Involvement of Aquaporin 9 in Osteoclast Differentiation*

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Aquaporins (water channels) selectively enhance water permeability of membranes. Since osteoclast differentiation includes a dramatic increase in cell volume, we hypothesize that aquaporin(s) is/are critical for the formation of the multinucleated osteoclast from its mononuclear precursor. Our studies employ two cell models, bone marrow macrophages (BMMs) and the murine macrophage-like cell line, RAW264.7, as osteoclast precursors. Receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) and macrophage-colony-stimulating factor or RANKL alone were used to induce osteoclast differentiation in BMMs or RAW264.7 cells, respectively. We first used qualitative reverse transcription (RT)-PCR to examine which of the aquaporins are expressed in osteoclasts and in their precursor cells. Out of the 10 aquaporins examined, only aquaporin 9 (AQP9) was expressed in osteoclast-lineage cells. AQP9 has unique aqueous pore properties mediating the passage of a wide variety of non-charged solutes in addition to water. Western analyses using specific antibodies revealed a higher AQP9 level in RANKL-treated than in untreated cells. Quantitative real-time RT-PCR analyses also demonstrated higher AQP9 mRNA levels in RANKL-treated cells. Finally, we examined the effect of phloretin, an AQP9 inhibitor, on RANKL-induced osteoclast differentiation. Cells were incubated with RANKL for 5 days, and phloretin was added for the last 2 days, when most fusion occurs. A dramatic reduction in osteoclast size and in the number of nuclei per osteoclast was observed in cultures containing phloretin. The inhibitor did not have a significant effect on the number and size of mononuclear phagocytes in cultures not treated with RANKL. Our results suggest a role for AQP9 in osteoclast differentiation, specifically in the fusion process.

The osteoclast is the principal, if not exclusive, resorptive bone cell and plays a central role in the formation, growth, and remodeling of the skeleton. This multinucleated cell is formed by the fusion of mononuclear progenitors of the monocyte/macrophage family (1–3). The differentiation of the osteoclast from its precursor cells requires the presence of osteoblasts or marrow stromal cells (1). Two factors expressed by the accessory cells, macrophage colony-stimulating factor and receptor activator of nuclear factor κB (NF-κB) (RANK)3 ligand (RANKL), are essential and sufficient to promote osteoclastogenesis (4, 5). Excessive osteoclastic activity leads to progressive loss of bone mass causing weakening of the skeleton, manifested by a variety of pathological conditions including osteoporosis. Reduced osteoclast activity results in the formation of overly dense bones, as found in osteopetrosis. Thus, regulation of osteoclastogenesis plays an important role in maintaining a healthy skeleton (1–3).

The volume of a sphere formed by fusion of several spheres (keeping the total surface the sum of the original surfaces) is greater than the total volume of the original spheres. Although the osteoclast and its precursors are not perfect spheres we hypothesize that at least qualitatively this phenomenon should occur when multinucleated osteoclasts are formed by the fusion of their precursors. Therefore, during osteoclastogenesis active synthesis of cytosol should take place. Since water is the main component of the cytosol, massive water influx is expected during osteoclastogenesis.

The discovery of aquaporins (water channels) in 1992 by Agre and colleagues (6) dramatically changed the concept of regulation of water transport through biological membranes. Aquaporins are ~30-kDa tetrameric proteins (7) characterized by six transmembrane-spanning helices, and both termini are cytosolic. Aquaporins are expressed across all organisms and control water transport in all cells (8–10). Eleven mammalian aquaporins have been identified so far and they have cellular and subcellular distributions in different organs that indicate probable functional roles. Studies in animals and humans have revealed that aquaporins participate in a wide range of physiological and pathological processes (7–10).

Our assumption that a massive water influx is required for the formation of the multinucleated osteoclast leads to our working hypothesis that aquaporin(s) is/are critical for this process. To this end, we examined the expression of aquaporins in osteoclasts and in their precursors. We identified aquaporin 9 (AQP9) as the only aquaporin that is expressed in osteoclast-lineage cells and whose level is markedly increased with differentiation. Moreover, phloretin, an AQP9 inhibitor, reduced osteoclast differentiation and decreased their size and nuclei number dramatically.

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TABLE 1
Primers used for RT-PCR analyses

| Gene   | Primer reverse | Primer forward | Tm (°C) |
|--------|----------------|----------------|---------|
| Actin  | TGCTGTGAAATCGTGA | TAGAGGGAATCACCTCTTGGA | 60.0    |
| GAPDH  | ATGGCTCAGGATTGAC | GAACCTGAATGTTGACAGGA | 57.7    |
| AQP0   | TGGGTACCTTTTGTCCTGG | CAGAGTTGAGTCCGAGAGAA | 58.7    |
| AQP1   | CAGGTGATGGCTCTTTATGCG | CACCGCTAATGAGCAGTCCTAC | 57.8    |
| AQP2   | TCTTGCTGATCGAGGAAGCAAG | CTCCGCTCTCTTCATCTTCA | 57.2    |
| AQP3   | CATGTGCTCTCTCTCTCCTC | TGTGGTATGCTGGGGTGAAT | 58.1    |
| AQP4   | TGGGTACCTTTTGTCCTGG | TGGCAGCTATCTCGGTGTC | 56.8    |
| AQP5   | CCTGTTGTTGGTGTTTTGCTGG | AACGAGGTGGTCTTGAGGCC | 60.0    |
| AQP6   | GAACCTCCACAATGACGCSC | TGGCGGATCTCTTCTATCGG | 59.9    |
| AQP7   | CTGTGATGGCTCTTTATGCG | CAGCTTCACATTCTCTTCCTC | 59.1    |
| AQP8   | GAGGCCAGTACGCTCTTAC | CATGCGGTCTGTGAAGTCG | 55.9    |
| AQP9   | CAGAGGTGGTCTTGAGGACAA | TGCTGGAAGGTGGACAGTGGA | 55.6    |

EXPERIMENTAL PROCEDURES

Cells—Primary bone marrow cells (BMMs) from long bones of 7–9-week-old BALB/c male mice and murine macrophage-like RAW264.7 cells were prepared as described previously (11). Cells were maintained at 37 °C in an incubator with 5% CO2 in a humidified atmosphere. To assess osteoclast differentiation BMMs (7 × 105/well in α-minimal essential medium containing 10% FCS and supernatant from CMG14-12 (1:20) as a source for macrophage-colony-stimulating factor) were incubated in 96-well plates (0.2 ml/well). Bacterially produced recombinant RANKL was added to induce osteoclast differentiation. Medium was changed on day 3 and osteoclast formation was evaluated on day 5 (see below).

Tartrate-resistant Acid Phosphatase (TRAP)—A commercial kit (catalog number 387-A, Sigma) was used according to the manufacturer’s instructions. TRAP-positive cells containing three or more nuclei were scored as osteoclasts.

RNA Extraction and cDNA Preparation—BMMs (2.5 × 105/plate) and RAW264.7 cells (3 × 105/plate) were plated in 35-mm tissue culture plates as described previously (11). Total cellular RNA was isolated using an EZ-RNA kit (Biological Industries, Beit Haemek, Israel) according to the manufacturer’s instructions. RNA from mouse kidney was received from Drs. Many and Silver. For RT-PCR, RNA was purified using a DNA-free RNA kit (Zymo Research). The RNA was treated with DNase (RQ1-RNase-free DNase, Promega, Madison, WI) and tested for being an essentially DNA-free by a PCR reaction dilutions: chicken anti-AQP9 antibody (1:1000), mouse anti-β-actin antibody (1:10,000), horseradish peroxidase-conjugated goat anti-mouse antibody (1:10,000), and horseradish peroxidase-conjugated rabbit anti-chicken antibody (1:10,000). Immunoactivity was assayed using an ECL kit (Pierce) according to the manufacturer’s instructions.

Surface Area Measurements—The surface area occupied by osteoclasts and their precursors was quantified using a computerized image analysis system (Olympus BX50, DP50, software: ImagePro Plus, Media Cybernetics).

Methylene Blue Uptake—Cells were seeded as for the osteoclast differentiation assay (see above). Monolayers were fixed and methylene blue uptake was measured as described (14).

Data Analysis—Experiments were repeated two to five times, and each experimental treatment was performed with six replications, unless stated otherwise. Data were analyzed with the JMP statistics package (version 4.1, from SAS Institute Inc., Cary, NC).

RESULTS

To study the role of aquaporins in osteoclast differentiation, we first examined which aquaporins are expressed in osteoclast and/or in their precursor cells. RNA was isolated from BMMs and RAW264.7 cells grown with or without RANKL. Using semiquantitative RT-PCR (Table 2) we found that out of 10 aquaporin genes examined, only AQP9, an aquaglyceroporin, was expressed in osteoclasts and their precursors. RNA preparations obtained from kidney were used as positive controls for certain aquaporins, validating that only AQP9 is expressed in osteoclast-lineage cells.

Quantitative real-time PCR was used to examine whether AQP9 expression is modulated during osteoclast differentiation. In Fig. 1A we show that the expression of AQP9 is markedly increased (about 10-fold) in osteoclasts (RANKL-treated BMMs) as compared with their precursors (BMMs that were not exposed to RANKL). While microscopic examination confirmed the osteoclastic phenotype of the RANKL-treated cells, we also measured levels of the established osteoclast marker, MMP9, to verify osteoclastic differentiation. Detailed kinetic analyses showed that the expression of both AQP9 and MMP9 was increased by RANKL already after 24 h of exposure to RANKL (~3- and 80-fold, respectively). It is of note that these changes were observed 2 days prior to any detectable fusion of the precursors. Maximal induction of AQP9 expression was
or without RANKL throughout the full 5 days of the experiment resulted in cell death. Therefore, cells were incubated with or without RANKL in preliminary analyses we found that the presence of phloretin, a specific AQP9 activity inhibitor (17), on RANKL-induced osteoclast differentiation. To examine whether AQP9 plays a role in this process, we studied the effect of phloretin on RANKL-induced osteoclast differentiation. Western blot analysis showed that AQP9 protein is expressed in osteoclast-lineage cells, and similar to the differential mRNA expression, we also found, using specific anti-AQP9-antibodies, that osteoclasts (RANKL-treated BMMs) express higher levels of AQP9 protein than the precursor cells (Fig. 2). Two bands (apparent molecular masses of 32 and 35 kDa) were observed, similar to previous observations using bile extracts from BMMs grown in the presence or absence of RANKL (100 ng/ml, 5 days) were analyzed using anti-AQP9 antibodies. Two independent samples are presented.

As seen in Fig. 3, RANKL induced TRAP-positive multinucleated cells also in the presence of phloretin (Fig. 3D). However, the size of the cells was markedly reduced in the phloretin-treated cells (compare B and D of Fig. 3). We also see in the figure that phloretin had no effect on the morphology of cells not treated with RANKL (compare A and C of Fig. 3). Thus, under these conditions phloretin does not exert toxic effects. To quantify the effect of phloretin on cell size, we used Image-pro software to measure the surface area occupied by the cells. We find in Table 3 that phloretin did not significantly affect the area occupied by mononuclear cells. In contrast, RANKL-induced cells treated with phloretin occupied ~10-fold less area than the control RANKL-induced Me2SO-treated cells. The number of nuclei/osteoclast was assessed in triplicate cultures, counting 100 osteoclasts in each well. The number of nuclei/osteoclast decreased from 18.5 ± 1.9 in the absence of phloretin to 6.1 ± 0.6 in the presence of the inhibitor. The number of osteoclasts/well did not differ significantly between control and phloretin-treated cultures. Thus, 3-fold less mononuclear cells were induced to undergo osteoclast differentiation in cultures containing phloretin. Of special interest is the comparison of area occupied by cells derived from phloretin-treated and untreated cultures with the same number of nuclei. Quantification of this phenomenon showed that phloretin-treated osteoclasts containing 18.0 ± 1.1 nuclei occupied 15% of the area occupied by untreated osteoclasts, which contained 19.2 ± 1.1 nuclei.

The effect of phloretin on methylene blue uptake is shown in Fig. 4. No effect was observed on mononuclear precursors (BMMs grown in the absence of RANKL). Methylene blue stains both nuclei and cytoplasm and is a reliable measurement of relative cell numbers when there is no difference in cell size and morphology between the examined populations (14). Thus, phloretin does not inhibit mononuclear cell proliferation and size. In contrast, a marked inhibition of methylene blue uptake is observed in RANKL-treated cells, reflecting the decreased cell size.

**DISCUSSION**

Although discovered only relatively recently, it is already clear that aquaporins are involved in several pathological situations. For example, hereditary nephrogenic diabetes insipidus is caused by mutations in AQP2 (18) and mutations in AQP0 cause congenital cataracts (10). In addition, enhanced expression of AQP4 was observed in brains of patients with inflammatory diseases (19), and AQP1 was found to be expressed in

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**TABLE 2**

**Expression of aquaporins in mouse cells and tissues**

RNA was prepared as described and semiquantitative RT-PCR analysis was performed using the primers described in Table 1. GAPDH and actin were used as the reference housekeeping genes. – and + denote the absence and presence, respectively, of the specific mRNA. For the RAW264.7 cells and BMMs the results represent both mononuclear and multinucleated (RANKL-treated) cells.

|          | RAW264.7 | BMMs | Kidney |
|----------|----------|------|--------|
| AQP1     | –        | –    | +      |
| AQP2     | –        | –    | +      |
| AQP3     | –        | –    | +      |
| AQP4     | –        | –    | +      |
| AQP5     | –        | –    | +      |
| AQP6     | –        | –    | +      |
| AQP7     | –        | –    | +      |
| AQP8     | –        | –    | +      |
| AQP9     | –        | –    | +      |
| AQP10    | –        | –    | +      |

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**FIGURE 1.** *Modulation of AQP9 and MMP9 by RANKL.* BMMs were incubated with or without RANKL. Quantitative real-time PCR using L32 as a reference gene was employed to quantify mRNA levels. AQP9/L32 and MMP9/L32 were arbitrarily given a value of 1. A, the relative increase after 5 days of incubation with RANKL. B, kinetic study of the expression level of AQP9 and MMP9. Bars represent the standard error of the mean of at least four independent replicates.

7-fold (6–12-fold in different experiments), while MMP9 maximal induction was ~4700-fold (Fig. 1B).

Western blot analysis showed that AQP9 protein is expressed in osteoclast-lineage cells, and similar to the differential mRNA expression, we also found, using specific anti-AQP9-antibodies, that osteoclasts (RANKL-treated BMMs) express higher levels of AQP9 protein than the precursor cells (Fig. 2). Two bands (apparent molecular masses of 32 and 35 kDa) were observed, similar to previous observations using bile and liver preparations (15, 16).

Thus, AQP9 expression is increased during osteoclast differentiation. To examine whether AQP9 plays a role in this process, we studied the effect of phloretin, a specific AQP9 activity inhibitor (17), on RANKL-induced osteoclast differentiation. In preliminary analyses we found that the presence of phloretin throughout the full 5 days of the experiment resulted in cell death. Therefore, cells were incubated with or without RANKL for 5 days, and phloretin (or Me2SO, the solvent of phloretin stock solution for control cells) was added to the medium for the last 2 days, when most fusion occurs.
normal human cornea and decreased in human corneas with endothelial disease but not in human corneas with non-endothelial corneal disease (20). These are a few of a growing number of examples pointing to the important physiological roles played by aquaporins.

We show here for the first time that a member of the aquaporin family, AQP9, plays a role in the differentiation of the bone-resorbing cell, the osteoclast. Previously, in two other examples, aquaporins were implicated in differentiation/development. The ontogeny and distribution of AQP7 and AQP8 in rat testis raised the possibility that these aquaporins are involved in testis development and spermatogenesis (21). It was also suggested that aquaporin proteins (3, 8, and 9) mediate trans-trophectodermal water movements during cavitation (22). Differentiation of the osteoclast includes fusion of mononuclear precursors to form the multinucleated osteoclast. The surface area and the volume of a sphere are proportional to the radius squared and cubed, respectively. Therefore, upon fusion of the precursors, the volume of the mature osteoclast increases more dramatically than its surface area and therefore must “add” new cytosol. Water being the major cytosolic component strongly suggests the use of aquaporin(s) for the enhanced water influx. We indeed find increased expression of AQP9 following induction of osteoclastogenesis by RANKL. The fact that the increase in AQP9 expression precedes the fusion is consistent with the hypothesis that this channel plays a role in the formation of a large multinucleated osteoclast, probably by mediating rapid water influx to enable the increase in cell volume. Moreover, phloretin, an efficient inhibitor of AQP9 (17, 23), reduces the size of RANKL-induced osteoclasts, probably by inhibition of the rapid water influx. Of special interest is the observation that osteoclasts with the same number of nuclei are much smaller in phloretin-treated cultures, suggesting a role for AQP9-mediated water influx in determining the size of the cell. However, phloretin may affect additional channels, for example it inhibits volume-sensitive and cyclic AMP-activated Cl7 channels (24). To inhibit AQP9 activity in a more specific manner we are currently investigating this point using SiRNA technology to specifically block the expression of the channel.

AQP9 is a unique aquaglyceroporin that, in addition to its water and glycerol transport properties, is also permeable to urea, mannitol, sorbitol, and other small solutes (17, 25). AQP9 is found in lung, liver, brain, spleen, peripheral blood leukocytes, and testes (17, 26), and we can now add osteoclasts to this list. The broad selectivity of AQP9 permits rapid and energetically efficient metabolite exchanges in cells. This characteristic is beneficial to osteoclasts that absorb and secrete large amounts of metabolites.

Our studies demonstrate a novel physiological role for aquaporin, the involvement in cell fusion and differentiation. Moreover, AQP9 is a likely candidate target for developing a novel strategy to inhibit osteoclastogenesis and may thus be beneficial in the treatment of a variety of diseases caused by enhanced osteoclastic bone resorption, such as osteoporosis and osteoarthritis.
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