The cellular mechanisms accounting for the progressive osteolysis associated with skeletal metastasis are unclear. Osteoclasts account for much of the bone resorption seen in the early stages of tumour metastasis (Galasko, 1976), but the contribution of other cells, principally tumour cells and tumour-associated macrophages (TAMs) within the tumour deposits in bone, is uncertain (Galasko, 1976; Galasko, 1982; Eilon & Mundy, 1978; Koeffler et al., 1978; Novak et al., 1984). This is largely because direct evidence of bone resorption by these cells has not clearly been demonstrated. However, there is now increasing evidence to show that mononuclear phagocytes derived from inflammatory and neoplastic lesions are capable of bone resorption (Athanasou et al., 1989; Athanasou et al., 1991; Athanasou et al., 1992), and that osteoclast-like cells can form directly from tissue macrophages (Udagawa et al., 1990).

Materials and methods

TAMs were isolated from five pneumonectomy specimens for lung carcinoma (three adenocarcinomas; two squamous carcinomas) by collagenase digestion and substrate adherence from 2 x 2 x 2 cm portion of tumour (McGee & Myrvik, 1981), and alveolar macrophages (AMs) from equal portions of lung uninvolved by tumour (Haskill, 1981). TAM or AM-containing cell suspensions in alpha minimal essential medium plus 10% foetal calf serum (Gibco) (MEM/FCS) were added to 6 mm wells (500 cells/well) containing either bone slices or glass coverslips, half of which had been seeded (1,000 cells/well) 24 h earlier with the mouse marrow stromal cell line ST2 (Riken cell bank, Japan) (Athanasou et al., 1989). Four bone slices (half previously seeded with ST2 cells) were studied for each variable, and cell cultures were incubated at 37°C (5% CO₂). After setting for 1 h, these were washed vigorously in MEM/FCS and placed in 16 mm wells containing αMEM/FCS. Dexamethasone (Sigma, UK) (10⁻² M) and 1.25 dihydroxy Vitamin D₃ (Roche, UK) (10⁻¹⁴ M) were added at the beginning of co-cultures with ST2 cells and at each change of medium (every 3 days). Adherent cells on bone slices were cultured for 24 h, 3, 7 and 14 days after which evidence of bone resorption was sought by scanning electron microscopy (SEM) (Chambers et al., 1984). The effect of PTH (NIBSC, UK) (10 IU/ml), PGE₂ (Sigma, UK) (10⁻¹² M) and conditioned medium (derived from culture of ST2 cells alone) on bone resorption by TAMs and AMs isolated from two of the above specimens was also examined. Cells cultured on glass coverslips for similar periods were assessed for several osteoclast characteristics viz. morphological response to salmon calcitonin (Rorer Pharmaceuticals, UK) 1 μg ml⁻¹ (Chambers & Magnus, 1982), acid phosphatase (AP) and tartrate-resistant acid phosphatase (TRAP) staining (Minkin, 1982) as well as indirect immunoperoxidase staining for CD14 (UCHM1) and CD68 (EBM/11) (Athanasou & Quinn, 1990) (macrophage-associated antigens) and epithelial cytokeratin (LP34) (Dakopatts a/s).

Results

Adherent mononuclear cells cultured on coverslips in the absence of ST2 cells were identified after 2 h and 24 h in culture as macrophages (TAMs) on the basis that they were acid phosphatase positive, TRAP negative, expressed monocyte/macrophage markers CD14 and CD68 and were negative for cytokeratins. Unlike osteoclasts, they were TRAP negative, CD14 positive (Athanasou & Quinn, 1990) and did not respond morphologically to calcitonin (Chambers & Magnus, 1982). No multinucleated cells were present amongst the isolated cells. Over the 14 day period of incubation, increased numbers of scattered TRAP positive cells, both mononuclear and multinucleated, were noted, but distinct cell clusters were not formed. In contrast, isolated TAMs co-cultured with ST2 cells showed more marked staining and earlier development of TRAP positivity with formation of a few small, relatively well-defined clusters of TRAP positive mononuclear cells which first appeared at about day 5 (Figure 1). By day 14, these clusters had grown larger and were composed almost exclusively of TRAP positive mononuclear and multinucleated cells.

On bone slices which contained TAMs but no ST2 cells, numerous ovoid or spindle-shaped cells (up to 25 μm diameter) were scattered over the bone slice. These had the SEM morphology of macrophages with numerous surface ruffles and widely spaced microvilli over the surface. After 3 days in culture, the bone slices, particularly around small collections of TAMs, showed poorly-defined roughening of the bone surface with exposure of mineralised collagen fibres (Figure 2a). These were more pronounced in cultures of longer duration, their extent being fully realised after the cells had been entirely removed from the bone surface. After 14 days in culture, occasional shallow surface of small diameter (generally less than 200 μm) were also noted (Figure 2b). These, however, were not a constant feature in all of our cultures. Large resorption pits were not
A collection of TRAP positive mononuclear and larger multinucleated TAMs forming an ST2 layer after 14 days incubation (× 100).

Figure 1

Lung adenocarcinoma-derived TAM with surrounding surface roughening after 3 days incubation. (Bar = 1 micron). (b) Shallow resorption pit with surrounding surface roughening on surface of bone slice on which lung adenocarcinoma-derived TAMs were cultured in absence of ST2 cells (14 days incubation). (Bar = 5 microns).

Figure 2

Large compound resorption pit on surface of bone slice on which TAMs were cultured for 14 days in presence of ST2 cells. (Bar = 10 microns).

Figure 3

Discussion

This study has shown that TAMs are capable of bone resorption. Macrophages are a major component of the host reaction to tumour infiltration and are often abundant in the vicinity of tumour metastases. Although individually most TAMs would appear to resorb less than osteoclasts, the collective effect of such macrophage-mediated low-grade resorption is likely in quantitative terms to be significant. However, it would appear that there is also a minor subpopulation of TAMs that are capable of differentiating into high-grade bone resoring cells in the presence of marrow stromal cells.

Two phases of osteolysis associated with tumour metastasis have been described (Galasko, 1976). In the early phase, osteoclasts predominate and bone resorption proceeds rapidly with the establishment of an osteolytic metastasis. In the later phase, the metastasis grows more slowly and few or
no osteoclasts are evident. Low-grade bone resorption by TAMs could account for the manner in which osteolysis proceeds in this later phase. Non-tumour derived AMs were similarly capable of surface resorption, an observation which accords with many previous studies which have provided indirect evidence that monocytes (Mundy et al., 1977), macrophages (Teitelbaum et al., 1979) and their fused products (Fallon et al., 1983) are capable of bone degradation. Extensive low-grade surface resorption by the numerous mononuclear phagocytes isolated in these studies may be the functional and morphological correlate of this phenomenon. We have previously shown that macrophages and macrophage polykaryons isolated from extraskeletal tumours containing large numbers of these cells are also capable of bone resorption (Athanasou et al., 1989; Athanasou, 1991b); such resorption is similar in type to that described in this study with formation of a few resorption pits and extensive low-grade surface resorption.

High-grade (osteoclast-like) lacunar bone resorption was only seen when TAMs were co-cultured with stromal cells. Unlike osteoclasts which resorb all the components of bone unaided by other cell types and do so within the first 24 h in culture (Chambers et al., 1984), TAM-associated bone resorption did not occur until 14 days in culture and only in the presence of stromal cells. At this time, clusters of heavily TRAP positive mononuclear and multinucleated cells are present on glass coverslips incubated in parallel with the bone slices. Stromal cells are not necessary for adsorption of AMs or TAMs onto bone slices as similar numbers of mononuclear phagocytes are isolated both in the presence and absence of ST2 cells. It appears that the stromal cells are necessary to influence the differentiation of a subpopulation of TAMs into bone-resorbing cells, as relatively few areas of lacunar resorption are present on bone slices relative to the number of TAMs isolated. Udagawa et al. have shown that murine monocytes and mature tissue macrophages, when co-cultured with ST2 stromal cells, can similarly fuse to form bone-resorbing cells. In our study, non-tumour-derived macrophages did not share the same capacity as TAMs to differentiate into high-grade bone-resorbing cells. A small proportion of DNA-synthesising mononuclear phagocytes, are known to form part of the macrophage pool in various body cavities and organs (van Furth, 1989). These proliferating mononuclear phagocytes are marrow derived and cannot be distinguished from resident macrophages. Pluripotent haemopoietic stem cells and certain committed progenitors are also known to be present in the peripheral circulation (Gold & Takaku, 1988). Within the cell population of TAMs isolated from tumours, there may be increased numbers of such relatively undifferentiated mononuclear phagocytes or stem cells; these may further differentiate in situ into cells capable of performing the highly specialised function of a cell of the mononuclear phagocyte system (MPS) appropriate to that tissue location. TAM differentiation into TRAP positive multinucleated cells which, like osteoclasts, the specialised MPS cell of bone, can form resorption pits may thus provide a mechanism whereby high-grade tumour osteolysis occurs in skeletal metastases.

Selective increase in carcinomas of numerous TAMs which form TRAP positive multinucleated cells also accords with the observation that in vitro transformation of blood monocytes to multinucleated cells is greatly increased in cancer patients and their relatives (Al Sumidiae et al., 1986). This may indicate that such cells are also present in the circulation. The manner in which stromal cells produce a suitable microenvironment for further differentiation of tumour-derived mononuclear phagocytes into bone-resorbing cells, the exact nature of these cells, and whether only a few or all tissue macrophages within a tumour are subject to this influence, remains to be determined. Absence of the effect of hormonal stimulation or conditioned medium alone on bone resorption by TAMs suggests that cell-cell contact may be important for this phenomenon to occur. The role of stromal cells in the further differentiation of TAMs also suggests that, at least in part, the diversity of macrophage heterogeneity may be determined by local tissue factors (Metcalf, 1984).

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Table 1 Summary of histochemical and functional characteristics of isolated and cultured (14 days) tumour-associated macrophages (TAMs) and alveolar macrophages (AMs) in the presence and absence of ST2 stromal cells, and comparison with those of osteoclasts

| Isolated AMs and TAMs (Days) | TAMs alone (ST2 cells) | TAMs + ST2 cells (Days) | AMs alone (Days) | AMs + ST2 cells (Days) | Osteoclasts* |
|-----------------------------|-----------------------|------------------------|-----------------|------------------------|-------------|
| TRAP                        | +                     | ++                     | +               | +++                    | ++          |
| Calcitonin response         | No reaction           | No reaction            | NA              | No reaction            | NA Inhibition |
| Bone resorption             | NA SR                 | SR LR                  | SR SR SR SR    | SR SR SR SR LR        |

* (Chambers et al., 1984).
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