Review

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The interconnected role of chemokines and estrogen in bone metabolism

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Summary **Over the past few decades, researchers have paid considerable attention to the relationship between estrogen and bone metabolism. Nevertheless, few studies have examined the potential role of chemokines in estrogen regulation of bone metabolism. Chemokines are members of a superfamily of low-molecular-weight chemoattractant cytokines. Various chemokines and their corresponding transmembrane G protein-coupled receptors play distinct roles in the functional regulation and homeostasis of the immune and skeletal systems. This review summarizes the evidence that chemokines and estrogen display cooperative behavior in the skeletal system, with a focus on the mechanisms by which estrogen regulates the chemotactic factors that affect bone metabolism. Chemokines appear to represent a novel area for further examination in order to develop new therapeutics to treat disorders of bone metabolism.**

Keywords: Chemokines, estrogen, bone metabolism, osteoblast, osteoclast, networks of the reproductive, endocrine, and immune systems and metabolic processes

1. Introduction

Bone homeostasis requires a balanced relationship between bone resorption and bone formation. An upset in bone homeostasis leads to a series of diseases including osteoporosis, osteomalacia, and osteosclerosis. Osteoblasts (OBs) and osteoclasts (OCs) are two distinct cell types that are mainly involved in bone homeostasis; both are influenced by many factors, such as cytokines, hormones, and growth factors. The receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) are the central cytokines involved in this process and they also affect several steps in the inflammatory response (*1*). Estrogen is a well-known regulator of bone metabolism and is also involved in immune function. Seventy-five years ago, Fuller-Albright noted that estrogen deficiency after menopause is associated with a decline in bone mineral density (BMD) and osteoporosis (*2,3*). Estrogen regulates bone metabolism through various pathways. Estrogen depletion involves a change in the levels of cytokines such as TNF-α, IL-1, IL-6, and IL-17, thus influencing the functioning of OBs, OCs, and T cells and consequently affecting bone metabolism (*4,5*).

The effect of cytokines on bone remodeling, especially those related to estrogen, has been examined and reviewed in other works, but little is known about the relationship between chemokines and estrogen in bone metabolism. This review will focus on the role and mechanism of chemokines on estrogen-regulated bone metabolism.

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2. Chemokines and chemokine receptors

Chemokines belong to a large family of small cytokines with a low molecular weight ranging from 7 to 15kDa, and chemokines are named according to their ability to induce directed chemotaxis of immune cells throughout the body under physiological and pathological conditions (*6*). Chemokines have been divided into four subfamilies, C, CC, CXC, and CX3C, according to the presence of four cysteine residues in the NH2-terminal part of the protein. The function of chemokines is mediated by the G protein-coupled receptor superfamily (GPCR) with seven transmembrane domains. Chemokines belonging to the CC- and CXC-subfamily are the best known chemokines thus far. The CC chemokine subfamily is mainly related to monocyte, lymphocyte, and natural killer cell chemotaxis, while CXC chemokines take part in the chemotaxis of neutrophils. The best known function of chemokines is their role in immune cell homeostasis and in the pro-inflammatory process. Homeostatic chemokines are generally involved in lymphocyte trafficking and localization and are usually produced constitutively. Pro-inflammatory chemokines are produced during infection, stimulating leukocyte chemotaxis and inducing the migration of leukocytes to injured or infected sites or activating cells to enhance the host inflammatory response (*6*). However, these two functions often overlap. Different chemokines play various roles in immunoreaction *via* their specific transmembrane G protein-coupled receptors. For example, CXCL1(KC) and CXCL2(MIP-2) participate in the first line of innate immunity by attracting neutrophils, and CCL5(RANTES) act on monocytes and macrophagocytes (*7*). In addition, chemokines play roles in cell proliferation, differentiation, activation, immunological tolerance, cell movement, haematopoiesis, viral or cell interactions, neovascularization, cancer metastasis, and other activities (*8,9*).

Nearly 50 chemokines and 20 chemokine receptors have been identified thus far. Some chemokines are able to interact with multiple chemokine receptors while others interact with one distinct chemokine receptor. A series of downstream signals emerge after chemokines bind with their receptors, such as the activation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), p21-activated kinase (PAK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Depending on the type of inflammatory response and the nature of the pathogen and due to the dynamic pattern of expression of chemokine receptors in a temporal and spatial sense, chemokines are able to mediate complex biological functions with exquisite specificity (*10-37*). The receptors and immune functions of chemokines are summarized in Table 1.

3. Chemokines and bone

3.1. *Chemokines and OB*

Numerous chemokines have been found to be involved in the process of bone metabolism (*38-49*), as summarized in Table 2. OBs are derived from bone marrow mesenchymal stem cells (BMMSCs). Along with other factors, chemokines play a crucial role in the process of BMMSCs differentiating into OBs. Ascorbate, b-glycerophosphate (b-GP), and dexamethasone (DEX) profoundly influence the differentiation of OBs. The synthetic glucocorticoid dexamethasone (DEX) increases the production of CXCL8 and CXCL1 by human mesenchymal stem cells (hMSCs) during differentiation into an osteoblastic lineage. ERK and p38 mitogen-activated protein kinase (MAPK) pathways are involved in subsequent activation of G-coupled receptors. A study has reported that OBs may also secrete CXCL8 and CXCL1, stimulating the differentiation of OBs and thus playing an autocrine role in bone metabolism (*50*). Chemokine and cytokine expression in MSCs were analyzed with a protein array during the differentiation of OBs. The levels of IL-6, MCP-1 (CCL2), and MIP-1B(CCL4) decreased and the levels of IL-10, IL-12, FGF-basic, and VEGF all increased with a lineage commitment towards mature OBs (*51*).

CXCL10 and CXCL13 were highly expressed in bone biopsies of patients following trauma. At the same time, high levels of their corresponding receptors-CXCR3 and CXCR5 were expressed in human OBs. While CXCR3/CXCL10 and CXCR5/CXCL3 have little effect on the proliferation of OBs, this finding suggests their involvement in the maturation of OBs and bone formation (*52*).

The receptors for CXCL8, CXCL9, CXCL10, and CCL20 have been found to be expressed in human primary OBs. Interestingly, those four chemokines are found to be elevated in the blood serum of patients with rheumatoid arthritis (RA) and expression of CCL20 and CCR6 increases in their OBs (*53*). However, a study by Pathak *et a*l. found that CXCL8 and CCL20 did not significantly inhibit OB proliferation or gene expression of matrix proteins. Although these chemokines did not directly enhance osteoclastogenesis, they did enhance IL-6 gene expression and protein production by OBs that in turn enhanced OB-mediated osteoclastogenesis (*54*). Experimental data suggests that the chemokines CXCL12 and CXCL13 increase the proliferation of OBs isolated from patients with osteoarthritis (OA) and that the expression of these chemokines is found mainly in areas of bone remodeling, providing another clue to their function. These chemokines also up-regulate the expression of collagen type I (a marker of OB differentiation) mRNA but they have no effect on alkaline phosphatase in OBs from patients with OA (*55*).

Table 1. The roles of chemokines in the immune system *(continued on next page)*

Chemokines	Other Name	Receptor	Receptors/Distribution in immune cells	Functions in immunity	*Ref.*
CXCL1	GROα	CXCR1, CXCR2	Neutrophils, monocytes, mast cells, basophils, dendritic cells, CD8 T cells, natural killer cells	Neutrophil trafficking	(*7*)
CXCL2	GROβ	CXCR2	Neutrophils, monocytes, mast cells, basophils, dendritic cells, natural killer cells	Neutrophil trafficking	(*7*)
CXCL3	GROγ	CXCR2	Neutrophils, monocytes, mast cells, basophils, dendritic cells, natural killer cells	Neutrophil trafficking	(*11*)
CXCL4	PF4	CXCR3	Basophils, Th1 cells, CD8 T cells, natural killer cells, Treg cells	Monocyte activation	(*12*)
CXCL5	ENA-78	CXCR2	Neutrophils, monocytes, mast cells, basophils, dendritic cells, natural killer cells	Neutrophil trafficking	(*7*)
CXCL6	GCP-2	CXCR1, CXCR2	Neutrophils, monocytes, mast cells, basophils, dendritic cells, CD8 T cells, natural killer cells	Neutrophil trafficking	(*13*)
CXCL7	NAP-2	CXCR2	Neutrophils, monocytes, mast cells, basophils, dendritic cells, natural killer cells	Neutrophil trafficking	(*7*)
CXCL8	IL-8	CXCR1, CXCR2	Neutrophils, monocytes, mast cells, basophils, dendritic cells, CD8 T cells, natural killer cells	Neutrophil trafficking	(*14*)
CXCL9	MIG	CXCR3	Basophils, Th1 cells, CD8 T cells, natural killer cells, Treg cells	Th1 response, Th1, CD8 and NK trafficking	(*15*)
CXCL10	IP-10	CXCR3	Basophils, Th1 cells, CD8 T cells, natural killer cells, Treg cells	Th1 response, Th1, CD9 and NK trafficking	(*15*)
CXCL11	I-TAC	CXCR3, CXCR7	Basophils, Th1 cells, CD8 T cells, natural killer cells, Treg cells	Th1 response, Th1, CD10 and NK trafficking	(*15*)
CXCL12	SDF-1	CXCR4, CXCR7	Widely expressed	Bone marrow homing	
CXCL13	BCA-1	CXCR5	Basophils, CD8 T cells	B cell and Tfh positioning in lymph nodes	(*16*)
CXCL14	BRAK	Unknown		Macrophage homing to the skin	(*17*)
CXCL15		Unknown			
CXCL16	SR-PSOX	CXCR6	Th1 cells, Th17 cells, natural killer cells, plasma cells	NKT and ILC migration and survival	(*18*)
CXCL17	DMC	Unknown			
CCL1	I-309	CCR8	Dendritic cells, monocytes, macrophages, Th2 cells, Treg cells	Th2 and Treg trafficking	(*19*)
CCL2	MCP-1	CCR2	Monocytes, macrophages, Th1 cells, basophils, natural killer cells	Monocyte trafficking	(*20*)
CCL3	MIP-1α	CCR1, CCR5	Dendritic cells, monocytes, macrophages, natural killer cells, Th1 cells, TH17 cells, Treg cells, neutrophils, basophils	Macrophage-NK migration; T cell/DC interaction	(*21*)
CCL4	MIP-1β	CCR5	Dendritic cells, monocytes, macrophages, natural killer cells, Th1 cells, TH17 cells, Treg cells	Macrophage-NK migration; T cell/DC interaction	(*21*)
CCL5	RANTES	CCR1, CCR3, CCR5	Dendritic cells, monocytes, macrophages, natural killer cells, Th1 cells, Th2 cells, TH17 cells, Treg cells, neutrophils, basophils, eosinophils, mast cells	Macrophage-NK migration; T cell/DC interaction	(*22*)
CCL7	MCP-3	CCR1, CCR2, CCR3	Neutrophils, monocytes, macrophages, Th1 cells, basophils, dendritic cells, eosinophils, Th2 cells, mast cells, natural killer cells	Monocyte mobilization	(*23*)
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5	Neutrophils, monocytes, macrophages, Th1 cells, basophils, dendritic cells, eosinophils, Th2 cells, mast cells, natural killer cells, TH17 cells, Treg cells	Th2 response	(*24*)
CCL9	MIP-1γ	CCR1	Neutrophils, monocytes, macrophages, Th1 cells, basophils, dendritic cells	T cell, NK cell, and myeloid cell migration	(*25*)
CCL11	Eotaxin	CCR3	Eosinophils, basophils, Th2 cells, mast cells, dendritic cells	Eosinophil and basophil migration,Th2 response	(*26*)
CCL12	MCP-5	CCR2	Monocytes, macrophages, Th1 cells, basophils, natural killer cells	Monocyte trafficking	(*20*)

Table 1. The roles of chemokines in the immune system *(continued)*

Chemokines	Other Name	Receptor	Receptors/Distribution in immune cells	Functions in immunity	*Ref.*
CCL13	MCP-4	CCR1, CCR2, CCR3	Neutrophils, monocytes, macrophages, Th1 cells, basophils, dendritic cells, eosinophils, Th2 cells, mast cells, natural killer cells	Th2 response	(*26*)
CCL14	HCC-1	CCR1	Neutrophils, monocytes, macrophages, Th1 cells, basophils, dendritic cells		(*26*)
CCL15	HCC-2	CCR1, CCR3	Neutrophils, monocytes, macrophages, Th1 cells, basophils, dendritic cells, eosinophils, Th2 cells, mast cells	Hematopoietic progenitor cell adhesion and migration	(*27*)
CCL16	HCC-4	CCR1, CCR3	Neutrophils, monocytes, macrophages, Th1 cells, basophils, dendritic cells, eosinophils, Th2 cells, mast cells	Macrophage activator	(*28*)
CCL17	TARC	CCR4	Th2 cells, Th17 cells, Treg cells, monocytes, basophils, CD4 & CD8 T cells	Th2 response, Th2 cell migration, Treg, homing to the lungs and skin	(*26*)
CCL18	PARC	Unknown		Th2 response, marker of AAM, homing to the skin	(*26*)
CCL19	ELC	CCR7	Dendritic cells (mature), T cells, basophils	T cell and DC homing to lymph nodes	(*29*)
CCL20	MIP-3α	CCR6	Th17 cells, natural killer cells, Treg cells	Th17 response, B cell and DC homing to gut-associated lymphoid tissue	(*30*)
CCL21	SLC	CCR7	Dendritic cells (mature), T cells, basophils	T cell and DC homing to lymph nodes	(*29*)
CCL22	MDC	CCR4	Th2 cells, Th17 cells, Treg cells, monocytes, basophils, CD4 & CD8 T cells	Th2 response, Th2 cell migration, T reg migration	(*10*)
CCL23	MPIF-1	CCR1	Neutrophils, monocytes, macrophages, Th1 cells, basophils, dendritic cells	Monocyte and macrophage trafficking	(*31*)
CCL24	Eotaxin-2	CCR3	Eosinophils, basophils, Th2 cells, mast cells, dendritic cells	Eosinophil and basophil migration	(*32*)
CCL25	TECK	CCR9	Basophils, dendritic cells	T cell homing to the gut, thymocyte migration	(*33*)
CCL26	Eotaxin-3	CCR3	Eosinophils, basophils, Th2 cells, mast cells, dendritic cells	Eosinophil and basophil migration	(*32*)
CCL27	CTAK	CCR10	T cells, IgA+ plasma cells	T cell homing to the skin	(*34*)
CCL28	MEC	CCR10	T cells, IgA+ plasma cells	T cell and IgA plasma cell homing to the mucosa	(*21*)
XCL1	Lymphotactin	XCR1	Dendritic cells, CD4+ T cells, NK cells	CD8(+) T cell cytotoxicity	(*35*)
XCL2	SCM-1β	XCR1	Dendritic cells	Cross-presentation by CD8+ DC	(*36*)
CX3CL1	Fractaline	CX3CR1	Monocytes, macrophages, Th1 cells, dendritic cells, natural killer cells	NK, monocyte, and T cell migration	(*37*)

3.2. *Chemokines and OCs*

In vitro, OCs differentiate from bone marrow cells and they require co-incubation with macrophage-colony stimulating factor (M-CSF) and receptor-activator of NF-κB ligand (RANKL) (*56*). A systematic study by Chambers *et al*. found that the chemokine ligands CCL9, CCL22, CXCL13, and CCL25 and the chemokine receptors CCR1, CCR3, and CX3CR1 were highly expressed in OCs and that RANKL strongly induced the expression of CCL9, CCR1, and CCR3 while inhibiting the expression of CCR2, CCR5, and CCR7 in OCs (*57*).

Inflammatory cytokines may stimulate OBs or other types of active cells and release CCR1 chemokines, including CCL3, CCL5, and CCL7. These chemokines stimulate the recruitment and development of pre-OCs and promote the migration of premature OCs (*58*). CCL3 also prolongs the survival of mature OCs through NF-κB signals (*59*). CCR5 is also a chemokine receptor for CCL3, CCL5, CCL7, and CCL8. The amount of tartrate-resistant acid phosphatase (TRAP)-positive OCs and the expression of OC markers - cathepsin K, metalloprotease 13 (MMP13), and RANKL - were significantly higher in CCR5-deficient mice ($CCR5^{-/-}$) treated for orthodontic tooth movement than in wild-type mice (WT). The expression of two osteoblastic differentiation markers - runt-related transcription factor 2 (RUNX2) and OCN - and the expression of interleukin 10 (IL-10), bone resorption regulators, and OPG was lower in $CCR5^{-/-}$ mice. Thus, CCR5

Table 2. The functions of chemokines in bone metabolism

Chemokines	Receptors	Main functions in bone metabolism	*Ref.*
CXCL1	CXCR1, CXCR2	Recruits osteoclast precursors	(*38*)
CXCL2	CXCR2	Generates osteoclasts (OCs)	(*39*)
CXCL5	CXCR2	Mobilizes HSCs	(*40*)
CXCL7	CXCR2	Stimulates the formation of OCs	(*41*)
CXCL8	CXCR1, CXCR2	Mediates the differentiation of hMSCs into OBs, enhances osteoblast-mediated osteoclastogenesis	(*50,53*)
CXCL9	CXCR3	Stimulates the formation of OCs	(*42*)
CXCL10	CXCR3	Mediates the differentiation of OCs	(*65*)
CXCL11	CXCR3, CXCR7	Reduces osteoclastogenesis	(*43*)
CXCL12	CXCR4, CXCR7	Increases the proliferation of OBs, promotes osteoclastogenesis, mediates the differentiation of OCs, recruits OC precursors, facilitates the homing of HSC	(*50,54,65,68,80*)
CXCL13	CXCR5	Increases the proliferation of OBs	(*54*)
CXCL16	CXCR6	Osteoblast migration	(*44*)
CCL2	CCR2	Mediates the differentiation of OBs, recruits OC precursors, promotes osteoclastogenesis	(*51,64*)
CCL3	CCR1, CCR5	Stimulates the recruitment and migration of premature OCs, prolongs the survival of OCs, augments the differentiation of osteoclasts, inhibits the differentiation of OBs	(*57,58,72*)
CCL4	CCR5	Mediates the differentiation of OBs	(*51*)
CCL5	CCR1, CCR3, CCR5	Stimulates the recruitment and migration of premature OCs	(*57*)
CCL7	CCR1, CCR2, CCR3	Stimulates the migration of premature OCs	(*57*)
CCL8	CCR1, CCR2, CCR3, CCR5	Stimulates the migration of premature OCs	(*45*)
CCL9	CCR1	Mediates the differentiation of OCs	(*56*)
CCL17	CCR4	Recruits osteoclast precursors	(*46*)
CCL18	Unknown	Recruits osteoclast precursors	(*47*)
CCL19	CCR7	Reduces osteoclastogenesis	(*43*)
CCL20	CCR6	Enhances osteoblast-mediated osteoclastogenesis	(*53*)
CCL21	CCR7	Recruits osteoclast precursors	(*48*)
CCL22	CCR4	Recruits osteoclast precursors	(*46*)
CCL23	CCR1	Recruits osteoclast precursors	(*49*)
CX3CL1	CX3CR1	Stimulates the formation of OCs	(*41*)

might be an inhibitor of alveolar bone resorption by down-regulating OC function during orthodontic tooth movement (*60*). A study reported finding CCR5 in osteolysis (*61*), while treatment with a CCR5 antagonist resulted in the abrogation of osteolysis induced by multiple myeloma (*62*) and improvement of arthritis-related bone loss (*63,64*). CCR2 binds to CCL2, CCL7, CCL8, and CCl3 and is down-regulated during the differentiation of OCs. The OCs in CCR2-knockout mice decreased in both quantity and quality, so osteoporosis did not occur in CCR2-knocknout mice after ovariotomy. After activation of CCR2 in OC progenitor cells, nuclear factor-κB (NF-κB) and extracellular signal–related kinase 1 and 2 (ERK1/2) signaling were activated while more RANK was expressed by progenitor cells, making them more susceptible to RANK ligand-induced osteoclastogenesis (*65*).

During the differentiation of OCs, the expression of CXCL10 and CXCL12 and their receptors CXCR3 and CXCR4 increased significantly (*66*). Stromal cell-derived factor 1a (SDF-1a), also known as CXC chemokine ligand 12 (CXCL12), is also produced by stromal cells, OBs, and OCs in multiple myeloma (MM) (*66-68*), and its receptor CXCR4 is a regulator of bone resorption. A study found that gambogic acid (GA) down-regulated CXCR4 mRNA expression in MM cells by suppressing the binding of NF-kB to a CXCR4 promoter. GA inhibits osteoclastogenesis mediated by MM by suppressing SDF1a/CXCR4 signaling pathways (*69*). A study by Diamond *et al*. suggested that the expression of SDF-1a increased in a murine model of myeloma, inducing a marked increase in OCs and bone loss (*70*). The chemokine CXCL12 may recruit OC precursors and increase the production of MMP-9 and increase the bone function of OCs in bone resorption (*71*) while CXCL10 in bone plays an important role in OBs and the recruitment and proliferation of T cells (*66*).

3.3. *Different chemokine axes and bone metabolism*

CCR1-CCL3-related signaling pathways (axes) play a crucial role in bone metabolism. Up-regulated levels of Runx2, Atf4, Osteopontin, and Osteonectin in $Ccr1^{-/-}$ mice resulted in less potential for OB differentiation. The co-culturing of $CCR1^{-/-}$OBs with OC precursors failed to induce OCs. $CCR1^{-/-}$ mice had impaired differentiation and functioning of OBs and OCs mainly as a result of the modulation of RANK-RANKL-mediated interaction (*72*). Mice treated with Met-RANTES, an antagonist of CCR1, displayed alveolar bone remodeling. CCR1 expression by OBs is significantly greater in patients with myeloma bone diseases (MBD) compared to healthy controls. The chemokine cytokine ligand 3(CCL3), a pro-inflammatory protein named macrophage inflammatory protein 1-alpha (MIP-1α), binds to CCR1 and it inhibits

the differentiation, proliferation, and osteogenic potential of OBs by impairing mineralization activation, it decreases levels of OCN, Runx2, and osterix (Osx), and it stimulates OC activity in MBD. Stimulation with CCL3 antibody can increase the levels of OCN, Runx2, and Osx, partially restoring the activity of OBs (*73*). CCL3 suppresses the activity of OBs mediated by ERK activation and a decrease in the osteogenic transcription factor osterix (*74*). CCL3 (MIP-1α) is an osteoclastogenic C–C chemokine, and bone resorption in $CCL3^{-/-}$ mice decreased with low levels of RANK, RANKL, and TNF-α after mechanical loading (*75*). CCL3 up-regulates the expression of RANKL by OBs, it increases the transcription of TNF-α, it induces OC–OB interaction, it augments the differentiation of OCs, and it consequently increases bone resorption (*76*). Mechanical loading, such as orthodontic force, up-regulated the expression of CCL3 and CCR1 in alveolar bone and soft periodontal tissues, thus affecting the recruitment, differentiation, and activation of OC precursor cells and OBs and resulting in bone remodeling (*58,77*). However, CCL3 had no effect on bone loss associated with periodontal disease (*78*). CCR5 is another receptor for CCL3 and is related to up-regulation of infection-related bone loss in periodontal disease and the prevention of bone resorption induced by mechanical loading (*79,80*).

CXCL12/CXCR4 is another important pathway that plays a key role in maintaining skeletal homeostasis. CXCR4 and its only ligand SDF-1/CXCL12 are expressed by many cells types, including stromal cells, OCs, and OBs. CXCR4 is a type of OB-specific chemokine receptor. CXCR4/CXCL12 facilitates the homing of HSC to bone narrow; disruption of CXCR4/CXCL12 results in a decrease in HSC homing that is lethal in embryos (*81*). When CXCR4 is deleted by Cre-Loxp technology in mature OBs in mice, bone mass decreases and alterations appear in cancellous bone structure. In mature OBs, CXCL12-CXCR4 signaling regulates osteoprogenitor and OC precursor populations, but it also plays a multifunctional role in regulating bone formation and resorption in mature OBs (*82*). In a model of bone metastasis, mice with CXCR4 null hematopoietic cells had an increase in bone resorption, the perimeter of OCs, and bone loss. After the disruption of CXCR4, the differentiation of OCs accelerated and the resorption of bone increased (*83*). The functions of chemokines in bone remodeling processes differ depending on the agent or disease that induced their production.

4. Estrogen modulates the expression of chemokines

4.1. *C chemokines*

A study found that ER-$\alpha^{-/-}$ human breast cancer does not respond to hormonal therapy and that it generally has a poor prognosis. Epigenetic regulation, including DNA methylation and histone deacetylation, is a common mechanism resulting in ER gene silencing. The pharmacologic inhibitors 5-aza 2'deoxycytidine (AZA) and Trichostatin A (TSA) alter this mechanism by stimulating ER mRNA and expression of its functional protein. A study by Keen *et al*. found that the expression of two genes - lymphotactin (XCL1) and protein phosphatase 2A (PP2A) - appeared to be related to ER expression. PP2A is an upstream determinant of ER expression, while XCL1 is downstream and responsive to ER expression. A study found that the expression of XCL1 was down-regulated in the presence of AZA/TSA and ICI 182,780 (*84*). In conclusion, estrogen receptors suppress XCL1 expression.

4.2. *CC chemokines*

Estrogen is known to significantly affect immunity and inflammatory autoimmune-mediated diseases. Estrogen regulates immunity and inflammatory autoimmune-mediated diseases by altering the secretion of chemokines from activated splenocytes or other lymphocytes. However, the roles of estrogen differ in different tissues and cells. Estrogen significantly promotes the expression of spleen MCP-1 (CCL2), MCP-3 (CCL7), MCP-5 (CCL12), eotaxin (CCL11), and stromal cell-derived factor-1(SDF-1; CXCL12) by endothelial cells but has little effect on dermal endothelial cells (*85*). However, estrogen reduces the levels of MCP-1 in human coronary artery endothelial cells (HCAECs) (*86*). Estrogen significantly increased the levels of some specific chemokines such as MIP-1a and MCP-1/JE in mammary cells *in vivo* and *in vitro* (*87*), while the expression of chemokines MCP-1/JE and MIP-1a did not change in murine monocytes co-cultured with estrogen or tamoxifen (*88*). A study has found that both estrogen and tamoxifen significantly down-regulate the expression of CCR2 by murine monocytes and that they down-regulate the expression of CXCR3 by murine monocytes to a lesser extent at the same time (*88*). Estradiol inhibited CCL20 secretion at 48 hr in freshly isolated and polarized uterine epithelial cells of BALB/c mice, regardless of whether keratinocyte growth factor (KGF) was present or not (*89*). Raloxifene is a selective estrogen receptor modulator (SERMs) and a promising treatment for experimental autoimmune encephalomyelitis (EAE). In animals with EAE, raloxifene decreased IL-1β and it induced the expression of CCL20 in reactive astrocytes, thus promoting Th17 cell migration (*90*).

4.3. *CXC chemokines*

Estrogen and epidermal growth factor (EGF) significantly promote the release of the angiogenic chemokine CXCL8 in human breast carcinoma MCF-7 cells. When MCF-7 breast carcinoma cells were co-cultured with EGF and estrogen, the expression of CXCL8 increased up to two-fold in comparison with

culturing with estrogen or EGF alone, indicating the additive effects of estrogen and EGF (*91*). Estrogen is thought to be a key regulator of CCL20 and CXCL1 in the upper female reproductive tract since it decreases the secretion of CCL20 but it directly increases the levels of CXCL1 in uterine epithelial cells (*92*). Estrogen enhances KGF-induced CXCL1 secretion in freshly isolated and polarized uterine epithelial cells at 24 hr (*89*). In Foxp3-deficient mice with EAE, estrogen (17b-estradiol, E2) suppressed the expression and proliferation of CCL2 and CXCL2 but it enhanced the secretion of interleukin-10 (IL-10) and IL-13 by myelin oligodendrocyte glycoprotein (MOG)-35-55-specific spleen cells. E2 treatment suppresses the expression of CCR6 in spleen and lymph node T cells while it increases the levels of IL-17, interferon-γ, and TNF-α, suggesting that E2 could provide protection against EAE even in the absence of Foxp3+ Treg cells. E2 treatment increased the expression of several chemokines and receptors, including CXCL13 and CXCR5 (*93*). Theiler's murine encephalomyelitis virus (TMEV) induces demyelination in susceptible strains of mice (SJL/J). In this immunopathological process, increased expression of CXCL10 by astrocytes is induced by the inflammatory cytokines IL-1α, IFN-γ, and TNF-α, causing demyelination. However, 17β-estradiol or selective estrogen receptor modulators (SERMs) combined with estrogen receptor-α inhibited the expression of CXCL10 in astrocytes (*94*).

4.4. *CX3C chemokines*

In order to examine the effect of estrogen on the cytotoxic response to an early-stage infection with the bronchitis (IB) virus in hen oviducts, Nii *et al*. designed an experiment in which they inoculated the oviductal magnum lumen of White Leghorn hens with attenuated IB virus (aIBV group) in the egg-laying phase and with its vehicle (control group) in the molting phase (*95*). Twenty-four hours later, the oviductal isthmus and uterus were collected to examine the expression of cytokines, including CXCL12, CX3CL1, and IFN-γ. The level of expression of CXCL12, CX3CL1, and IFN-γ was significantly higher in the aIBV group in the egg-laying phase and M-EB hens compared to the control group. Nii *et al*. concluded that estrogen enhanced the expression of CX3CL1 in hen oviducts infected with the IB virus. Another study suggested that genistein, a polyphenolic nonsteroidal isoflavonoid with estrogen-like activity, strongly suppressed TNF-α-induced expression of CX3CR1 in monocytes (*96*).

5. The mechanism by which estrogen modulates chemokines

In experiments where spleen endothelial cells were cultured with estradiol, estradiol up-regulated the level of estrogen receptor alpha (ER-α) 2.9-fold and it down-regulated the level of estrogen receptor beta (ER-β) 2.1-fold. In dermal endothelial cells, however, levels of both ER-α and ER-β decreased. When spleen endothelial cells were co-cultured with tamoxifen (one of ER antagonists) or ICI 182,780, none of these estradiol-mediated effects on splenic chemokines were noted, indicating that estrogen selectively regulates chemokines through estrogen receptors (*85,97*). In HCAECs, estrogen at a concentration of 10-8M reduced the levels of MCP-1, down-regulating MCP-1 mRNA and protein expression 30% (*86*). Raloxifene and tamoxifen also inhibited the expression of MCP-1 mRNA and protein. They induced a concentration-dependent inhibition of MCP-1 expression and production in cultured HCAECs. Treatment with estrogen or raloxifene and tamoxifen was ineffective at changing levels of MCP-1 expression in human umbilical vein endothelial cells (HUVECs) (*86*). In cultured mammary cells and murine mammary tissue, estrogen significantly down-regulates the levels of specific chemokines - MIP-1a and MCP-1/JE - compared to baseline levels, and estrogen also suppresses expression of JE/monocyte chemoattractant protein 1(MCP-1/JE) mRNA in murine macrophage cells (*87*). A study by Janis *et al*. reported no change in the expression of the chemokines MCP-1/JE and MIP-1a in murine monocytes, regardless of whether those cells were co-cultured with estrogen or tamoxifen (*88*). However, estrogen may affect the functioning of chemokines by decreasing the chemotaxis of monocytes to MCP-1/JE (*88*). The effects of estrogen on chemokines, including MCP-1/JE and MIP-1a, seem to be cell line-dependent. CXCL8 (interleukin-8), a member of the CXC family, is overexpressed in ERα-negative breast cancer cell lines while a high level of CXCL8 expression in tumors is closely correlated with the activating protein-1 (AP-1) pathway and somewhat correlated with the NF-κB pathway (*98*). The inhibitory effect of estradiol or raloxifene on CCL20 secretion and function was reversed by administration of an estrogen receptor antagonist designated ICI 182,780 (*89,90*). ERα antagonists mediate CCL20 and CXCL1 secretion while ERβ does not. Several studies have indicated that treatment of uterine epithelial cells with Y134 (ERα and ERβ-SERMS) markedly enhanced CCL20 production and inhibited CXCL1 production, while treatment with PHTPP (ERβ specific SERMS) had no effect on either CCL20 or CXCL1 (*92*). The findings above indicate that estradiol plays an important role in mediating chemokine secretion.

6. The relationship between chemokines and estrogen in regulation of bone metabolism

Estrogen has been found to down-regulate the expression of cytokines that are known to enhance

osteoclastogenesis, such as IL-1 and IL-6. Estrogen deficiency also increases the amount of TNF-α producing T cells, thus promoting the expression of RANKL in OBs and facilitating osteoclastogenesis (*99*).

Ovariectomized (OVX) mice are a model that can be used to examine the effect of estrogen on bone metabolism. Fat from OVX mice was found to secrete high levels of MCP-1 (CCL2) and an ovariectomy was found to induce increased formation of OCs (*100*). CCR2 is a receptor for CCL2 that is expressed by various hematopoietic cells. In OVX mice, CCR2 was up-regulated in preosteoclasts; when estrogen was deficient, CCR2 induced the expression of RANK, thus promoting osteoclastogenesis that led to bone loss. In contrast, bone loss was inhibited in $CCR2^{-/-}$ mice after an ovariectomy. The absence of CCR2 increased the number of preosteoclasts, but these preosteoclasts did not counteract the protection provided by CCR2, so CCR2 plays a crucial role in the differentiation of OCs (*65*). Estrogen down-regulated the expression of CCR2 in monocytes and the levels of CCL2, but CCL2 increased after menopause (*101,102*). An estrogen deficiency up-regulated the expression of CCR2 preosteoclasts through both nuclear factor-κB (NF-κB) and extracellular signal–related kinase 1 and 2 (ERK1/2) signaling and an estrogen deficiency promoted osteoclastogenesis.

Estrogen is an important immunomodulatory agent that regulates the immune system, thus affecting bone metabolism. Regulatory T cells (Treg cells) suppress bone resorption and the differentiation of OCs in bone marrow. The CXCL12-CXCR4 pathway is critical to Treg cells migrating to and staying in bone marrow. An estrogen deficiency decreases the expression of CXCR4 in Treg cells and it significantly reduces the Treg cell population in bone marrow in OVX mice with little effect on CXCL12. An estrogen deficiency down-regulates the Treg cell population in bone marrow by inhibiting CXCR4 expression in Treg cells and Treg cell trafficking, thereby preventing Treg cells from suppressing the differentiation of OCs and eventually leading to bone loss in OVX mice (*103*).

In EAE, estrogen down-regulates IL-17 production and it inhibits Th17 differentiation through estrogen receptor α (ERα) in T cells (*102,104-106*). An estrogen deficiency increases the amount of Th17 cells in bone marrow and IL-17 levels. IL-17 promotes the expression of RANL, TNF-α, and IL-6 and TRAP-positive cells while blocking IL-17 pathways, resulting in little bone loss in OVX mice (*107*). IL-17 plays a crucial role in inflammation-induced bone loss by stimulating osteoclastogenesis, so IL-17 is thus the driving force in some autoimmune diseases that involve bone metabolism, such as RA in particular. IL-17 up-regulates synovial fibroblasts to produce CXCL8, which attracts neutrophils to the joints and thus enhances joint inflammation in RA (*108,109*). The CCR6-CCL20 pathway is expressed in Th17 cells, and this pathway facilitates the migration of Th17 cells to the site of inflammation (*110*). Estrogen augments the expression of CCR6 and CCL20 by Th17 cells in lymph nodes (LNs) through ERα, preventing the migration of Th17 cells to joints in established arthritis and resulting a higher level of Th17 cells in LNs and a lower level in joints (*111*). Estrogen affects the migration of Th17 cells *via* the CCR6 and CCL20 pathways while Th17 cells produce IL-17 to stimulate the expression of CXCL8, thus affecting the migration of neutrophils.

7. Conclusion

Chemokines play an important role in estrogen-regulated bone metabolism and may serve as a novel area for further examination in order to develop new therapeutics to treat diseases of bone metabolism.

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