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Induction of Chemokines and Chemokine Receptors CCR2b and CCR4 in Authentic Human Osteoclasts Differentiated With RANKL and Osteoclast Like Cells Differentiated by MCP-1 and RANTES

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Abstract Chemokines MCP-1 and RANTES are induced when authentic bone resorbing human osteoclasts differentiate from monocyte precursors in vitro. In addition, MCP-1 and RANTES can stimulate the differentiation of cells with the visual appearance of osteoclasts, being multinuclear and positive for tartrate resistance acid phosphatase (TRAP+). We show here that MIP1α is also potently induced by RANKL during human osteoclast differentiation and that this chemokine also induces the formation of TRAP+ multinucleated cells in the absence of RANKL. MIP1α was able to overcome the potent inhibition of GM-CSF on osteoclast differentiation, permitting the cells to pass through to TRAP+ multinuclear cells, however these were unable to form resorption pits. Chemokine receptors CCR2b and CCR4 were potently induced by RANKL (12.6- and 49-fold, $P = 4.0 \times 10^{-7}$ and 4.0×10^{-8}, respectively), while CCR1 and CCR5 were not regulated. Chemokine treatment in the absence of RANKL also induced MCP-1, RANTES and MIP1α. Unexpectedly, treatment with MCP-1 in the absence of RANKL resulted in 458-fold induction of CCR4 ($P = 1.0 \times 10^{-10}$), while RANTES treatment resulted in twofold repression ($P = 1.0 \times 10^{-4}$). Since CCR2b and CCR4 are MCP-1 receptors, these data support the existence of an MCP-1 autocrine loop in human osteoclasts differentiated using RANKL. J. Cell. Biochem. 97: 512–518, 2006. © 2005 Wiley-Liss, Inc.

Key words: osteoclast; chemokines; MIP1α; MCP-1; RANTES

Chemokines are a superfamily of small cytokines that play an important role in intercellular signalling [Rollins, 1997]. Chemokines are subdivided into different families, primarily CC and CXC chemokines, based on the position of the first two cysteine residues [Gao et al., 1993]. These families have known involvement in the development of several cell types, immune responses, inflammation and allergies [Horuk, 2001]. Many of the ligands in the CC chemokine family have the capacity to interact with and share multiple chemokine receptors [Horuk, 2001]. Chemokines are potent agents for attracting monocytes and macrophage-like cells to sites of inflammation. In relationship to bone disease, chemokines are discussed as candidates for involvement in bone loss in various inflammatory diseases through the recruitment of osteoclast precursors [Choi et al., 2000]. Osteoblasts are proposed as a source of chemokines [Yu et al., 2004], however new data suggests the possibility that chemokines are produced by osteoclasts for autocrine and paracrine signals during osteoclast differentiation [Kim et al., 2005].

Osteoclasts are large, multinuclear cells that stain positive for tartrate resistant acid phosphatase (TRAP) and express cathepsin K (CTSK), an osteoclast specific protease. Osteoclasts differentiate from haematopoietic precursors of monocyte/macrophage lineages [Sakiyama et al., 2001], under the influence of receptor activator of NFκB ligand (RANKL), a member of the TNF receptor superfamily that is

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expressed on the surface of stromal cells and osteoblasts [Takahashi et al., 1999]. Human osteoclast differentiation can be promoted in vitro using soluble recombinant RANKL in the presence of macrophage colony stimulating factor (M-CSF), replacing the need for stromal cell co-cultures.

In mouse, CCL9 or macrophage inflammatory protein 1 gamma (MIP1γ) is the major chemokine expressed by osteoclasts [Lean et al., 2002]. We showed by array [Day et al., 2004] and real-time PCR [Granfar et al., 2005] that "regulated on activation normal T-cell expressed and secreted" (RANTES) and the chemokine receptor CCR2 were induced during human osteoclast differentiation. Furthermore, in human osteoclasts, we have shown that monocyte chemotactic protein 1 (MCP-1) along with RANTES are potently induced by RANKL and that both MCP-1 and RANTES induced the formation of TRAP+, multinuclear cells from M-CSF-treated monocytes in the absence of RANKL [Kim et al., 2005]. Such TRAP+ multinuclear cells produced by chemokines have many phenotypic features in common with osteoclasts but are not capable of bone resorption activity, possibly due to a lack of induction of cathepsin K. In the presence of RANKL, MCP-1 accelerates the process of human osteoclast differentiation [Kim et al., 2005].

Macrophage inflammatory protein 1 alpha (MIP1α) is a CC chemokine in the RANTES family, which is reported to act directly on osteoclast progenitors and enhance osteoclast formation [Choi et al., 2000; Han et al., 2001]. In this study, we add MIP1α to the list of chemokines induced by RANKL in human osteoclast differentiation. We also examine the effects of MIP1α in osteoclast differentiation and its capacity to reverse GM-CSF mediated repression of osteoclast differentiation.

The necessary components of an autocrine loop are the receptor and the ligand. We have established that RANKL induces two potent chemokines that also have RANKL-independent effects on mononuclear cells. In this article, we test the hypothesis that RANKL treatment results in an autocrine loop by inducing receptors for MCP-1 and RANTES, during osteoclast differentiation. Furthermore, the effects of chemokine treatment in the absence of RANKL on chemokine receptor expression are also investigated.

METHODS

Preparation and Culture of Human Monocytes

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation, as previously described [Day et al., 2004]. PBMCs were plated at 10^6 cells per cm^2 and non-adherent cells removed by washing in normal saline. Cells were cultured in minimal essential media (supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin [Invitrogen, Carlsbad, CA]), 5% CO_2 supplemented with 25 ng/ml of M-CSF and 20 ng/ml of RANKL to induce osteoclast formation. GM-CSF, MCP-1, MIP1α and RANTES were used at 25 ng/ml. GM-CSF, RANKL, M-CSF MCP-1, RANTES and MIP1α were purchased from Peprotech (Rocky Hill, NJ). All data are based on a minimum of three replicate experiments performed independently on different occasions, unless otherwise stated. All blood samples were derived from the same four donors (the authors). All cultures were performed for 21-days.

After 21 days, cells were fixed in acetone, citrate and formaldehyde solution and stained for TRAP using leukocyte acid phosphatase staining kit (Sigma-Aldrich, St. Louis, MO). TRAP positive cells that had three or more nuclei were considered multinuclear. Cell counts were made blinded to treatment groups. Bone-resorption assays were performed on dentine slices in 96-well plates, as described in Hodge et al. [2004]. Dentine slices were sputter coated with gold and observed by scanning electron microscopy.

RNA Studies

At 21 days, cultures were lysed using 4 M guanidium isothiocyanate, 1% lauryl sarcosine and total RNA pelleted through a 5.7 M cesium chloride, 100 mM EDTA cushion by ultracentrifugation in a Beckman SW41 rotor at 27,000 rpm for 16 h [Sambrook et al., 1989]. Total RNA was converted into cDNA using ImProm-II Reverse Transcriptase (RT, Promega) and oligo dT primer. Primers for quantitative PCR assays are described in Table I. Quantitative PCRs were performed using SYBR Green I Supermix (Bio-Rad, Hercules, CA) in a Bio-Rad i-Cycler [Kim et al., 2005] measuring copies per ng total RNA using quantitative standards and cycling

TABLE I. Primers for Quantitative Real-Time Gene Expression Assays

Gene	Forward	Reverse
18s rRNA	CTTAGAGGGACAAGTGGCG	ACGCTGAGCCAGTCAGTGTA
MCP-1	TCGCGAGCTATAGAAGAATCA	TGTTCAAGTCTTCGGAGTTTG
MIP1α	CTATGGACTGGTTGTTGCCA	AGGGGAACTCTCAGAGCAAA
RANTES	GAGCTTCTGAGGCGCTGCT	TCTAGAGGCATGCTGACTTC
CCR1	TTCCTGTTCACCCATGAGTG	AAGGGGAGCCATTTAACCAG
CCR2a	CATAGCTCTTGGCTGTAGGA	GTGAAGCCAGACGTGTGATT
CCR2b	AACAAACACGCCTTCCACTG	GTCAAAGTCTCTACCCACAG
CCR4	CTTATGGGGTCATCACCAGT	AGTAGGTATGGTTGCGCTCA
CCR5	ACCAAGCTATGCAGGTGACA	GAACAGCATTTGCAGAAGCG
CTSK	TGAGGCTTCTCTTGGTGTCCATAC	AAAGGGTGTCATTACTGCGGG
TRAP	GACCACCTTGGCAATGTCTCTG	TGGCTGAGGAAGTCATCTGAGTTG

All primers are written 5′–3′.

conditions as described previously [Granfar et al., 2005].

Statistical Analysis

Analysis of variance of gene expression data (ln transformed) with Fisher's post-hoc *t*-test was used to determine significance of effects. Data are presented as mean values ± SE copies per ng total RNA.

RESULTS

RANKL Induces Chemokines

We previously reported that RANKL induces MCP-1 and RANTES during human osteoclast differentiation from monocyte precursors [Kim et al., 2005]. The absolute and relative levels of mRNA for chemokines in RANKL and M-CSF-treated osteoclasts were compared to macrophage-like cells treated with M-CSF alone. Figure 1A shows absolute mRNA content in copies per ng of total RNA from a series of five independent experiments. These data verify the induction of MCP-1 and RANTES reported in Kim et al. [2005]. MCP-1 and RANTES were induced 10.5- and 36-fold, with $P = 8.6 \times 10^{-7}$ and 1.9×10^{-10}, respectively. We report for the first time that the chemokine MIP1α, is also induced by RANKL during human osteoclast differentiation from monocyte precursors with 22-fold induction, ($P = 4.2 \times 10^{-10}$).

Regulation of Chemokine Receptors by RANKL

CC chemokine receptors capable of interacting with MCP-1, RANTES and MIP1α were tested for RANKL regulation. In comparison to M-CSF-treated cells, RANKL and M-CSF resulted in the significant induction of chemokine receptors CCR2b and CCR4, (12.6- and 49-fold, $P = 4.0 \times 10^{-7}$ and 4.0×10^{-8}, respectively), that are capable of interaction with MCP-1. In contrast, the receptors for RANTES and MIP1α were not induced (CCR1 and CCR5; 1.0- and 1.4-fold, $P = 0.96$ and 0.22, respectively) (Fig. 1B). CCR2a was not induced by

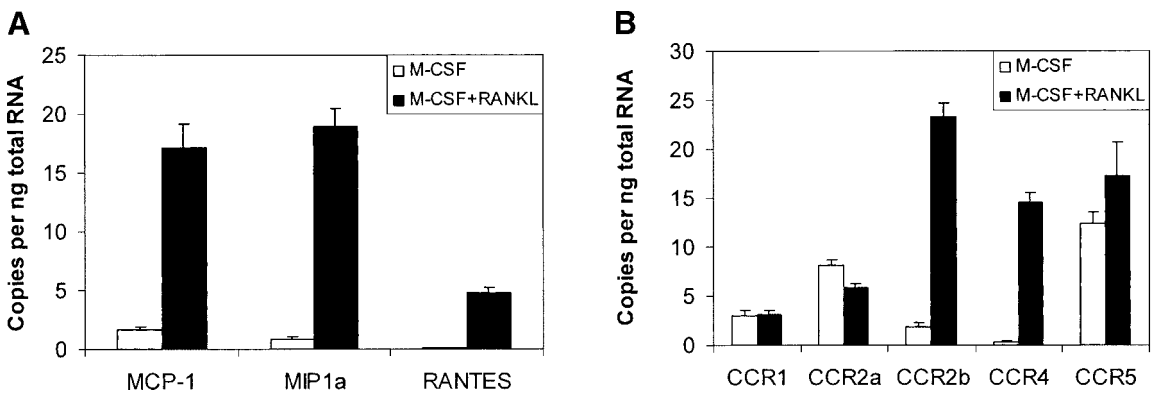

Fig. 1. **A**: Induction of CC chemokine genes by RANKL. Graph shows the comparison in mRNA content measured by quantitative real-time PCR of MCP-1, MIP1α (MIP1a) and RANTES in macrophage like cells (cultures treated with M-CSF alone, white columns) and osteoclasts (cultures treated with M-CSF+RANKL, black columns). **B**: Expression analysis of chemokine receptors in macrophage like cells compared to osteoclasts (white and black columns as in panel A). Error bars are SE from replicate experiments.

RANKL ($P = 0.29$). These data suggest that receptors for MCP-1, but not RANTES or MIP1α are induced by RANKL during osteoclast differentiation. Despite the lack of regulation by RANKL of the RANTES family receptors CCR1 and CCR5, reasonable amounts of the mRNA for CCR1 and CCR5 are present in osteoclasts, making it difficult to discount autocrine functions of RANTES and MIP1α. Since RANKL regulates both MCP-1 and its possible cognate receptors, these data provide evidence for an autocrine function of MCP-1 in osteoclasts.

Effect of MIP1α on Osteoclast Biology

We hypothesised that MIP1α would behave similarly to RANTES rather than MCP-1 in osteoclast biology. In the presence of RANKL and M-CSF, MCP-1 enhanced osteoclast formation, whereas RANTES did not [Kim et al., 2005]. Continuous exposure of RANKL and M-CSF-treated cells to 25 ng/ml GM-CSF completely suppressed osteoclast formation, resulting in TRAP negative mononuclear cells with phenotypic characteristics of dendritic cells [Kim et al., 2005]. We previously demonstrated that MCP-1 and RANTES treatment was able to overcome this potent suppression of human osteoclast formation and resulted in a TRAP+, multinuclear phenotype, although only MCP-1 treatment was able to recover the formation of authentic osteoclasts capable of bone resorption activity. Furthermore, chemokine treatment with M-CSF alone leads to TRAP+ multinuclear cells that are similar to osteoclasts but are unable to resorb bone. When tested in this assay for the recovery of osteoclast formation, MIP1α was able to recover TRAP+ and multinuclear phenotypes from GM-CSF repression, but was unable to recover bone resorption activity (Fig. 2A). Similar to RANTES [Kim et al., 2005], MIP1α did not have a significant enhancing effect on osteoclast formation in RANKL and M-CSF-treated cultures (Fig. 2E) although the number of TRAP+ multinuclear cells in cultures treated with MIP1α and M-CSF was substantially higher than M-CSF alone treated cultures. MIP1α, in the absence of RANKL, behaved similarly to MCP-1 and RANTES (in the absence of RANKL), producing TRAP+ multinuclear cells that had the appearance of small osteoclasts. These osteoclast-like cells were negative for bone resorption. Although TRAP mRNA levels were not significantly different from those in authentic osteoclasts ($P = 0.78$), mRNA levels for cathepsin K (CTSK) were 28-fold lower ($P = 2.3 \times 10^{-3}$) in the TRAP+ multinucleated cells derived from treatment with MIP1α and M-CSF (Fig. 2F).

Regulation of Chemokines and Receptors by Chemokines

MCP-1, RANTES and MIP1α all induce TRAP+ multinuclear cells in the absence of RANKL. Cells were treated with either MCP-1 or RANTES in the presence of M-CSF and the expression of chemokines and their cognate receptors were examined at 21 days. MCP-1 treatment resulted in the dramatic induction of MIP1α and RANTES expression (45- and 90-fold with $P = 1.3 \times 10^{-10}$ and 8×10^{-12}, respectively) when compared to M-CSF alone treatment (Fig. 3A). MCP-1 was induced to a minor but significant extent (4.2-fold with $P = 2.4 \times 10^{-4}$) by MCP-1 treatment. In marked contrast, MCP-1 expression was induced 31-fold by RANTES treatment ($P = 5.0 \times 10^{-8}$). MIP1α and RANTES were significantly induced by RANTES treatment (12.7- and 21.6-fold, $P = 1.4 \times 10^{-8}$ and 5.1×10^{-10}, respectively), but to a lesser extent than that observed with MCP-1 treatment. These data are consistent with feedback inhibition by the ligand through its receptor, limiting the production of the cognate ligand. In other words, RANTES family members (MIP1α and RANTES) are more potently induced by MCP-1 than by RANTES. Similarly, RANTES induces MCP-1 more potently than MCP-1 induces its own expression.

Chemokine receptor expression after treatment with chemokines MCP-1 and RANTES followed the same pattern as observed with RANKL treatment in that CCR1 and CCR5 were expressed but not significantly regulated, while CCR2 and CCR4 were profoundly regulated. CCR4 was induced 458-fold by MCP-1 treatment ($P = 1.0 \times 10^{-10}$) and repressed two-fold by RANTES ($P = 1.0 \times 10^{-4}$). CCR2b was induced 26-fold by MCP-1 ($P = 8.9 \times 10^{-8}$) and 8.0-fold by RANTES ($P = 1.2 \times 10^{-5}$). CCR2a was induced 3.5-fold by MCP-1 ($P = 0.001$) and repressed 10-fold by RANTES ($P = 1.1 \times 10^{-5}$).

DISCUSSION

In this study, we provide evidence that chemokines MCP-1, RANTES and MIP1α are

induced by RANKL in human osteoclasts. MIP1α is induced to a similar extent to MCP-1 and RANTES, and although MIP1α can induce TRAP+ multinuclear cells in the absence of RANKL, these cells are not osteoclasts and are incapable of bone resorption. We had proposed that such cells represent a stage in the process of recruitment and activation of osteoclast precursors that are attracted to the site of RANKL-RANK signalling. Osteoclasts form by fusion of precursors, and this fusion event appears to rely on chemokines, since MCP-1 and RANTES [Kim et al., 2005] and MIP1α (this study) are potent inducers of TRAP+ multinuclear cells that resemble osteoclasts in appearance. In the in vivo situation RANKL is presented through cell-cell contact to a RANK+ osteoclast precursor. A cell stimulated by RANKL then produces soluble chemokines that are known attractants of monocytes. Subsequently, monocytes may be attracted to the site of fusion to form an osteoclast.

Chemokines have complex relationships between receptors and ligands, with many overlapping specificities [see Mahalingham and Karupiah, 1999; Horuk, 2001]. CCR2 [Loetscher et al., 1996] and CCR4 [Power et al., 1995] act as MCP-1 receptors. CCR2 exists in two splicing variant forms, CCR2a and CCR2b, which differ in the carboxyl terminus [Sanders et al., 2000]. CCR2b is reported to be the major cell surface form in monocytes [Tanaka et al., 2002] and, as monocytes differentiate into macrophages, total CCR2 content is reported to decrease [Wong et al., 1997]. CCR2b is induced strongly by RANKL, suggesting that this isoform may be the MCP-1 receptor in osteoclasts. The fact that CCR4 is induced

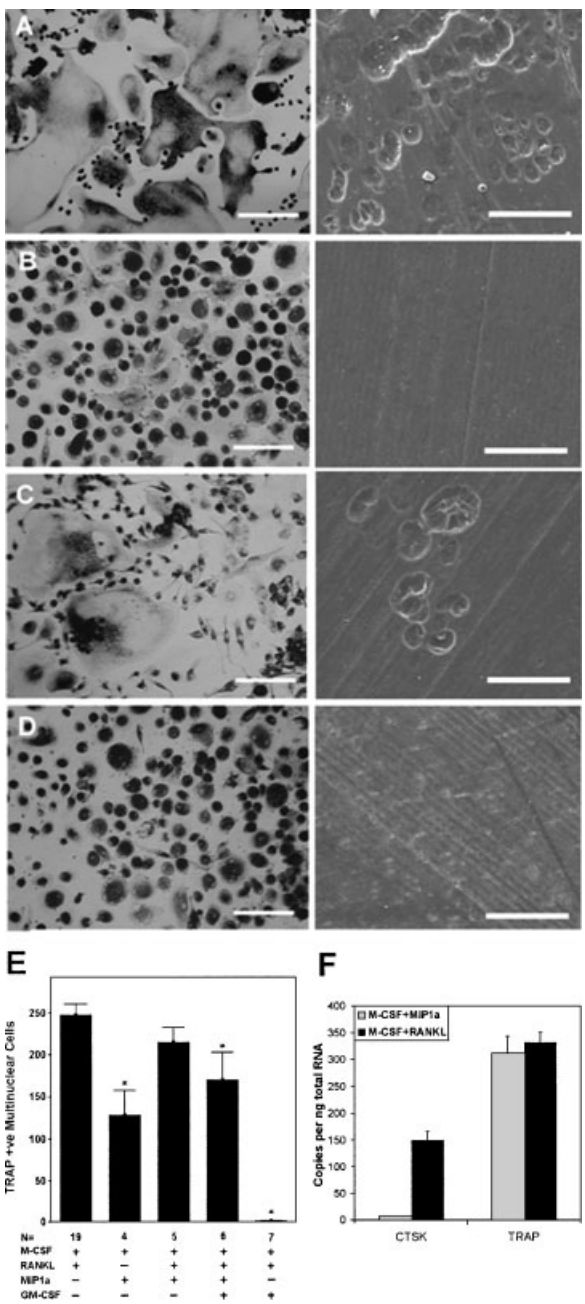

Fig. 2. MIP1α effects on cells in culture. For sections A–D, figures are light micrographs of TRAP-stained cells grown on plastic (left panels) and electron micrographs of resorption pits of cells grown on dentine slices (right panels) for different treatments. **A**: M-CSF and RANKL treated cells produce TRAP+ multinuclear osteoclasts (left) that are positive for bone resorption activity (pits in right panel). **B**: MIP1α and M-CSF-treated cells are TRAP+ multinuclear cells with the appearance of osteoclasts (left panel), although of reduced size in comparison to those in section A. Right panel shows these cells are negative for bone resorption activity. **C**: MIP1α in the presence of RANKL and M-CSF, results in TRAP+ multinuclear osteoclasts (left) that are positive for bone resorption (right). **D**: MIP1α treatment recovers the TRAP+ multinuclear phenotype from potent suppression by GM-CSF (left panel). Although cells are TRAP+ and multinuclear, they are negative for bone resorption (right panel). GM-CSF suppresses osteoclast formation, resulting in mononuclear TRAP- cells with a dendritic cell phenotype (image not shown). Bar is 100 microns in all panels. **E**: Counts of TRAP+ multinuclear cells per square centimetre after treatments as indicated. N indicates the number of independent experiments. Asterisk represents a significant difference at $P < 0.05$ compared to control osteoclasts (M-CSF- and RANKL-treated cells). GM-CSF suppresses the formation of TRAP+ multinuclear cells. **F**: Osteoclast marker genes TRAP and capthepsin K (CTSK) in TRAP+ multinuclear cells differentiated by treatment with MIP1α and M-CSF (grey striped columns) compared to authentic osteoclasts differentiated by treatment with RANKL and M-CSF (black columns); CTSK is expressed at a substantially lower level in MIP1α-treated cells (striped columns) compared to osteoclasts. In contrast, TRAP was expressed at similar levels in both cell types.

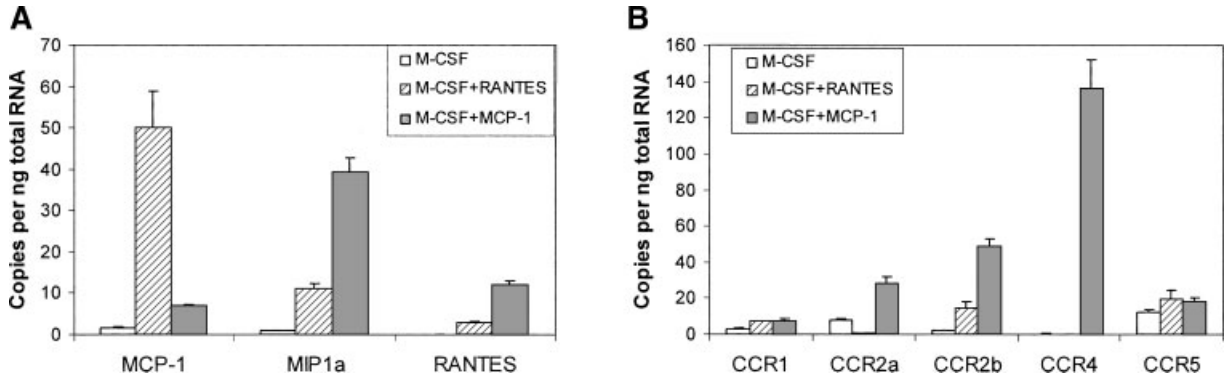

Fig. 3. Regulation of chemokines and their receptors by MCP-1 and RANTES treatment in the absence of RANKL. **A**: Strong regulation of MCP-1, RANTES and MIP1α is observed after treatment with either MCP-1 (grey columns) or RANTES (striped columns) compared to M-CSF treatment alone (white columns). MCP-1 treatment in the absence of RANKL induces RANTES and MIP1α, whereas RANTES treatment potently induces MCP-1. **B**: Receptors for MCP-1 (CCR2a, CCR2b and CCR4) are potently induced by MCP-1. In contrast, receptors for RANTES family members (CCR1 and CCR5) are not regulated.

substantially by MCP-1 suggests that chemokine exposure results in the induction of chemokine receptor gene expression. The induction of MCP-1 receptors by MCP-1 is consistent with an autocrine loop. In this scenario, an MCP-1 autocrine loop ramifies the effect of chemokines. CCR4 is reported as a MCP-1 receptor [Power et al., 1995], but it also responds strongly to CCL17 and CCL22 [Imai et al., 1997, 1998], chemokines that we have not examined. The relative contributions of MCP-1, CCL17 and CCL22 in osteoclasts are yet to be determined.

Although receptors for MCP-1 were RANKL induced, receptors for RANTES family members (RANTES and MIP1α) were not induced by RANKL. Despite this fact, reasonable levels of mRNA for these receptors were present. Clearly, treatment of cells with RANTES and MIP1α in the absence of RANKL resulted in strong induction of MCP-1. These facts, coupled with previous data on the effects of RANTES indicate that it is likely that RANTES and MIP1α have a role in osteoclast differentiation. Although MIP1α had no apparent effect on osteoclast differentiation, we show in this paper that MIP1α can induce the formation of TRAP+ multinuclear cells in the absence of RANKL. Our data is in contrast to the reported effects of MIP1α in mouse RAW264.7 cells and in mouse bone marrow derived monocytes [Oba et al., 2005], where MIP1α enhanced osteoclast formation in a manner similar to that which we reported for MCP-1 in human osteoclasts [Kim et al., 2005]. Despite these minor differences, CC chemokines enhance osteoclast differentiation in human and mouse models.

Combined with our previous observations on the effects of MCP-1 on human osteoclasts, these data add weight to the possibility that MCP-1 acts in an autocrine loop in human osteoclast differentiation. CCR4 is substantially induced by both RANKL and MCP-1. We show here that CCR2b is potently induced by both RANKL and by MCP-1. CCR2a is less influenced, suggesting that CCR2b and CCR4 may be of primary importance to MCP-1 signalling in osteoclasts. Further analysis of chemokine regulation in osteoclasts is warranted.

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