Presence of osteoclast-like multinucleated giant cells in the bone and nonostotic lesions of Langerhans cell histiocytosis

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Langerhans cell histiocytosis (LCH) is a disease that can involve one or multiple organ systems characterized by an accumulation of CD1a+ Langerhans-like cells as well as several other myeloid cell types. The precise origin and role of one of these populations, the multinucleated giant cell (MGC), in this disease remains unknown. This work shows that in three different lesional tissues, bone, skin, and lymph node, the MGCs expressed the characteristic osteoclast markers, tartrate-resistant acid phosphatase and vitronectin receptor, as well as the enzymes cathepsin K and matrix metalloproteinase-9. Although, in bone lesions, the osteoclast-like MGCs were only CD68+, in the nonostotic sites, they coexpressed CD1a. The presence of osteoclast-like MGCs may be explained by the production of osteoclast-inducing cytokines such as receptor activator of nuclear factor κB ligand and macrophage colony-stimulating factor by both the CD1a+ LCH cells and T cells in these lesions. As osteoclast-derived enzymes play a major role in tissue destruction, the osteoclast-like nature of MGCs in all LCH lesions makes them a potential target for the treatment of this disease.
Thus, it is clear that the cellular environment plays a crucial role in cell differentiation. In this report, we demonstrate that the cytokine environment of LCH lesions may allow local formation rather than attraction of osteoclast-like MGCs. The local formation may explain the coexpression of CD1a observed on osteoclast-like MGCs in nonbone lesions as the normal osteoclast precursors are likely to be absent in these tissues. So, although the phenotype of the osteoclast-like MGCs was more normal in bone lesions, it seems likely that this population must contribute a large part of the chronic tissue destruction in all LCH lesions. Thus, the osteoclast-like nature of MGCs provides a rationale for the successful treatment of LCH patients with antiosteoclast therapy.

Figure 1. Phenotypic characterization of MGCs in LCH lesions for osteoclast markers. (A) MGCs in bone (left) and skin (right) LCH lesions were TRAP⁺. (B) Triple-color immunofluorescent staining for CD68, CatK, and VNR in an LCH bone lesion (left) and a skin lesion (right). (C) Double immunofluorescent staining for CD68 and another osteoclast marker, MMP-9, in an LCH bone lesion. Original magnifications, (A) 220; (B) 290; and (C) 270.
RESULTS AND DISCUSSION

MGCs in LCH lesions phenotypically express osteoclast markers

Although the different types of MGCs all have a hematopoietic precursor, the osteoclast has very distinct functional and phenotypic characteristics (3). Thus, to clarify whether the MGCs observed in LCH lesions are indeed of an osteoclast-like phenotype, we performed multicolor immunohistochemical analysis for the typical osteoclast markers, CD68, tartrate-resistant acid phosphatase (TRAP), vitronectin receptor (VNR), and the enzymes cathepsin K (CatK) and matrix metalloproteinase–9 (MMP-9; Table I and Fig. 1).

CD68, a marker of the monocyte–macrophage lineage cells, was used to detect MGCs in LCH lesions. CD68 was observed in 13 out of the 15 LCH bone biopsies analyzed. Importantly, MGCs were also found in nonostotic LCH lesional sites, namely the lymph node (4/4) and the skin (3/7). Five out of seven nonostotic lesions that contained MGCs stained positive for TRAP (Fig. 1 A, right), an enzyme present in osteoclastic vesicles that fuse with endocytic vesicles containing the bone matrix degradation products. This enzyme induces the release of reactive oxygen species that destroy the matrix components of the bone (16). 9 out of the 13 bone lesions also showed TRAP positivity on the CD68+ MGCs (Fig. 1 A, left). Triple immunofluorescent staining for CD68, VNR, and CatK showed that all the bone (Fig. 1 B, left) and lymph node lesions (not depicted) with MGCs were VNR and CatK+. In contrast, one out of three skin lesions containing MGCs was positive for VNR and CatK (Fig. 1 B, right). A further enzyme characteristic of osteoclasts, MMP-9, was also present on the CD68+ MGCs in all bone lesions (Fig. 1 C). Moreover, MMP-9 was also expressed in the MGCs of skin (1/3) and lymph node lesions (4/4). CatK and MMP-9 are proteases.

Table I. Characterization of MGCs in LCH lesions for osteoclast markers, osteoclast-secreted enzymes, and osteoclast-inducing environment

| Lesional site | MGCs in lesion | Lesional environment |
|--------------|----------------|---------------------|
|              | Phenotypic markers of osteoclasts | Osteoclast-secreting enzymes | Osteoclast-inducing cytokines and receptor |
|              | CD1a CD68 TRAP VNR CatK MMP-9 | M-CSF RANKL RANK |
| Bone         |                |                     |
| b1           | – + + + + + + + + + | # # # |
| b2           | – + + – + + + + + | – # # |
| b3           | – – – + + + + + | # # # |
| b4           | – + + + + + + + + | # # # |
| b5           | – + + + + + + + + | – # # |
| b6           | – + + + + + + + + + | # # # |
| b7           | – + + + + + + + + + | # – # |
| b8           | – – – – – – – | # # # |
| b9           | – + + – + + + + | # – # |
| b10          | – + + + + + + + | # – # |
| b11          | – + + + + + + + | # # # |
| b12          | – + + + + + + + + + | # – # |
| b13          | – + + – + + + + | – # # |
| b14          | – + + + + + + + + + | # # # |
| b15          | – + + + + + + + + + | # # # |
| Skin         |                |                     |
| s1           | + + + + + + + + + | # # # |
| s2           | – – – – – – – | # – # |
| s3           | – – – – – – – | ND – # |
| s4           | – – – – – – – | ND ND – |
| s5           | – + + – – – – | ND ND ND |
| s6           | – + – – – – – | – # # |
| s7           | – – – – – – – | – – – |
| Lymph node   |                |                     |
| l1           | + + + + + + + + | # # ND |
| l2           | ND + + + + + + | # # # |
| l3           | – + + + + + + + | # # # |
| l4           | + + + + + + + | – # # |
involved in the degradation of organic components from the bone matrix, such as type I collagen and other matrix proteins (17, 18). VNR is a receptor for the integrin vitronectin commonly found in osteoclasts and likely to be involved in the interaction between the osteoclast and the bone matrix (19). Thus, the expression of typical osteoclast markers as well as characteristic osteoclast-secreted enzymes by the MGCs in LCH lesions confirms that these cells are indeed osteoclast-like MGCs.

**Possible origin of MGCs in LCH lesions**

The presence of these osteoclast-like giant cells in LCH bone lesions is perhaps not that unusual as this is the normal tissue site for osteoclasts, which, through their resorbing activity, help to maintain the normal homeostasis of the bone (6). However, even in the ostotic LCH lesions, these osteoclast-like cells were present in relatively higher numbers than in normal bone and appeared to be “floating” within the cellular infiltrate of the lesion, whereas normally close contact with bone would be expected. In contrast, the finding of osteoclast-like cells in nonostotic LCH sites raises the question of their origin. To investigate this, we performed triple immunofluorescent stainings for the LC marker, CD1a, the macrophage marker, CD68, and CatK to more clearly identify the MGCs. In all bone lesions, the CatK+ osteoclast-like cells coexpressed the macrophage marker CD68. In none of the ostotic lesions did these osteoclast-like MGCs express CD1a (Table I and Fig. 2 A). This finding suggests that the MGCs in bone LCH display the features of a normal osteoclast. In contrast, in one out of three skin and two out of four lymph node lesions that contained osteoclast-like cells, the MGCs expressed both CD68 and CD1a (Table I and Fig. 2 B). Hence, although both the osteoclast-like giant cells in bone as well as in nonbone lesions expressed CD68, only the giant cells in skin and lymph node coexpressed CD1a. This unusual phenotype of these osteoclast-like giant cells in skin LCH has been reported before in a single case without any further characterization (20). The majority of nonostotic lesions studied were in fact from patients without additional bone lesions. This excludes the possibility that the MGCs were derived from bone lesions. Therefore, it is likely that the lesional environment induces the local formation of the osteoclast-like MGCs even in unusual sites, such as these nonostotic LCH sites. This, together with the fact that the normal precursors of osteoclasts are likely to be absent from these sites, may result in osteoclast-like MGCs derived from a different origin (e.g., CD1a+ cells). Alternatively, the CD1a+ expression by MGCs in these sites may be due to induced expression of CD1a at a later stage.

To better understand the likely mechanisms of MGC formation in LCH lesions, we looked at the expression of intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule expressed by monocytes upon fusion to form MGCs (21) and Ki-67, a nuclear protein associated with somatic cell proliferation (22). We found that in all LCH lesions, the MGCs displayed strong membrane staining for ICAM-1. In contrast, the MGCs were consistently negative for the proliferative marker Ki-67 (unpublished data). However, there was a high expression of Ki-67 in other cells in the lesions that we and others have shown previously to be largely due to the CD1a+ LCH cells (23, 24). These findings suggest that the osteoclast-like MGCs present in LCH lesions may be formed by the fusion of resident monocytes–macrophages rather than by cell division. Cytokines such as IFN-γ, which has previously been shown to be expressed in LCH lesions (25), are well-known inducers of ICAM-1 expression and, thus, may initiate the fusion of monocytes and macrophages to form MGCs. Thus, MGCs seem to be intrinsic to LCH lesions and specific factors within the well-

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**Figure 2. Phenotypic difference in osteoclast-like MGCs in bone versus nonbone lesions.** Triple immunofluorescent staining for the monocyte lineage marker, CD68, DC marker, CD1a, and the enzyme CatK was performed in LCH bone (A) and skin (B). The osteoclasts in LCH skin lesions clearly expressed CD1a as well as CD68 and CatK (B). In contrast, LCH bone lesions never expressed CD1a (A). Original magnification, 260.
characterized “cytokine storm” in LCH lesions are responsible for their formation.

**The osteoclast-inducing cytokines RANKL and M-CSF are highly expressed in LCH lesions**

As shown by in vitro studies, the environment in which the mononuclear cells present determines their differentiation into the various mononuclear phagocyte system-derived cells. Similarly, the tissue site and environment may have a large influence on the cellular composition of LCH lesions. Previous work by our group and others has clearly shown the presence of a cytokine storm in LCH lesions (25). For instance, factors involved in osteoclastogenesis such as IL-1, IL-6, and TNFα are highly expressed. In the present report, we have extended the analysis of cytokines to those specifically involved in the induction of osteoclast differentiation. One such cytokine involved in osteoclast induction is RANKL. In 24 LCH lesions studied for RANKL expression, 17 were found to be positive. We found that this cytokine was not expressed by the endothelial cells and macrophages, as assessed by triple staining combining RANKL with the CD31 and CD68 markers, respectively (unpublished data). Instead, triple staining for RANKL, CD1a to identify the LCH cells and the T cell marker CD3, revealed that the majority of CD1a+ LCH cells and T cells in close proximity to the LCH cells expressed RANKL (Fig. 3 A). Thus, both the CD1a+ LCH cells and T cells contribute to osteoclastogenesis through up-regulated RANKL and, thus, provide a mechanism for the potentiation of osteoclast formation and bone resorption in LCH lesions.

One key feature of osteoclast differentiation is the interaction between RANKL and its receptor, RANK, commonly expressed by the osteoclast precursor cells. We looked at the presence of RANK receptor on CD68+ and CD1a+ cells by triple immunofluorescent staining. All the lesions that showed expression of RANKL were also positive for RANK, which was expressed by a high proportion of CD1a+ cells and to a lesser extent by CD68 cells as shown in Fig. 3 B. The expression of RANK by CD1a+ cells as well as the presence of its ligand by activated T cells in LCH lesions is also important, as this interaction is known to induce a survival signal to DCs (26).

Furthermore, we looked at the expression of another cytokine known to be involved in osteoclast differentiation, M-CSF. M-CSF is normally produced by osteoblasts and/or stromal cells and is involved in the differentiation of osteoclasts from an early stage. We found it to be expressed by the MGCs and strikingly also by CD1a cells in 11 out of 15 LCH bone lesions. Interestingly, we found that this cytokine

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**Figure 3. Expression of cytokines known to be involved in osteoclast differentiation in bone and skin LCH.** Two- and three-color immunofluorescent stainings were performed for the cytokines RANKL and its receptor RANK and M-CSF. (A) Representative picture of an LCH bone lesion showing that the majority of the CD1a+ LCH cells (blue) express RANKL (green). This colocalization results in a turquoise color. In addition, many of the neighboring T cells (red) also expressed RANKL (green). This colocalization resulted in a yellow color. (B) The CD1a+ LCH cells (red) also expressed RANKR (green). This colocalization results in a yellow color in the merged image. (C) Representative picture of an LCH skin lesion showing that the osteoclast differentiation cytokine, M-CSF (green), was expressed by the CD1a+ LCH cells (red). This colocalization resulted in a yellow color in the merged image. In contrast, normal LCs (indicated by arrows) in the epidermis (E) did not express any M-CSF. Original magnification, (A, B, and C) 270.
showed characteristic CD1a by immunohistochemistry using S-100 and CD1a as markers. All biopsies embedded tissue from 26 patients with a diagnosis of LCH were identified.

MATERIALS AND METHODS

To date, only a few case reports, including one we authored, have provided support for the hypothesis that the MGCs in LCH lesions express various matrix-degrading enzymes suggestive of the resorbing capacity of the MGCs in LCH lesions. Such a role would also help explain the predominant symptom of bone pain suffered by patients with LCH bone lesions. The present report has provided support for the hypothesis that the excessive bony destruction found in LCH lesions is likely mediated by osteoclast-like giant cells. Therefore, these cells are a potential target in LCH lesions.

To date, only a few case reports, including one we authored, have indicated the use of bisphosphonates as a successful treatment of bone LCH (28). However, all of these case reports lack the fundamental background for the rationale. Bisphosphonates appear to act when administered at therapeutic doses only in bone, which is probably due to their specific affinity to this tissue. This group of compounds is known to have an inhibitory effect on the number and activation of osteoclasts (29). Thus, this study has provided a rationale for the use of bisphosphonates in the treatment of LCH patients.

Rationale for the use of bisphosphonates in the treatment of LCH

Due to the lack of fresh biopsy material, it was not possible to perform functional studies such as the use of dentine discs to determine the resorbing capacity of the MGCs in LCH lesions. However, the finding that the MGCs in LCH lesions are expressing various matrix-degrading enzymes supports the hypothesis of a destructive role for these cells in LCH lesions. Such a role would also help explain the predominant symptom of bone pain suffered by patients with LCH bone lesions. The present report has provided support for the hypothesis that the excessive bony destruction found in LCH lesions is likely mediated by osteoclast-like giant cells. Therefore, these cells are a potential target in LCH lesions.

Single enzymatic staining for TRAP. TRAP staining was performed using a combination of solutions that include naphthol-AS BI phosphate, dimethylformamide, tartaric acid, acetate buffer, veronal buffer, sodium nitrite, and paranaseoline. Tissue sections were deparaffinized, rehydrated, and incubated with the reactive solution for 20 min. After washing with distilled water, the tissue sections were counterstained with Mayer's hematoxylin and mounted using Histomount media (National Diagnostics). We would like to thank Dr. R. Jaffe for supplying some of the LCH skin lesions used in this work. C. da Costa is supported by Fundação para a Ciencia e a Tecnologia, Portugal. This work was partly funded by The Histiocytosis Association of America.

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REFERENCES

1. Egeler, R.M., and G.J. D’Anngio. 1995. Langerhans cell histiocytosis. J. Pediatr. 127:1–11.
2. Favara, B.E., and R. Jaffe. 1994. The histopathology of Langerhans cell histiocytosis. Br. J. Cancer. 23:S17–S23.
3. Anderson, J.M. 2000. Multinucleated giant cells. Curr. Opin. Hematol. 7:40–47.
4. Byrd, T.F. 1998. Multinucleated giant cell formation induced by...
IFNγ/IL-3 is associated with restriction of virulent Mycobacterium tuberculosis cell to cell invasion in human monocyte monolayers. Cell. Immunol. 188:89–96.
5. McNally, A.K., and J.M. Anderson. 1995. Interleukin-4 induces foreign body giant cells from human monocytes/macrophages: differential lymphokine regulation of macrophage fusion leads to morphological variants of multinucleated giant cells. Am. J. Pathol. 147:1487–1499.
6. Tetculeba, S.L. 2000. Bone resorption by osteoclasts. Science. 289: 1504–1508.
7. Murch, A.R., M.D. Grounds, C.A. Marshall, and J.M. Papadimitriou. 1995. Interleukin-4 induces formation of human monocyte/macrophage fusion and macrophage mannose receptor expression. J. Immunol. 158:3385–3390.
8. Kudo, O., Y. Fujikawa, I. Itonaga, A. Sabokbar, T. Torisu, and N.A. Athanasou. 2002. Proinflammatory cytokine (TNFalpha/IL-1alpha) gene products in Langerhans cell histiocytosis. J. Pediatr. Hematol. Oncol. 24:727–732.
9. Ruco, L.P., A. Stoppani, D. Vitolo, S. Uccini, and C.D. Baroni. 1993. Expression of adhesion molecules in Langerhans' cell histiocytosis. Histopathology. 23:29–37.
10. Shankar, G., I. Davison, M.H. Helfrich, W.T. Mason, and M.A. Horton. 1993. Integrin receptor-mediated mobilisation of intracellular calcium in rat osteoclasts. J. Cell Sci. 105:61–68.
11. Tsurukai, T., N. Udagawa, K. Matsuzaki, N. Takahashi, and T. Suda. 2000. Roles of macrophage-colony stimulating factor and osteoclast differentiation factor in osteoclastogenesis. J. Bone Miner. Metab. 18: 177–184.
12. Paquette, R.L., N.C. Hsu, S.M. Kiertscher, A.N. Park, L. Tran, M.D. Roth, and J.A. Glasy. 1998. Interferon-alpha and granulocyte-macrophage colony-stimulating factor differentiate peripheral blood monocytes into potient antigen-presenting cells. J. Leukoc. Biol. 64:338–367.
13. Duperrié, K., A. Eljaafari, C. Dezutter-Dambuyant, C. Bardin, C. Jacquet, K. Youeda, D. Schmitt, L. Gebuhrer, and D. Rigal. 2000. Distinct subsets of dendritic cells resembling dermal DCs can be generated in vitro from monocytes, in the presence of different serum supplement. J. Immunol. Methods. 238:119–131.
14. Verhaeghe, G., J. Kleijn, X. Paliard, W.Z. Wei, and A. Galy. 2000. Dendritic cells generated from CD34+ progenitor cells with flt3 ligand, c-kit ligand, GM-CSF, IL-4, and TNF-alpha are functional antigen-presenting cells resembling mature monocyte-derived dendritic cells. J. Immunother. 23:48–58.
15. Serum-Delpart, C., S. Arnaud, P. Jurdic, S. Natuf, M.F. Grasset, C. Soulais, C. Donnenget, O. Destaing, A. Rivollier, M. Perret, et al. 2002. Flk3+ macrophage precursors commit sequentially to osteoclasts, dendritic cells and microglia. BMC Immunol. 3:15–25.
16. Halleen, J.M., S. Kaisanen, J.I. Salo, S.V. Reddy, G.D. Roodman, T.A. Hentunen, P.P. Lehenkan, H. Kuja, P. Vihko, and H.K. Vaananen. 1999. Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase. J. Biol. Chem. 274:22907–22910.
17. Bossard, M.J., T.A. Tomaszkiew, S.K. Thompson, B.Y. Amegadzie, C.R. Hanning, C. Jones, J.T. Kurtuly, D.E. McNulty, F.H. Drake, M. Gowen, and M.A. Levy. 1996. Proteolytic activity of human osteoclast cathepsin K. Expression, purification, activation, and substrate identification. J. Biol. Chem. 271:12517–12524.
18. Okada, Y., K. Naka, K. Kawamura, T. Matsumoto, I. Nakano, N. Fujimoto, H. Sato, and M. Seki. 1995. Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase B) in osteoclasts: implications for bone resorption. Lab. Invest. 72:311–322.
19. Shankar, G., I. Davison, M.H. Helfrich, W.T. Mason, and M.A. Horton. 1993. Integrin receptor-mediated mobilisation of intracellular calcium in rat osteoclasts. J. Cell Sci. 105:61–68.
20. Duperrié, K., A. Eljaafari, C. Dezutter-Dambuyant, C. Bardin, C. Jacquet, K. Youeda, D. Schmitt, L. Gebuhrer, and D. Rigal. 2000. Distinct subsets of dendritic cells resembling dermal DCs can be generated in vitro from monocytes, in the presence of different serum supplement. J. Immunol. Methods. 238:119–131.