Selective RARγ Agonists inhibit heterotopic ossification by blocking chondrogenesis but not osteogenesis

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Introduction: Heterotopic ossification (HO) consists of formation of ectopic bone masses following major traumas or invasive surgery. Occurrence of HO lesions is observed in as many as 15 % of total hip arthroplasty patients and over 60 % in wounded soldiers with blast-injured limbs or other serious wounds. HO also follows brain or spine injury. Current treatment options for HO are irradiation and/or NSAID. These treatments are partially effective, but have considerable side-effects including delay of wound healing, malignant tumor formation and bleeding from gastro-intestinal system. Thus, there is an urgent need to develop new and effective therapies for HO.

The process of HO lesion formation nearly recapitulates endochondral ossification. Retinoic acid (RA) is a potent inhibitor of chondrogenesis and has been tested to block pathological endochondral ossification in HO, but failed in clinical trials because of inconsistent effects and general toxicity. RA exerts its function via the three nuclear retinoic acid receptors (RAR) α, β and γ. Upon RA binding, unique combination of RAR and RXR heterodimers regulate a variety of different biological processes.

Since each RAR isoform regulates unique set of target genes, we tested the following working hypotheses: (1) particular RAR isoform(s) play a dominant role in RA-induced anti-chondrogenic activity and if this is the case, (2) activation of such RAR by selective agonists could effectively block ectopic bone formation with moderate side effects compared to the more serious effects caused by RA. This study is conducted to test the above working hypotheses and investigate the mechanisms of action of anti-chondrogenic signals mediated by the RARs.

Materials and Methods: Floxed RARα and β, and RARγ knockout were kindly provided by Dr. Pierre Chambon (INSERM, France). Prx1-Cre mice were purchased from Jackson Laboratories. Selective RARα (NRX195183) and RARγ agonist (NRX194647) were gift from Dr. Rosh Chandraratna (Nurex). Other selective RAR agonists were purchased either from Tocris USA or Atomax Chemicals. In vitro chondrogenesis; Micromass culture of limb bud mesenchymal cells was used to study the effect of retinoid signaling on chondrogenesis. Limb bud mesenchymal cells were isolated from E11 embryos of CD-1, RARα and β double deficient, or RARγ null mice. Twenty µl of cell suspension (1 x 10^5 cells/ml) were spotted onto the culture dish and maintained in 3% FBS DMEM and F12 (1:1). Cultures were treated with various RAR agonist or DMSO (vehicle) alone at final 0.1%. Cells were stained with Alcian blue and has been tested to block pathological endochondral ossification in HO, much earlier (within two weeks) than in control mice which still contained both cartilage and bone by 2 weeks. These data indicate that RARγ may normally function as a negative modulator of cartilage formation during bone repair in postnatal mice. To further characterize RARγ signaling in endochondral ossification, mouse primary osteoblasts and MC-3T3 E1 osteoblast cell line cells were treated with RARγ agonist. Interestingly RARγ agonist affected neither proliferation nor alkaline phosphatase activity of those cells, suggesting that the compound does not interfere with osteogenic cell differentiation. In the last experiment, we tested various selective RAR agonists in our mouse HO model. Large ectopic HO-like cartilaginous masses formed in control mice by day 7 and were replaced by bone by day 14. However, cartilage and bone formation was completely prevented in mice receiving γ-agonist (0.4 to 4.0 mg/kg/day) by gavage; inhibition was about 95% measured by BV/TV ratios (p<0.01). There were no appreciable γ-agonist side effects in terms of body weight, liver and kidney function and articular cartilage phenotype. As expected, we observed partial HO inhibition in companion mice receiving RA (12 mg/kg/day), but this treatment caused serious side effects including skin and articular cartilage damage. The inhibition of HO by RARγ agonist was not observed in RARγ null mice.

Discussion: Our data demonstrate that RARγ is the major negative regulator for chondrogenesis and that activation of RARγ function by a selective agonist effectively blocks chondrogenesis. In our HO animal model, RAR gamma agonist completely suppresses BMP-induced heterotopic ossification with no major side effects and was far more potent that the RA alone strategy we reported previously (J. Orthop. Res. 28:271-277, 2010). Thus, our study strongly indicates that selective pharmacologic activation of RARγ represents a novel, powerful and seemingly safe treatment against HO.

Results: To identify the RAR signaling pathway that has the greatest anti-chondrogenic activity, we first prepared micromass cultures of E11.5 mouse embryo limb mesenchymal cells and treated with a pan-agonist (RA) or selective RARα or RARγ agonist. Numerous cartilage nodules formed in controls, but few formed in RA-treated cultures and none in RARγ agonist-treated cultures. Selective RARγ agonist partially inhibited chondrogenesis. To further analyze the roles of individual RAR in chondrogenesis, we prepared limb bud cell cultures from RARα and β double deficient mice or RARγ null mice and treated each with RA. Both RARα and β double KO and RARγ null limb bud cell cultures formed cartilage nodules in the absence of RA. RA completely inhibited chondrogenesis in RARγ and β double KO cell cultures. In contrast, RA inhibited cartilage nodule formation only 25-30% in RARγ null limb bud cell cultures. These findings indicate that RAR gamma is the major RAR for anti-chondrogenic activity. To examine the roles of RARγ signaling in endochondral ossification, we created 1 mm diameter bone defect in tibia of 6 week-old control or RARγ null mice and compared the endochondral bone repair process. Histological analysis revealed that the bone defect was initially filled with cartilaginous tissue and then by endochondral bone. In RARγ null mice, the defect was almost completely filled with new bone much earlier than in control mice which still contained both cartilage and bone by 2 weeks. These data indicate that RARγ may normally function as a negative modulator of cartilage formation during bone repair in postnatal mice. To further characterize RARγ signaling in endochondral ossification, mouse primary osteoblasts and MC-3T3 E1 osteoblast cell line cells were treated with RARγ agonist. Interestingly RARγ agonist affected neither proliferation nor alkaline phosphatase activity of those cells, suggesting that the compound does not interfere with osteogenic cell differentiation. In the last experiment, we tested various selective RAR agonists in our mouse HO model.