OSTEOCLASTOGENESIS AND LYMPH NODE ORGANOGENESIS
IN DIFFERENT SPECIES OF EGYPTIAN RATS

BY
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Abstract

The development of lymphoid organs depends on the correct expression of several molecules within a defined timeframe during ontogeny. Although this is an extremely complex process, with each secondary lymphoid tissue requiring subtly different signals, a common framework for lymphoid development is beginning to emerge. Bone remodeling is tightly regulated by a molecular trial composed of OPG/RANK/RANKL. The receptor activator of RANKL (localized on osteoblasts) enhances osteoclastogenesis via interaction with its receptor RANK (localized on osteoclasts), whereas osteoprotegerin (OPG) (produced by osteoblasts) inhibits this osteoclastogenesis by binding to RANKL. The RANK provides critical signals necessary for lymph node organogenesis and osteoclast differentiation. The TNF family molecule OPGL has been identified as a potential osteoclast differentiation factor and regulator of interactions between T cells and dendritic cells in vitro. Thus OPGL is a new regulator of lymph node organogenesis and lymphocyte development and is an essential osteoclast differentiation factor in vivo. So, the result of this study showed that lymph node organogenesis appears to require adequate quantity of RANKL, and this significant level can apparently persist despite marked overexposure of the soluble RANKL inhibitor OPG.

Keywords: Osteoclastogenesis, Osteoprotegerin, RANK, RANKL, Bone remodeling, Organogenesis, Lymphocyte development, Lymph node, Rats.

Introduction

The lymphatic system occupies the very important position in physiology, pathology and clinical medicine, as is attested by its relation to tissue drainage, digestion, infection and inflammation, malignancy and edema (Bekker et al., 2001; Hou et al., 2011). However, the increase in knowledge along this line depends considerably on the development of data concerning the anatomy and distribution of the entire system (Higgins, 1925). Nopajaroonsoi et al. (1971) considered the lymph nodes as the highly specialized immune-competent organs with distinct topographic and cellular characteristics, which are altered in a specific manner in response to different forms of stimulation. Lymph nodes of some rodent species have been extensively studied by light and electron microscopy and their histological features are well known (Nopajaroonsoi et al., 1971).
The essential role of RANKL in the formation, function and survival of osteoclasts is well established (Lacey et al., 2000). This TNF family member is required for the existence of osteoclasts and for the resorption of bone, as shown by the total absence of osteoclasts in knockout mice that lacks either RANKL (Kong et al., 1999) or its receptor RANKL (Dougall et al., 1999; Li et al., 2000). Analysis of these knockout animals also revealed an essential role for RANKL in the formation of lymph nodes (Pettit et al., 2001; Redlich et al., 2002; Campagnolo et al., 2002; Stolina et al., 2005; 2007). Genetic ablation of RANKL was associated with lymph node agenesis, although Peyer’s patches appeared normal and splenic lymphoid areas remained intact (Anderson et al., 1997; Kong et al., 1999; Bachman et al., 1999). These observations suggest that the immune phenotype of RANK and RANKL knockout mice might be largely restricted to lymph node agenesis are the consequence thereof. However, it is also possible that RANKL plays an ongoing role in the function of the developed immune system (Lacey et al., 2000; Bekker et al., 2001; Hou et al., 2011). RANKL inhibition is an experimental therapeutic approach for chronic bone loss conditions such as osteoporosis and rheumatoid arthritis, so it is important to characterize the potential for immune-modulatory effects during RANKL inhibition, we attempted to address these issues by studying the effect of the life-long RANKL inhibition on the development of the immune system and on organogenesis of the lymphoid organs in the three tested species of rats.

As the TNF super-families have been grown to more than two dozen combined members over the past 30 years, their involvement in interactions between immune cells, with regard to the events governing cellular differentiation, activation, and survival have been well established (Lacey et al., 2000; Yasuda et al., 1998). Identification of TNF superfamily cytokine; TRANCE (RANKL/OPGL/ODF/TNFSF 11) that interacts with two receptors—one functional, TRANCE-R (RANK/TNFRSF 11), and one decoy, OPG is a survival factor for activated osteoclast differentiation and the activation, making TRANCE signaling crucial for proper bone homeostasis, and a potential therapeutic to get in disease such as osteoporosis, osteolytic metastatic cancer, arthritis and periodontitis (Dougale et al., 1999; Kong et al., 1999; Kim et al., 2000). Importantly, the positive role that TRANCE has in activating the immune system appears to significantly contribute to pathologic bone loss. These observations have spurred intense study of the various ways in which the immune system can influence bone. Furthermore, TRANCE has also been demonstrated to play essential roles in the developmental processes leading to both lymph node formation, and the expansion and function of mammary gland during pregnancy and lactation. Thus, TRANCE is quickly emerging as a cytokine of significant importance to further understanding unique aspects of mammalian biology (Naito et al., 1999; Lories et al., 2001;
Hofbauer and Heufelder, 2001; Thiell et al, 2002; Walsh and Choi, 2003). Characterization of the functions of TRANCE and its receptors have contributed significantly to the emergence of a new field of study, the osteoimmunology, directed at examining the interplay between active immunity and maintenance of bone homeostasis (Yun et al, 1998; Jones et al, 2002). Bone remodeling is tightly controlled by OPG and RANKL; however, the ratio of OPG/RANKL is considered to better reflect environmental signals. A high ratio of OPG/RANKL is indicative of promoting bone formation while a low ratio favors bone resorption. Such imbalance has been encountered in some osteolytic lesions where RANKL is up-regulated as well as in osteoblastic disorders where OPG is up-regulated (Hofbauer and Heufelder, 2001; Stolina et al, 2003; Boyce and Xing, 2008).

This study aims to give a comparative account on lymph node distribution in the three tested species of rats. This is the basis for an attempt to consider the question, whether the comparative anatomy of the lymphoid system might contribute to taxonomy of this group. It is believed, though that the physiologic effects of TRANCE very likely extend beyond the scope of the current study.

Materials and Methods

All studies and experimented procedures followed the guiding principles in guide for the care and use of laboratory animal and were approved by the Institutional Animal Care using Committee of Cairo University, Egypt.

Three species of rats from two different families were used in the present study; with one previously studied species, Rattus norvegicus have been investigated at first as control model.

Family: Arvicolidae
Genus: Clethrionomus: Micortus agrestis (Linnaeus 1761)
Family: Muridae
Genus: Arvicanthis: Arvicanthis niloticus (Desmarest 1822)
Genus: Acomys: Acomys cahirinus (Desmarest 1891)
Genus: Rattus: Rattus norvegicus (Berkenhout 1769)

Morphology of the lymph nodes: At least 10 specimens from each species were killed by ether overdose and dissected directly within few minutes. By the aid of binoculars, regional lymph nodes were examined in all mentioned species. Sketches with the location of the nodes were prepared indicating location, relative size and number of lymph nodes on both sides of the body. A general model of the animal features was drawn and repeated for the different aspects with the delineation of their characteristic lymph node distribution. The nomenclature of lymph nodes is according to Job (1922), Sanders and Florey (1940). The specimen of the adult laboratory rat (R. norvegicus) was investigated as control model of the lymph node distribution.

Biochemical assays for sera: The whole blood aliquots were allowed to clot to produce serum. Concentration of selected cytokines, RANKL, osteocalcin and OPG were measured using
the commercially available, species-specific Luminex Ab-immobilized microbial kits (Linco Research). The murine OPG assay showed > 95% cross-reactivity with recombinant rat OPG standard whereas the murine RANKL assay showed ~ 40% cross-reactivity with recombinant rat RANKL standard. All OPG and RANKL data were generated using these recombinant rat protein standard curves as suggested previously.

Bone histology: Tibiae harvested from each species were fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) for 24h. Samples were decalcified in 10% EDTA (pH 7.5) at 4°C for 2 weeks and paraffin embedded. Five micron longitudinal serial sections from three representative levels were cut from each specimen and stained with hematoxylin and eosin for routine examination.

Statistical analysis: All the tests were performed with the statistical package of Social Science (SPSS) version 15.0 (SPSS, USA). All statistical analysis was carried out at the 5% level of significance (P < 0.05). Data were expressed as mean ± S.E. Comparisons were made using T-test.

Results

Bone mass phenotype: Histological analysis of long bones in A. niloticus confirmed the occurrence of mild osteopetrosis. The long bones were osteopetrotic in appearance, with the epiphysis, metaphysis and diaphysis showing accumulation of cartilage and bone that almost filled the marrow space. The mid-diaphysis bone density was similar to that in the metaphysis, indicating that little, if any resorption of bone occurring. There was no evidence of periosteal bone modeling adjacent to the growth plates. These surfaces appeared smooth in contrast to the eroded surfaces at the site in A. niloticus. Moreover, the columnar structure of chondrocytes was disorganized at the epiphyseal growth plate (Fig. 1A, B). This is consistent with the shortened femurs seen on histological studies. Osteopetrosis was also evident in histological sections of vertebral bodies (Fig. 1C, D). The calvarias of Micortus agrestis was thinner than in other species, and the marrow spaces were reduced. A. cahirinus developed rounded bones, possibly because of the osteopetrotic changes in the vertebral skeleton. Moreover, in histological sections there were no cells showing osteoclast morphology in any of the bones studied (Fig. 1E, F).

Anatomical distribution of lymph nodes: The location and distribution of lymph nodes were consistent in individual animal of every species used in the present study, despite occasional variation in size and number of nodes. The results of this study were compared with lymph nodes in the adult laboratory rat, R. norvegicus which have been extensively studied by Tilney (1971) (Tab. 1). The lymph node groups of the three tested species of rats used in the present study were classified into somatic nodes which drain skin and underlying musculature, and visceral nodes which drain primarily the thoracic, abdominal and pelvic organs.
**R. norvegicus:** The superficial cervical group (1) is represented by only one node which displays two lobes. This is also demonstrated in the posterior cervical group (4), but it is relatively larger in size. The facial group (2) is demarcated by three rounded nodes; one of them is lobulated into two lobes. The internal jugular group (3) is represented by three small rounded nodes at each side of the trachea. The brachial group (5) is demonstrated as two relatively large oval-shaped nodes; one of them is divided into two lobes. The axillary group (6) is detected as two nodes; one is relatively large oval-shaped and the other one is small rounded node. The inguinal group (7) comprises four to six small rounded nodes, which are enclosed within a fat sheath. The superior mesenteric group (12) comprises five relatively large oval-shaped nodes found in the front of the stomach and enclosed with a fat sheath. The gluteal (9), iliac (10) and inferior mesenteric (13) nodes have not been observed in *A. niloticus.*

**A. niloticus:** The nodes have a yellowish-orange color. Among twenty individual studied, some variation was detected (Tab. 1). The superficial cervical group (1) is represented as two nodes; one large oval or small rounded node at each side of the body with the exception of both the popliteal (8) and gluteal (9) groups which are recognized as two relatively large oval-shaped nodes (Tab.1).

**M. agrestis:** The lymph nodes are characterized by their reddish-white color. Their number and location have been described among twenty individuals (Tab. 1). Generally, the superior mesenteric group (12) has the highest number of nodes with a range between four to six large nodes. The number of other groups in this species is relatively small in comparison with the other investigated species. The outstanding difference between this species and the other mentioned two species comprises the absolute absence of the inferior mesenteric (13) lymph nodes. The superficial cervical group (1) forms two equal size nodes at each side of the body. The posterior cervical (4) group also varies from one to three smaller nodes. The axillary (6) group is represented by three rounded nodes at each side of the body; two are relatively larger than the other one. The brachial group (5) occurs as three rounded nodes. The internal jugular group (7) is represented by two to five small rounded nodes enclosed within a fat sheath. The facial group (2) is represented as four extremely large nodes and two relatively small undivided nodes. The popliteal group (8) is represented as one extremely large node and the other one is relatively smaller undivided node. The renal group (11) is represented by three small rounded nodes at each side of the body.
oval-shaped node and two small nodes. The posterior cervical group (4) and the brachial group (5) are only one node at each side of the body; with the brachial group being the largest. The axillary group (6) occurs as relatively three to four small rounded nodes. The superior mesenteric group (12) is represented by four small rounded nodes found around the end of the stomach, and sometimes varied from five to six nodes. On the other hand, the renal (11) and the gluteal group (9) occur as two small rounded nodes. The inferior mesenteric group (13) is varied from one to two small rounded nodes. The facial (2), popliteal (8), inguinal (7) and iliac (10) lymph nodes have not been observed in *M. agrestis*.

*A. cahirinus*: The lymph nodes are characterized by their white color. The number of lymph nodes is relatively small in comparison with the other investigated species. In different location of these nodes, the maximum number of most of them is three, but they are of smaller size. The outstanding difference are marked by the absence of the facial, superior mesenteric, renal, iliac, inguinal, axillary and popliteal lymph nodes in *A. cahirinus* with observation of the inferior mesenteric nodes and absence of superior mesenteric lymph nodes (Tab. 1). The superficial cervical group (1) is represented as three nodes, one large oval-shaped and two small rounded at each side of the body. The posterior cervical group (4) and the internal jugular (3) vary from one to two small rounded nodes. The axillary group (6) is presented by three rounded nodes.

**Serum OPG/RANK levels:** The serum OPG concentration of the *R. norvegicus* and *A. niloticus* groups were significantly higher than those of the other groups (P<0.01, P<0.01, respectively) (Fig. 2A). Notably, the *M. agrestis* had a significantly higher level than those in the *A. cahirinus* group (P<0.05, P<0.05, respectively). Moreover, the *A. cahirinus* group had a significantly high level of OPG/RANK ratio than *A. niloticus* and *M. agrestis* (P<0.05) (Fig. 2B).

### Table 1: Variation in number lymph nodes in the different species of adult rats

<table>
<thead>
<tr>
<th>Lymph node group</th>
<th>Number (Range)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>R. norvegicus</em></td>
</tr>
<tr>
<td>(1) Superficial cervical</td>
<td>1(1-2)</td>
</tr>
<tr>
<td>(2) Facial</td>
<td>3(2-4)</td>
</tr>
<tr>
<td>(3) Internal jugular</td>
<td>3(2-3)</td>
</tr>
<tr>
<td>(4) Posterior cervical</td>
<td>1(1-2)</td>
</tr>
<tr>
<td>(5) Brachial</td>
<td>3(1-3)</td>
</tr>
<tr>
<td>(6) Axillary</td>
<td>2(2-3)</td>
</tr>
<tr>
<td>(7) Inguinal</td>
<td>5(4-6)</td>
</tr>
<tr>
<td>(8) Popliteal</td>
<td>2(1-2)</td>
</tr>
<tr>
<td>(9) Gluteal</td>
<td>2(2-3)</td>
</tr>
<tr>
<td>(10) Iliac</td>
<td>1(1-2)</td>
</tr>
<tr>
<td>(11) Renal</td>
<td>1(1-2)</td>
</tr>
<tr>
<td>(12) Superior mesenteric</td>
<td>4(4-6)</td>
</tr>
<tr>
<td>(13) Inferior mesenteric</td>
<td>4(4-5)</td>
</tr>
</tbody>
</table>

*Each group comprises at least 10 animals*
Fig.1: Histological changes in bone. A, B: Femur of *Arvicandhis niloticus* is shortened and cup-shaped. Morphology of growth plate and columnar organization of chondrocytes (asterix) distributed, and shaft of femur is filled with cartilage and bone (arrowhead). No evidence of periosteal bone modeling occurring next to grown plates. C, D: Osteopetrosis (black arrowhead) of *Micortus agrestis* vertebral bone. Note hematopoietic island localized along vertebra of *M. agrestis* rats (white arrowhead). E, F: Complete absence of osteoclasts in *Acomys cahirinus* rats. X40
Fig. 2: A- Serum level of OPG and RANKL; B- OPG/RANKL ratio in different species of adult rats.

Discussion
The random variations of the lymph node number within species of *A. niloticus, M. agrestis* and *A. cahirinus* were noted. Instability of the lymph nodes and number even in normal animals,
has been demonstrated in albino rat (Kindred, 1938), guinea pig (MacMillan, 1974), laboratory rat (Tilney, 1971), and gerbil. The changes occurring within the lymph nodes are believed to result largely from the disfunction of the normal lymphocytic drainages (Kindred, 1938). From the present results, it could be demonstrated the absence of some lymph node groups in some of the species investigated. However, the absence of these nodes is substituted by other groups, which drain the same region. For instance, the absence of the iliac lymph nodes in *M. agrestis* and *A. cahirinus* and is accompanied by the presence of the gluteal node. Both groups of lymph nodes drain the same parts of the body and have the same afferent drainage to the renal nodes. The approximate total absence of the inguinal group in *M. agrestis* and *A. cahirinus* which drain the gluteal area and lateral aspect of the tail (Tilney, 1971), is substituted by the presence of the gluteal nodes. The absence of the inguinal lymph nodes may be due to the fact that specimens are characterized by their short tail. However, this suggestion is not valid for *A. cahirinus* which has a long tail. Also, the inferior mesenteric group is only present in *M. agrestis* and *A. cahirinus* and not observed in *A. niloticus*. This group of lymph node has the same function as the superior mesenteric group. These observations support the suggestion that the disappearance some lymph node groups, which are substituted by the other groups, and share the same function, may be due to the evolutionary stage of these species and the time appearance of them in such taxa (Stolina *et al.*, 2003). Moreover, the appearance of certain lymph node in the individual of the same species may be related to the occasional need of the body to these nodes as has been suggested earlier (Job, 1922; Bloom and Fawcett, 1968).

From the data obtained in the present study, it seems evident that there is a definite plan in the structure and arrangement of the lymph nodes in species with a wide degree of variation in number of nodes. According to Grau (1974), lower species of mammals (e.g. rodents) have fewer lymph centers, most of which lie in the trunk, whereas peripheral centers, as in the head or extremities may be lacking in particular species to varying degree. However, Gulland (1894) stated that the lymph nodes are few and small in rodents, more numerous in carnivores and better developed in man than in other forms. Lymph nodes of rats, in general, are encapsulated by a thick layer of connective tissue fibers with subcapsular sinuses underneath. The demarcation of cortex and medulla are more prominent than in mice. Primary lymphoid nodules are present in the cortex. Moreover, the predominance of the cortex over the medulla in the posterior cervical, axillary and gluteal nodes was characterized by the increase of their lymphocyte population. The variations in the boundaries between the cortex and the medulla as demonstrated in the posterior cervical, axillary and gluteal nodes might reflect variations in the importance of the immunological reactions undergone by
the cortical part. This phenomenon was postulated in different animal models (Zwerina et al, 2004).

Little information is available in the literature regarding the immunoregulatory properties of OPG. However, OPG is expressed by B cells and DC (Aubin and Bonneley, 2000) and contributes to B cell development (Hou et al, 2011), mice lacking OPG seem to show inability to sustain an antigen specific IgG3 production as the only defect (Stolina et al, 2003). On the other hand, data have been published indicating that the RANKL-RANK system, in addition to being critical for lymphocyte and lymph node development, may contribute to the immune response by stimulating T cells and DC functions (Suda et al, 1999; Bachmann et al, 1999; Josien et al, 1999).

The RANKL-RANK interaction is central to bone metabolism, and treatment with OPG, which blocks this interaction has dramatic effects on bone metabolism in animals and humans (Bekker et al, 2001; Stolina et al, 2003; 2007; Ko et al, 2010; Hou et al, 2011). An expected finding in OPGL/-/- mice has been that these mice completely lack all lymph nodes (Kong et al, 1998). This lack of lymph nodes is not due to a homing defect of lymphocytes into nodes (Kong et al, 1999). The essential role of RANKL in the formation, function and survival of osteoclasts is well established (Lacey et al, 2000). This TNF family member is required for the existence of osteoclasts and for resorption of bone, as shown by the total absence of osteoclasts in knockout mice that lacks either RANK (Kong et al, 1999) or its receptor RANKL (Dougall et al, 1999; Li et al, 2000). Analysis of these knockout animals also revealed an essential role for RANKL in the formation of lymph nodes (Pettit et al, 2001; Redlich et al, 2002; Campagnuolo et al, 2002; Stolina et al, 2005; 2007).

With respect to postmenopausal osteoporosis, there is a report indicating that RANKL and OPG, which is a protein pertaining to the TNF receptor, are key factors mediating the differentiation of osteoclasts (Bae and Kim, 2010). There is also a study reporting that the increase of RANKL implies an increase of bone turnover related to bone fracture and activation of osteoclast precursors (Schett et al, 2004). In addition, OPG competitively binds with RANKL instead of RANK, thus interfering with the interaction of RANK and RANKL (Hofbauer and Heufelder, 2001). The concentration ratio of OPG/RANKL is thus important in preserving bone mass, helping to maintain an appropriate balance of bone remodeling (Aubin and Bonneley, 2000; Lories and Luyten, 2001). If this balance becomes skewed, metabolic bone diseases such as osteoporosis can result (Lindberg et al, 2001). Concomitant with an increase in the OPG/RANKL concentration ratio, metabolism of bone resorption can be hindered, and it is reported that this is due to the consequence of the OPG/RANKL ratio in the osteoblasts (Bae and Kim, 2010; Koet al, 2010; Hou et al, 2011).

One interesting implication of the present study is that OPGL secreted
from the three tested species of rats may directly modulate osteoclastogenesis and the activity of mature osteoclasts. As mutant mice that lack T cells still have normal bone cavities and tooth eruption (Mombaerts et al, 1993), T cells are probably not required for normal bone homeostasis. However, the local inflammation within the bone, as a result of metastasis, infections and fractures, or joint inflammation of arthritis attracts T cells which could then actively participate in bone remodeling through production of OPGL. The role of OPGL producing T cells in the bone resorption remains to be elucidated. Inhibition of OPGL function might allow the treatment T cell mediated arthritis, a condition that leads to bone destruction and crippling. Collectively, the present data showed that the lymph node organogenesis and osteoclast differentiation are regulated by the TNF-family cytokine OPGL.

Conclusion
These results suggest that the inhibition of RANKL during organogenesis does not significantly influence the development of the rodent immune system. These results are consistent with the possibility that many of the immune related changes in animals that lack RANK or RANKL may be secondary to the absence of lymph nodes and/or the hematopoietic challenge associated with markedly reduced bone marrow space.

Consequently, the lymph node formation appears to require the adequate quantity of RANKL, and this significant level can apparently persist despite marked overexpression of the soluble RANKL inhibitor OPG.

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