Estrogen receptor-related receptor-α (ERR-α) is dysregulated in inflammatory arthritis

E. Bonnelye 1, N. Laurin 1, P. Jurdic 2, D. A. Hart 3 and J. E. Aubin 1

Objectives. Subchondral bone loss is a characteristic feature of inflammatory arthritis. Recently, estrogen receptor-related receptor-α (ERR-α), an orphan nuclear receptor, has been found to be involved in activation of macrophages. We hypothesized that ERR-α which is expressed and also functional in articular chondrocytes, osteoblasts and osteoclasts, may be involved in rodent models of inflammatory arthritis.

Methods. Erosive arthritis was induced in DBA/1 mice by injection of type II collagen in Freund’s complete adjuvant. RNA was isolated from the bone and joints and expression of ERR-α and cartilage (GDF5 and Col2a1) and bone [bone sialoprotein (BSP) and osteocalcin (OCN)] markers was analysed by semi-quantitative PCR.

Results. We report for the first time that the expression of ERR-α is down-regulated early in bone and later in joints of mice with type II CIA. Concomitantly, temporal changes were observed in GDF-5 and Col2a1 expression in joints following both initial injection and booster injection of type II collagen. Similarly, down-regulation of ERR-α mRNA expression in subchondral bone in mice with induced joint inflammation was also paralleled by down-regulation of markers of bone formation (BSP, OCN).

Conclusions. These data suggest that dysregulation of ERR-α expression may precede and contribute to the destruction of cartilage and bone accompanying inflammatory arthritis.

Key Words: Joint, Bone, Rheumatoid arthritis, Nuclear receptor, Estrogen receptor-related receptor.

Introduction

RA is a chronic inflammatory autoimmune disease characterized by invasive synovial hyperplasia leading to progressive joint and bone destruction [1]. Bone erosion most frequently occurs at sites of inflammation and cartilage–pannus and bone–pannus interfaces, with involvement of multiple cell types including synovial cells, immune cells, osteoclasts and articular chondrocytes.

The nuclear receptor superfamily comprises both ligand-dependent molecules such as the estrogen receptors (ERs), and a large number of so-called orphan receptors for which no ligand has yet been identified [2]. Three orphan receptors, ER-related receptor α (ERR-α), ERR-β and ERR-γ (NR3B1, NR3B2 and NR3B3, respectively, according to the Nuclear Receptors Nomenclature Committee, 1999), share similarity with ER-α and ER-β (NR3A1 and NR3A2, respectively) [3, 4]; however, they do not bind estrogens [5–9]. Indeed, sequence alignment of ERR-α and the ERs reveals a high similarity (68%) in the 66 amino acids of the DNA-binding domain but only a moderate similarity (36%) in the ligand-binding domain which may explain the fact that ERR-α recognizes the same DNA-binding elements as ERs but does not bind estrogens [10]. Based on its ability to recognize similar DNA sequences as the ERs and the ability of estrogens to stimulate ERR-α gene expression in several tissues (uterus, heart, bone, etc.), ERR-α has been proposed to modulate estrogen signalling [11–16]. In addition to modulation of ER-mediated transcriptional activity, ERR-α regulates fatty acid oxidation and the adaptative bioenergetic response [17–19]. Moreover, ERR-α is involved in cartilage and bone formation in vitro [20–22], and an ESRRA gene regulatory variant associated with increased ERR-α expression has been associated with increased bone mineral density in pre-menopausal women [23, 24]. Very recently, ERR-α has also been described to be involved in macrophage activation [25].

Due to ERR-α expression in bone and articular chondrocytes in adult rats and its function in bone and cartilage formation in vitro, and its function in immune cells, we hypothesized that ERR-α may be regulated in rodent models of inflammatory arthritis.

Materials and methods

Mouse model of collagen-induced inflammatory arthritis

The protocol was given ethical approval by the University of Calgary Animal Care Committee. Male DBA/1 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) at the age of 8 weeks. The animals were housed locally in the University of Calgary Animal Care Centre until 12–14 weeks of age to acclimatize and reach an age when induction of arthritis by collagen II is more uniform. This model of inflammatory arthritis is widely used [26, 27] and animals injected with collagen II in adjuvant (adjuvant + type II collagen) develop an RA-like disease based on histological analysis. Groups of animals were injected with either pyrogen-free phosphate buffered saline (PBS, controls), Freund’s complete adjuvant (FCA, adjuvant controls) or 100 μg collagen II in FCA (experiments) via the base of the tail on day 0. Emulsions of collagen II (bovine, catalogue number IMAD, Chondrex, Inc., Redmond, WA, USA) and FCA (catalogue number IMBII, Chondrex, Inc.) were prepared with a Polytron homogenizer according to the supplier’s protocols (Chondrex, Inc.). In specific experiments, groups of animals were boosted with the appropriate preparation at week 5. At the time of sacrifice (1, 2, 3, 5 and 7 weeks post-treatment), animals were anaesthetized, bled for serum and then terminated by cervical dislocation.
Production of Flt3 ligand cells and macrophage or dendritic cell differentiation

Flt3 ligand cells (FL cells) were generated in vitro as previously described [28]. Briefly, total bone marrow cells from C57BL/6 mice (IFRA-CREDO, Lyon, France) were seeded at 5 × 10⁵ cells/ml in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies, Cergy-Pontoise, France) supplemented with 15% fetal calf serum (FCS, Dutcher, Brumath, France) and 5 ng/ml human FL (R&D Systems, Lille, France). After 6 days of culture, non-adherent cells were collected and designated as FL cells. For macrophage production, FL cells were cultured for 4 days in the presence of 20 ng/ml of M-CSF. Dendritic cells (DCs) were obtained from FL cells cultured in RPMI 1640, supplemented with 10 mM Hepes, 2 mM l-glutamine complemented with 5 ng/ml murine GM-CSF and 10 ng/ml murine TNF-α (Peprotech, Rocky Hill, NJ, USA) [28].

RT-PCR

Total RNA was extracted with Trizol reagent from mouse joints and bones, DCs, FL cells and macrophages. Briefly, the epithyses of the mouse distal femurs and proximal tibiae (joints) were dissected from the metaphyseal and diaphyseal bones (bones) (Fig. 1); marrow was flushed quickly from bones with ice cold PBS until bones were whitened. Both joints and bones were immediately snap-frozen in liquid nitrogen, pulverized over liquid nitrogen using a mortar and then extracted with Trizol reagent. Samples of total RNA (1.5–5 μg) were reverse-transcribed using oligo dT and the first-strand synthesis kit of Superscript™ II (Invitrogen, Carlsbad, CA, USA). PCR was performed with primers specific for Col2a1 (collagen type II chain) [21], osteocalcin (OCN) and bone sialoprotein (BSP) [22], GDF-5 [29], c-fms (MM_FMSCR), (collagen type II chain) [21], osteocalcin (OCN) and bone sialoprotein (BSP) [22], GDF-5 [29], c-fms (MM_FMSCR), L32 and ERR-α (Peprotech, Rocky Hill, NJ, USA) [28]. Primers were sequenced for verification. All primers used are shown in Table 1.

Results

ERR-α expression is decreased in the joints and subchondral bone in type II CIA in mouse

To ask whether ERR-α dysregulation may play a role in conditions in which cartilage integrity is lost, e.g. in joints undergoing destruction as seen in inflammatory arthritis, erosive arthritis was induced by injection of type II collagen in FCA (adjuvant + type II collagen) into DBA/1 mice [26, 27]. Semi-quantitative RT–PCR of RNA from joints 7 weeks after initiation of treatment revealed a dramatic decrease in ERR-α expression in adjuvant-injected (Fig. 2A and B; samples 5–8) and adjuvant + type II collagen-injected (Fig. 2A and B; samples 9–12) mice compared with control mice injected with PBS (Fig. 2A and B; samples 1–4). The decrease in ERR-α expression was even more striking when mice were boosted with the same treatments 5 weeks after the first injection (Fig. 2A and B; samples 13–16). Given our previous observations on a functional role of ERR-α in osteoblasts and bone [22] and the changes that occur in subchondral bone in erosive arthritis [30], we also assessed ERR-α gene expression in subchondral bones from the same treated mice. Although no differences were seen in ERR-α gene expression in adjuvant-treated mice ((Fig. 2C and D; samples 5–8) compared with PBS-treated control mice (Fig. 2C and D; samples 1–4), ERR-α expression was significantly inhibited in mice injected with adjuvant + type II collagen (Fig. 2C and D; samples 9–12) and in mice boosted at 5 weeks (Fig. 2C and D; samples 13–16).

ERR-α expression is down-regulated late in joints and early in bone in type II CIA

To determine whether changes in ERR-α occur acutely and are early indicators of joint destruction or reflect loss of chondrocytes in the later stages of disease, ERR-α expression was assessed over a time course beginning at 1 week post-injection. We first confirmed that an inflammatory response was present at all time-points, as seen by the robust increase in M-CSF receptor c-fms [31] expression in injected compared with non-injected joints (Fig. 3G and H). Consistent with results reported above, ERR-α expression was significantly reduced in joints 7 weeks after initial induction [mice boosted at 5 weeks; Fig. 3A and B (samples 1–4)] and in bone [22] and the changes that occur in subchondral bone in erosive arthritis [30], we also assessed ERR-α gene expression in subchondral bones from the same treated mice. Although no differences were seen in ERR-α gene expression in adjuvant-treated mice ((Fig. 2C and D; samples 5–8) compared with PBS-treated control mice (Fig. 2C and D; samples 1–4), ERR-α expression was significantly inhibited in mice injected with adjuvant + type II collagen (Fig. 2C and D; samples 9–12) and in mice boosted at 5 weeks (Fig. 2C and D; samples 13–16).

Table 1. Primers and conditions used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>PCR cycles</th>
<th>T, °C</th>
<th>Reference</th>
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<tr>
<td>ERR-α</td>
<td>5’ : CAGGAAAGTGAATGCCCCAGG</td>
<td>25</td>
<td>50</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>3’ : TCTTGACAACAAATACATT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L32</td>
<td>5’ : CATGGTGCCCTCCGGCGCTCCT</td>
<td>23</td>
<td>50</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>3’ : CATTTCTTGGCTGGTAGGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDF-5</td>
<td>5’ : TGGGACAGCTGAGATCATGCC</td>
<td>30</td>
<td>50</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>3’ : ACGATGTCCTCGTACTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-fms</td>
<td>5’ : CTTGGGAGATCTTCCTGGTGG</td>
<td>26</td>
<td>58</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>3’ : TAGAGACGGCCAGAGCTCGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col2a1</td>
<td>5’ : GCCCTCGCTGGAGGAGATGATC</td>
<td>30</td>
<td>50</td>
<td>MM_FMSCR</td>
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<tr>
<td></td>
<td>3’ : CTCGATCTGCTCGACGGGCTG</td>
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<tr>
<td>BSP</td>
<td>5’ : GCAGTCTCTCTTCTAGTCG</td>
<td>27</td>
<td>55</td>
<td>[22]</td>
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<tr>
<td></td>
<td>3’ : CTGACCTCTGAGGCTATAGAG</td>
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<tr>
<td>OCN</td>
<td>5’ : AGGACCCTGTCCTGACG</td>
<td>27</td>
<td>55</td>
<td>[22]</td>
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<td></td>
<td>3’ : AAGGGTGGTTCACAGATATG</td>
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<tr>
<td>MHC II</td>
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<td></td>
<td>3’ : GGACTTGTACAGCTGCTCC</td>
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Bonnetelye et al., 2008.
16–19] compared with PBS controls [Fig. 3A and B; samples 1–4]. There was a non-significant trend towards reduced ERR-α expression at 1, 2 and 3 weeks post-injection (Fig. 3A and B; samples 5–8, 9–12 and 13–15, respectively). We next assessed expression, in the same samples, of mRNA for articular chondrocytes (GDF5, a TGF-β/BMP superfamily member [32]) and for proliferating chondrocytes (Col2a1). Compared with PBS controls (Fig 3C and D; samples 1–4), there was a striking down-regulation of GDF5 gene expression at 1 and 2 weeks (Fig. 3C and D; samples 5–8 and 9–12) and at 7 weeks (mice boosted at 5 weeks; Fig. 3C and D; samples 16–19) but a recovery to control levels at 3 weeks (Fig. 3C and D; samples 13–15). Col2a1 expression, on the other hand, was increased at 1, 2 (Fig. 3E and F; samples 5–8 and 9–12) and 7 weeks [mice boosted at 5 weeks; Fig. 3E and F (samples 16–19)] and inhibited at 3 weeks (Fig. 3E and F; samples 13–15) compared with PBS controls (Fig. 3E and F; samples 1–4).

Interestingly, and in contrast to what was seen in cartilage in the same mice, ERR-α gene expression was significantly down-regulated in subchondral bone at all time-points post-injection (Fig. 4A and B). Expression of the osteoblast associated markers, BSP and OCN, in the same samples was also significantly decreased at 1, 3 and 7 (after boost at 5 weeks) weeks [Fig. 3C and D (samples 5–8); Fig. 3E and F (samples 5–8)] compared with PBS controls [Fig. 4C and D (samples 1–4); Fig. 4E and F (samples 1–4)]; both markers tended towards control levels at 2 weeks [Fig. 4C and D (samples 9–12); Fig. 4E and F (samples 9–12)].

ERR-α mRNA is expressed in monocyte-derived cells and precursors

We have previously shown that ERR-α is expressed in monocyte-derived cells such as osteoclasts [13], suggesting that it may

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**Fig. 2.** Expression of ERR-α in joints and femur samples from mice with inflammatory arthritis induced by injection of type II collagen. RNA was extracted at 7 weeks separately from joints or bones (minus bone marrow) from each of four mice injected with PBS as a control (samples 1–4), adjuvant alone (samples 5–8) or adjuvant and collagen type II (adjuvant + type II collagen) once at day 0 (samples 9–12) or twice (booster injection at 5 weeks; samples 13–16). Semi-quantitative RT-PCR was done separately on RNA from joint samples (A) and femur samples (C) from each mouse. The mean ± s.d. of the pooled joint samples (n = 4) (B) and femur samples (n = 4) (D) is also shown (ANOVA, P < 0.05; Student’s t-test post-tests *P < 0.05, **P < 0.01, PBS control vs injected).
also be expressed by other cell types derived from the same haematopoietic lineage, including macrophages (M) or DCs, both of which are known to be crucial in the inflammation process in RA. Since RNA extracted from the joints also includes a contribution from the pannus, we therefore hypothesized that the late down-regulation of ERR-α/C11 observed in joints in type II CIA may reflect ERR-α/C11 expression in haematopoietic cells. Consistent with this, we found that ERR-α mRNAs is expressed in early monocyte precursors (FL cells) obtained from mouse bone marrow after 6 days of expansion with FL, in mature DCs and macrophages (M) (Fig. 5A and B). FL cells are haematopoietic precursors that can differentiate into macrophages, osteoclasts or DCs depending on the cytokines used during cell cultures and exhibit immature monocyte characteristics including expression of the M-CSF receptor, c-fms (Fig. 5A) [28]. As cell lineage controls, mRNA levels for the c-fms marker (expressed by FL cells and
macrophages) and the immunoreceptor, MHCII (known to be very abundant in DCs) are also shown (Fig. 5B).

Discussion

ERR-α expression in fetal and adult growth plate and bone suggests that it may be involved not only in the formation but also in the maintenance and integrity of cartilage/bone throughout the lifetime of the organism. In keeping with this hypothesis, we showed previously that ERR-α/C11 is expressed in osteoblasts and chondrocytes and appears to have an anabolic function in both bone and cartilage [21, 22, 24]. This is consistent with the results reported here in which we found that ERR-α expression was significantly decreased at all times in adjuvant + type II collagen-injected mice compared with PBS controls; BSP and OCN were also decreased at all times (except BSP at 2 weeks; ANOVA $P < 0.01$, $P < 0.05$ and $P < 0.0001$, respectively; Student’s $t$-test post-tests $^* P < 0.05$, $^** P < 0.01$, $^*** P < 0.001$, PBS control vs treated).

In spite of some animal to animal variation in levels of molecules quantified, statistically significant differences in ERR-α and cartilage and bone markers were seen with treatment condition and time. The temporal changes observed in GDF-5 (articular chondrocyte marker) and Col2a1 (marker of articular and growth plate chondrocytes) expression following both the initial injection and the booster injection of collagen type II are similar to the changes seen in the phenotype of articular chondrocytes during osteoarthritic cartilage degeneration, i.e. transition towards a more fetal cellular phenotype [34, 35]. Although some aspects of the chondrocyte phenotype appear to ‘recover’ at intermediate times between initial and booster injections (see the 2–3 week time-points), ERR-α expression remains depressed at all times, suggesting that the chondrocyte phenotype is irreversibly modified by the inflammatory stimulus and that ERR-α may be a marker of this modification.

Fig. 4. Early decrease of ERR-α/C11 expression in bone destruction in type II CIA. ERR-α and marker expression during a time-course experiment in RNA extracted from femoral bones from mice injected with PBS (samples 1–4) (after 4 weeks; mid-way between start and end points of treated mice), or adjuvant + type II collagen after 1 week (samples 5–8), 2 weeks (samples 9–12), 3 weeks (samples 13–15) and at 7 weeks (booster at 5 weeks; samples 16–19). Semi-quantitative RT-PCR was done separately on RNA from bone samples from each mouse (A, C and E). The mean ± S.E. of the pooled bone samples ($n = 4$) is also shown (B, D and F). Results are shown for expression of ERR-α (A and B) and osteoblast markers [BSP (C and D) and OCN (E and F)]. PCR products in each case were normalized to L32. ERR-α expression was significantly decreased at all times in adjuvant + type II collagen-injected mice compared with PBS controls; BSP and OCN were also decreased at all times (except BSP at 2 weeks; ANOVA $P < 0.01$, $P < 0.05$ and $P < 0.0001$, respectively; Student’s $t$-test post-tests $^* P < 0.05$, $^** P < 0.01$, $^*** P < 0.001$, PBS control vs treated).
However, it is also likely that the overall ERR-α levels reflect the complexity of the changes in phenotype of both the articular and growth plate chondrocytes in which ERR-α is expressed. Further, and as previously mentioned, the RNA extracted from the joints also includes a contribution from the pannus, which is known to contain macrophages and multinucleated tartrate-resistant acid phosphatase-positive cells (osteoclast-like cells) identified at erosive surfaces in joints of mice with CIA [36, 37]. We previously showed that ERR-α is expressed in osteoclasts in vivo and in vitro (i.e., the mouse leukocyt monocyte macrophage culture line, RAW, before and after differentiation; [13]). Although a functional role for ERR-α in osteoclasts has not yet been elucidated, we have shown that ERR-α is a potent regulator of the osteoprotegerin (OPG)/receptor activator of nuclear factor-kappa B ligand (RANKL) ratio [38–40] in osteoblasts in vitro [13], indicating that ERR-α can regulate osteoclast development at least indirectly and may therefore predict a parallel role in joint destruction in RA. Moreover, recently, ERR-α had been associated with activation of macrophages [25]. Concomitant to these results, we confirm ERR-α expression in macrophages and found its presence in DCs suggesting that ERR-α may also play a role in the inflammation in RA. It should be noted that ERR-α mRNA levels were also decreased in the joints following exposure of the mice to Freund’s adjuvant alone. As adjuvant alone does not lead to a T-lymphocyte-dependent, progressive and erosive arthritis [41, 42], other mechanisms must be operative. These may relate to macrophage activation and down-regulation of ERR-α and/or release of soluble factors from such activated macrophages which could contribute to the apparent down-regulation of ERR-α expression by other cell types. This is a potentially important point since the mice receiving adjuvant + type II collagen would have the induction of the T-lymphocyte arthritis superimposed on a background of adjuvant-induced changes. However, while the changes in expression of ERR-α mRNA in joint tissues was similar in adjuvant and adjuvant + type II collagen-treated mice, only in the latter were changes detected in ERR-α mRNA levels in the subchondral bone.

The down-regulation of ERR-α mRNA expression in subchondral bone (bone from which marrow had been removed; see Materials and methods section) in mice with induced joint inflammation, paralleled by down-regulation of markers of bone formation (BSP, OCN), are notable for the rapidity of response of subchondral osteoblasts to joint inflammation. These data suggest that dysregulation of ERR-α expression may precede and contribute to the development of the osteopenia accompanying RA. Our data on bone after initial and booster injections also suggest that osteoblast function parallels closely the presence or loss of the inflammatory stimulus. The fact that osteoblast markers, but not ERR-α itself, recovers in bone again suggests the interesting possibility that ERR-α is a useful marker of early changes in subchondral bone in inflamed joints. Moreover, the acute decrease in ERR-α expression in bone after induction of joint inflammation may participate in stimulation of osteoclast development and, consequently, the destruction of joint cartilage and bone. This is consistent with the fact that RANKL is now considered a therapeutic target in arthritis, as OPG treatment prevents bone loss at inflamed joints and has partially beneficial effects on cartilage destruction in arthritis models [43].

ERR-α has recently been shown to be activated by IFN-γ and to be a key effector of IFN-γ in innate response to bacterial pathogenesis [25]. As a result, mice lacking ERR-α are susceptible to infection by intracellular pathogens. IFN-γ is also known to play a critical role in RA as an anti-inflammatory molecule, especially during the initial phase of RA [44], and both inflammatory and degenerative arthritis parameters (cartilage and bone destruction) are significantly exacerbated in the absence of IFN-γ [45]. The role of IFN-γ—as an anti-inflammatory, chondroprotective and anti-osteoclastogenic molecule—support the importance of ERR-α activation in immune cells, articular chondrocytes and osteoclasts in RA.

In conclusion, our data suggest that ERR-α expression level may be useful as a diagnostic marker for diseases characterized by destruction of cartilage and bone such as that seen in inflammatory arthritic conditions. Together with our analyses of ERR-α function in osteogenesis and chondrogenesis, they also point towards modulation of ERR-α activity as a therapeutic strategy in skeletal diseases.

**Rheumatology key messages**

- ERR-α is down-regulated in bone and joints of mice with type II CIA.
- ERR-α dysregulation may precede and contribute to the cartilage and bone destruction accompanying inflammatory arthritis.

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