasts. It is now generally accepted that the osteoclast, a giant multinucleated cell, is the universal agent of cellular bone resorption and its bone destructive structure is the membranous ruffled border formed on the bone-contacting side of the resorbing cell.

The ruffled border was originally observed by light microscopy as an intricate fringed, interfacial border between the bone-facing surface of the osteoclast and the resorbed bone matrix by Kolliker (1873) who named it the "brush border" and considered it as the bone destructive organ of osteoclasts (Kolliker, 1889). The suggested resorptive function of the brush border was, however, disputed by both Leriche and Policard (1926) and Ham and Gordon (1952) who thought that the brush border was merely the fringe of collagen fibres on the resorbed bone surface, rather than a structure of the osteoclast. Scott and Pease (1956) for the first time provided convincing evidence, by transmission electron microscopy (TEM), that the brush border was indeed the osteoclast cytoplasmic structure related to bone resorption. They found that where the surface of an osteoclast was in contact with the resorbed mineralized bone matrix, the plasma membrane presented a number of continuous membrane foldings or ruffles fanning out towards the resorbed bone matrix. They also found that between these membrane foldings there were extracellular channels, 6.5-8.5 nm in width, in continuity with cytoplasmic vacuoles. The interwinding channels and vacuoles contained apatite crystals and collagen fibre fragments, suggesting bone resorption activities.
These findings were further confirmed by later investigators who studied the brush border by TEM and adopted the name the "ruffled border" used by Scott and Pease (1956). According to their studies, the foldings are between 50-150 nm in thickness (Dudley and Spiro, 1961; Hancox and Boothroyd, 1963) and several micrometers in length (Cameron, 1972). The ruffled border is surrounded by the peripheral zone of the cytoplasm which is poor in organelles but rich in contractile filaments (Scott and Pease, 1956; Hancox and Boothroyd, 1963; Cameron, 1972). This peripheral plasma zone, later named the "sealing zone" (Schenk et al., 1967), is firmly attached to the bone surface (with an interface zone of 0.2-0.5 nm thickness) to segregate the extracellular space from the resorption area under the ruffled border (Lucht, 1972a,b; Holtrop and King, 1977). Between the ruffled border and the underlying eroded surface of solid bone matrix, is an extracellular compartment, in which the membranous foldings of the ruffled border end (Holtrop and King, 1977; Marchisio et al., 1984). This sub-compartment, which is highly acidified, and enriched with a variety of enzymes, is the functional equivalent of a large secondary lysosome responsible for dissolving solid bone matrix (Baron, 1989, 1990; Marks, 1989; Vaes, 1968, 1988).

Knowledge of osteoclast ultrastructural morphology has been mainly based on TEM observations. Although scanning electron microscopic (SEM) studies of in vitro osteoclasts have been reported, using cultured bone tissue (Horton et al., 1984), and cultured osteoclasts (Boyle et al., 1984; Chambers et al., 1984; Jones et al., 1984; Chambers, 1985; Helfrich and Mieremet, 1988), we are aware of only two SEM studies of in vivo osteoclast morphology. Jones and Boyle (1977) were the first who studied osteoclasts from frontal bones of fetal rat and demonstrated the heterogeneous three dimensional morphology of osteoclasts in vivo. Recently, de Saint-Georges et al. (1989) reported another SEM study of femoral bone, exhibiting osteoclasts which appeared to be connected one to another by long pseudopodia. These in vivo SEM studies revealed a wide variation in size, shape, surface appearance and membranous structure of osteoclasts. Thus, osteoclast morphology is more complicated than initially inferred from two-dimensional TEM studies. The present SEM study of in vivo osteoclasts, using a freeze-fracturing method, demonstrates the cell-bone matrix interfacial morphology and is particularly focused on in situ structures of the ruffled border area.

Materials and Methods

Young adult male Wistar rats (150-180 grams) were used in this study. The animals were sacrificed by cervical dislocation. The femora from both sides were dissected and the soft tissues on the bone surface were removed. The epiphyses were removed, using a 3/4 inch (1.9 cm) diamond disc driven by a dental engine, to isolate the diaphyses. The marrow tissue of the diaphyses was then flushed out with 0.1 M sodium cacodylate buffer (pH 7.2-7.4) using a 5 cc 16G syringe. The resultant diaphyseal bone shaft was immersed in 2 % paraformaldehyde/2.5 % glutaraldehyde fixative overnight and subsequently washed three times with, and stored, in 0.1 M sodium cacodylate buffer at 4°C. Tissues were prepared for light microscopy and scanning electron microscopy as described below.

Light microscopy

The femoral diaphyses were split longitudinally into two pieces using a razor blade, and decalcified in 45% formic acid and 20% sodium citrate for 1 week. The tissues were then washed, dehydrated in a graded alcohol series, cleared in methyl salicylate, infiltrated and embedded in paraffin. Sections of 5 µm thickness were cut, Hematoxylin-Eosin stained and examined by light microscopy.
Scanning electron microscopy (SEM)

Previously fixed femoral diaphyses were immersed in liquid nitrogen, without cryopreservatives, for 2-3 minutes to allow spontaneous fracture into 3-5 pieces. The tissue fragments were then postfixed in 1% osmium tetroxide dissolved in 0.1 M cacodylate buffer (pH 7.2-7.4) for 1 hour, dehydrated through six concentrations of ethanol (from 50% to 100%). The dehydrated samples were loaded into a critical point drying unit (Ladd Research Industries Inc., Burlington, VT) and flushed with carbon dioxide for four periods of 5 minutes each, to replace the alcohol in the samples. Then the carbon dioxide was evaporated by heating to 41°C at a pressure of 1,300 lb/in² (8.96 x 10⁶ Nm⁻²). After this drying procedure and mounting, the samples were loaded into a Polaron E5100 SEM coating unit (International Scientific Instruments Inc., Santa Clara, CA) equipped with an E5500 thickness monitor and sputter-coated (15-20 kV) with gold to a thickness of 10-15 nm. Endosteal, trabecular, and fractured cortical surfaces were examined for resorption areas in an Hitachi 2500 SEM (Nissei Sangyo Canada, Rexdale, Ontario) at 10 kV.

Results

Light microscopic observation

Decalcified histological sections clearly showed the sites of osteoclastic resorption at the trabecular, endosteal, periosteal surfaces and inside the intracortical canals in rat femoral bone (Fig. 1). The cytoplasm of the osteoclasts was abundant, irregularly shaped, varying in size, with typical "foamy" appearance. The number of the nuclei ranged from 2 to more than 10 with heterogeneity in shape and staining density. At the bone-contacting side, the "brush border" appeared to merge with the resorption surface and the cytoplasmic border could not be clearly distinguished. While most osteoclasts were seen to be approximately ovoid, some were seen to be large and irregularly shaped with multiple bone-contacting surfaces.

SEM observations

General morphology. SEM of freeze-fractured samples clearly showed resorption fields on both endosteal and trabecular surfaces. They usually consisted of shallow, overlapped, co-cavities and were characterized by scalloped edges which protruded out of the base of the fields. Although each femur had been vigorously washed as part of the preparatory procedure, in order to remove marrow cells, many osteoclasts remained adherent to the resorption surface. While the shape and size of the osteoclasts varied greatly, two distinguishable types of morphology could be clearly identified. The first (Fig. 2a) was relatively flat, approximately ovoid with highly fimbriated border and having a diameter in the range between 20-40 µm. The whole cell was in close contact with bone surface and usually fully accommodated and adapted to a resorption lacuna. The second was greatly elongated, varying in size and shape, with many extended pseudopodia (Fig. 2b). This cell type usually exhibited multiple attachments to the mineralized bone surface through branched pseudopodia. The extended cytoplasmic portions between two bone-contacting sites were frequently seen to cross over other cells on bone surface. While these two general types differ in their shapes, they are commonly characterized by microvilli on the dorsal membrane (Fig. 2).

The ruffled border. When the main bodies of the cells were fractured in the same plane as the under-
Figure 3. The ruffled border. a: An osteoclast is freeze-fractured to expose the in situ ruffled border (indicated by two arrows). Although the sealing zone can be seen as the peripheral portion of the cell, it cannot be clearly identified since the separation of the cell from bone surface has occurred due to dehydration. Bar = 3 µm. b: Close examination of the ruffled border seen in Fig. 3a, showing a number of individual filopodia which are almost uniform and regularly shaped. Many of them have been broken as a result of tissue processing, so that the distal portions remain buried in the underlying matrix. Bar = 1 µm.

lying bone, the relationship between the in situ ruffled border and the bone matrix was clearly visualized. The ruffled border area comprised filopodia, rather than continuous membranous ruffles. The individual filopodia originated either directly from the cell body or from a larger branch, and projected perpendicularly towards the bone surface (Figs. 3 and 4). They were relatively uniform, evenly shaped, approximately 150 nm in diameter and 2-3 µm in length (Fig. 3). These filopodia were found to have penetrated the bone matrix to a depth of approximately 1 µm. As shown in Fig. 4, in which the relationship between the ruffled border and apposed matrix was well preserved, many individual filopodia were inserted into, and tightly interdigitated with, the underlying bone matrix. Each filopodium was separated from its neighbours by the surrounding matrix and possibly acted as an individual local resorbing unit inside the bone matrix. Although the sealing zone could be assumed to be located at the peripheral region of the cells, it could not be clearly identified. In some cases, this penetrating ruffled border was seen to have separated from the underlying resorbed surface along with the whole body of the cell, as the result of shrinkage caused by critical point drying. In these areas, particulate matter was associated with the detached filopodia (Fig. 4d).

Pseudopodia-bone matrix interface. Some bone-contacting pseudopodia, longitudinally fractured along with the apposed bone matrix, also appeared to possess ruffled borders. In these cases, the bone-contacting face of the pseudopodia (Fig. 5) consisted of many small membranous branches which formed small channels and vacuoles containing segregated particulate matter. In addition, the bone matrix exhibited small resorption lacunae. Unlike the ruffled border under the main body of the cell, these pseudopodial membranous structures appeared as irregularly shaped branches which ran in different directions without clearly identifiable ends. They were closely apposed to the bone matrix, but did not penetrate the matrix below the bone surface.

Peripheral border-bone matrix interface. The fimbriated peripheral border of the ovoid shaped osteoclasts comprised individual filopodia similar to those seen at the ruffled border (Fig. 6a). These peripheral filopodia projected toward the bone surface. They were also approximately 2-3 µm in length and of uniform, 150 nm diameter, and were assumed to have been interdigitated with the protruding collagen tips of the resorbed matrix surface. In some areas (Fig. 6b) these filopodia were fractured due to tissue processing so that the distal portions remained buried below the bone surface, suggesting that they had penetrated the underlying mineralized matrix.

Discussion

Light microscopic examination revealed large, irregularly shaped, multinucleated osteoclasts, consistent
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with the accepted histological description of these cells (Weinmann and Sicher, 1955; Pritchard, 1972). The SEM results demonstrated that while the shape and size of the osteoclasts varied greatly, two general morphological types could be distinguished which were in agreement with the observations of Jones and Boyde (1977). The first was approximately ovoid in shape with a fimbriated border, closely adapted to the bone surface. The second was elongated and attached to the bone surface by pseudopodia. While the second type have been considered as mobile or inactive osteoclasts (Jones and Boyde, 1977), the present results suggested both cell types are active resorbing cells (see below). This suggestion is supported by a recent light microscopic study of Abe et al. (1990) who reported three types of osteoclast morphology in rat parietal bone, two of which were

Figure 4. Penetration of the ruffled border. a: An osteoclast (arrow) is freeze-fractured along with its underlying bone matrix (m). The ruffled border (indicated by two wavy arrows) is seen to be firmly attached to the bony surface. Bar = 3 µm. b: Higher magnification of the same osteoclast (Oc). The ruffled border is seen to be composed of clusters of filopodia (arrows) which are uniform, regularly shaped, and originate from larger branches (arrowheads). Bar = 600 nm. c: Filopodia (arrows) which have penetrated the underlying bone matrix (m) to a depth of about 1 µm. The bone surface line is indicated by a wavy arrow. Bar = 300 nm. d: In some areas, the filopodia have been separated from the bone surface (S) along with the cell (OC), but are still partly embedded and surrounded by matrix (m). Bar = 1 µm.
similar to those described herein, all of which were considered actively resorptive. While both types of osteoclasts shown in the present study are commonly characterized by microvilli on the dorsal surface membrane, as shown by Jones and Boyde (1977), the satellite-like osteoclasts, with a smooth surface, reported by de Saint-Georges et al. (1989) were not observed in the present study. Such differences in cell surface morphology may reflect the functional state of the cells (Miller, 1977).

Jones and Boyde (1977) showed the structure of the ruffled border in cells detached from the bone surface. In the present SEM study, the freeze-fracture method enabled the exposure of the \textit{in situ} ruffled border at the fractured surface. The ruffled border comprised a number of individual filopodia which were evenly shaped, regularly arranged, 2-3 $\mu$m in length and 150 nm in diameter. These dimensions are consistent with the TEM studies of Dudley and Spiro (1961) and Hancox and Boothroyd (1963). These filopodia penetrated the mineralized bone matrix to a depth of about 1 $\mu$m. While the ruffled border, as described by Vaes (1988) and Baron (1989), acidifies the sub-compartment and releases lysosomal enzymes responsible for the removal of the matrix, the present results suggest that each filopodium might have acted as a single micro resorbing unit to penetrate the matrix. Thus, the penetration of the filopodia may represent the high functional efficiency of the osteoclasts, since it was mainly seen on the ovoid shaped osteoclasts which were fully attached to a resorption surface and have been previously identified as actively resorbing osteoclasts (Lutch, 1972a,b; Jones and Boyde, 1977; Baron \textit{et al.}, 1986; Fukushima \textit{et al.}, 1991). Similar individual filopodia were also seen at the peripheral border of the osteoclasts. However, it could not be determined whether these peripheral filopodia appeared...
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as the result of the shift of the ruffled border from central to the peripheral area due to the cell movement, or if they represented additional resorptive structures of the osteoclasts.

The membranous contacting structures were also observed at the ends of the pseudopodia of the osteoclasts. Since these cells were only attached to the bone surface at the extremities of the pseudopodia, they could possibly have been identified as detached inactive osteoclasts in TEM sections in previous studies (Lutch, 1972a, b; Fukushima et al., 1991). The contacting area of the pseudopodia was usually composed of membrane foldings which did not penetrate the matrix below the bone surface. However, the framework of the membranous foldings was closely apposed to the resorbed bone matrix and included many vacuoles containing particulate matter, suggesting its resorptive activity. Compared with the uniformly shaped, regularly arranged penetrating filopodia described above, these pseudopodial structures may represent the early stage of the ruffled border development. Based on these observations, it can be proposed that as soon as the elongated mobile cell gains partial attachment to bone surface by pseudopodia, the membrane at the ends of the pseudopodia may immediately engage resorptive activity by forming a frame of resorptive foldings. In this manner, osteoclasts may have more than one membranous resorption domain which has also been discussed by Taylor et al. (1989).

It has been schematized that, between the ruffled border and the underlying eroded surface of solid bone matrix, there is a large extracellular compartment in which the membrane foldings of the ruffled border end (Vaes, 1988; Marks, 1989; Baron, 1990). However, in the present study, a space between the endings of the filopodia of the ruffled border and the bone surface was seen only when the whole cell was detached from the bone surface, due to critical point drying. When the in situ micro-relationship between the ruffled border and bone matrix was well preserved (Figs. 4b and c), no visible space was seen to separate the filopodia from bone matrix. On the contrary, many filopodia penetrated the bone to a depth of 1 µm and were tightly interdigitated with the surrounding matrix. This penetration explains the light microscopic observation of the "brush border" which had merged with its apposed matrix (Fig. 1). Indeed, in reviewing TEM photomicrographs from previous studies (Scott and Pease, 1956; Hancox and Boothroyd, 1963; Cameron, 1972; Rhodin, 1974; Miller, 1977), the ruffled foldings were seen to closely interdigitate with demineralized collagen fringe without an obvious space between the endings of the membranous projections and the underlying substrate, although deep penetration into the bone matrix, as we demonstrate, has not been reported previously.

Conclusions

Three morphological types of osteoclast-bone interfacial relations were observed. In particular, the individual filopodia of the osteoclast ruffled border penetrated the bone surface to a depth, approximately 1 µm, greater than that previously shown with TEM. Thus, the common schematization of a large secondary lysosomal sub-compartment does not represent the real in vivo situation. In both the previous TEM and the present SEM results, many extracellular spaces are seen, within the ruffled border network as channels or vacuoles. Thus, a communicating network of these channels and vacuoles may represent the real sub-osteoclast compartment which functions as a secondary lysosome.

Acknowledgement

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Discussion with Reviewers

A. Nanci: The concept that osteoclasts can exist in many shapes and that their ruffled border consists of cytoplasmic extensions which may be filopodial in nature or sheath-like is already established in the literature (reviewed by Gay, 1992). Could the authors highlight the specific contributions of the present study?

Authors: Yes, we do not suggest that the description of osteoclast filopodia is unique to our study, although we would point out that Gay (1992) relied on the work of Holtrop and did not illustrate such details herself in the above review. Previous descriptions of osteoclast ruffled border filopodia were based solely on the TEM studies and thus we believe that our SEM observations reported here provide new data. However, the major observation which we make is not the presence of the filopodia per se but the depth to which they are obviously capable of penetrating the bone matrix. This was surprising to us as it was certainly contrary to the consensus opinion of osteoclast membrane penetration of bone, and also raised the fascinating question of how such apparently fragile cell appendages could be so intimately interdigitated with the host tissue. Since the appearance was exhibited in more than one area of an osteoclast, we also feel that reporting of these multiple sites of apparent resorption, in vitro, lends support to similar observations previously obtained from in vitro studies (see also response to next question).

A. Nanci: How frequent are osteoclasts with filopodia compared to the ones with pseudopodia? Since not all cells show a clear filopodial border, what are the bases for defining a filopodium as a resorbing unit?

Authors: We did not perform such numerical analyses ever, we suggest that individual filopodia which penetrate the bone surface may have acted as micro-resorbing
units because they were seen within the mineralized bone matrix. With respect to filopodia at the cell border, while our photomicrographs demonstrate the fractured ends of some filopodia that have remained within the bone, we do not imply that all filopodia were necessarily involved in resorption. Indeed, it is now accepted (Gay, 1992) that the same cell may be capable of exhibiting several behavioural patterns simultaneously (see also discussion with S. Jones below).

A. Nanci: In Figures 4b and 4c, at the magnification shown, some of the filopodia can actually be confused with the underlying collagen fibrils. Would a higher magnification be useful in distinguishing these structures by perhaps revealing cross-banding on collagen? 

Authors: The filopodia were distinguished from surrounding matrix by their finger-like shape and their continuity above the bone surface, with the main body of the cytoplasm. This distinction was also made clear by the similarity of the filopodia in Fig. 4 to those in Figs. 3 and 6. Indeed, if the filopodia shown in Fig. 4 were collagen fibres, they should have exhibited cross-banding as we have shown elsewhere (Fig. 9 in Zhou et al., 1994).

A. Nanci: Could the authors comment on why the sealing zone could not be clearly identified in their preparations?

Authors: In some cases (such as in Fig. 5), the field of view simply does not contain areas which could be assigned as sealing zones as shown by previous TEM studies. However, in other cases (such as in Fig. 4a), there are areas which could be considered to correspond to the sealing zone shown by TEM. Nevertheless, we should point out that the theory of the sealing zone has been questioned (Holtrop, 1991). The fact that the osteoclasts resorb bone while they are moving around [Chambers et al., 1984 (text reference); Kanehisa and Heersche, 1988] and also that electron dense markers were seen within the channels of the ruffled border as early as 5 minutes after their injection (Lucht, 1992), would seem to dispute the necessity of a seal for osteoclasts to resorb bone. In this regard, our demonstration of the deep penetration of individual filopodia (Fig. 4) suggest that they may function as isolated units without the need for a general cellular seal.

A. Nanci: What is the evidence that the cell in Fig. 2b is actually attached to bone at multiple sites and what is the nature of these attachment?

Authors: Clearly, when viewing the dorsal surface of cells in SEM, one cannot demonstrate actual attachment. However, the pseudopodia are clearly apposed to the bone surface while the main body of the cell is separated from bone surface by lining cells. Nevertheless, when cells are seen in contact with bone surface, they are commonly considered as attached cells in both SEM and TEM studies [Jones and Boyde, 1977; de Saint-Georges et al., 1989; Fukushima et al., 1991 (text references)].

D.B. Jones: Figure 4d shows a more classical picture of a ruffled border where the filopodia have clear frills. The structure seems to have broken away more cleanly from the surface than in Fig. 6a. Since the osteoclasts are very tightly attached to the surface, it seems that we are looking here at an artifact. I would like the authors to comment on this possibility.

Authors: We think that the wide separation in Fig. 4d between the ruffled border and the underlying bone surface is an artifact resulting from critical point drying. Also, some of the filopodia ends may have broken away which is more obvious in Fig. 6b where they remain buried in the underlying matrix. Therefore the artificial separation may partly depend on how firmly the cells are originally attached to the bone surface, which is probably related to their functional status.

S. Jones: The importance of this paper lies in the challenge to the concept of the annular clear zone as a sealant ring, and the proposal that a communicating network of channels and vacuoles within the ruffled border functions as a secondary lysosome. Do the authors agree that their hypothesis is supported by results from confocal microscopy of actively resorbing osteoclasts [Taylor et al., 1989 (text reference)], where vinculin-containing foci were demonstrated throughout the ruffled border zone, and could represent compartmentalized resorption sites?

A. Nanci: The authors describe that the surface in contact with bone shows channels and vacuoles. Are the cavities illustrated true membrane-enclosed vacuoles?

S.C. Miller: It is not clear at all how the authors are defining the extracellular space. Are the authors referring to a space between the ruffled border and the putative bone surface, or a true extracellular space? Such extracellular space appear to occur in the authors own illustrations. Are not the channels that contain "particulate matter" extracellular?

Authors: The results from the present SEM study showed no visible extracellular space between the ruffled border filopodia and the surrounding bone matrix which they penetrated (Fig. 4). Thus, the commonly described sub-compartment, between the ruffled border and the underlying bone surface, did not actually exist. Instead, within the ruffled border and the inner part of the cell body, many channels and vacuoles were seen which were apparently not completely enclosed by membrane but were, in fact, extracellular. These channels and vacuoles formed a communicating network which may function as a secondary lysosome. The work by Taylor et al. (1989, text reference), which revealed the extensive contacting foci throughout the ruffled border area from the cell body level down to the depth of the resorption lacuna, is confirmed by the present demonstration of individual penetration of the ruffled border filopodia, which could represent a network of locally compartmentalized resorption sites.

See over for Additional References. 
**Additional References**


