Folate receptor-mediated targeting of therapeutic and imaging agents to activated macrophages in rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by inflammation of the joints and destruction of cartilage and bone, often compromising both the quality and duration of life. The disease pathology is complex, involving the infiltration and activation of various populations of immune cells along with the release of destructive inflammatory mediators into the synovium of affected joints. Although it is still debatable whether activated macrophages are the primary promoters of RA, emerging data clearly show that the biological activity of this subset of inflammatory cells greatly contributes to both the acute and chronic stages of the disease. The further discovery of folate receptor expression on these activated (but not quiescent) macrophages in both animal models and human patients with naturally occurring RA has opened the possibility of exploiting folic acid to target attached drugs to this population of pathologic cells. Indeed, recent studies have shown that folate-linked imaging and therapeutic agents can be selectively delivered to arthritic joints, allowing both visualization and treatment of RA, with little or no collateral toxicity to normal tissues. This review will first summarize data documenting specific expression of the folate receptor on activated macrophages and then focus on the development of folate-targeted diagnostic and therapeutic agents for guided intervention into rheumatoid arthritis.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is characterized by destructive inflammation of both internal organs and joints, the latter typically manifested as damage to cartilage, bone, tendons, and ligaments. Statistics show that RA and related musculoskeletal disorders affect greater than 0.5–1% of the population worldwide, and it is predicted that one out of every five Americans will suffer from one of these disorders by 2020 [1]. Females are affected by RA three times more often than males, and the disease can start at any age, with a peak incidence at 50–60 years of age [2]. Tragically, ~80% of the affected population becomes disabled within 20 years of symptom onset, making RA the most common cause of disability in the workforce [3,4]. Not surprisingly, the total social and medical costs attributed to RA are predicted to exceed US$100 billion by 2020 [5,6]. While several anti-arthritic drugs are now available, many of these are very costly and have limited efficacy and/or undesirable side effects [7,8]. Therefore, researchers throughout the world are attempting to develop safer and more effective treatments that can selectively target the cellular and molecular mediators of the inflammatory disease.

2. Pathogenesis and treatment of RA

A large number of recent studies demonstrate that activated macrophages constitute the key effector cells in RA [9–12]. Although these reports do not exclude important roles for other immune cells, they clearly define a direct correlation between the level of macrophage activity and the observed joint inflammation, articular pain, and bone erosion. Molecularly, this correlation is explained by the fact that activated macrophages secrete multiple potent mediators of inflammation and tissue destruction, including proinflammatory cytokines (e.g. IL-1, IL-6, TNF-α), chemokines, prostaglandins, metalloproteinases, and reactive oxygen species [9,13]. Further, activated macrophages are known to participate in antigen presentation, and thereby they are thought to contribute to the activation and proliferation of antigen specific T-cells and their consequent destructive activities [9,14,15]. Fig. 1 lists many of the proinflammatory substances released by activated macrophages that may contribute to the symptoms of RA. This figure also lists a few of the currently available pharmaceutical agents that inhibit the indicated mediators of inflammation.

As inferred above, pharmaceutical scientists have devoted considerable effort to designing agents that can block most mediators of inflammation secreted by activated macrophages [9,16]. For example, Celebrex and Vioxx (celecoxib and rofecoxib, respectively) were developed to inhibit cyclooxygenase-2 and thereby block production of prostaglandins [17]. Enbrel and Remicade (etanercept and infliximab, respectively), in contrast, were developed to bind and neutralize TNF-α [18]. Kineret (anakinra), a recombinant form of IL-1 receptor antagonist, is administered to neutralize IL-1 [19], and the other drugs listed in Fig. 1, function to inhibit other inflammatory pathways of the activated macrophage. Unfortunately, several clinical studies now indicate that suppression of only a single mediator of inflammation will not achieve an adequate clinical response in a substantial number of RA patients. Consequently, physicians frequently prescribe combination therapies in an attempt to more fully alleviate the symptoms of the disease, such as joint discomfort and bone erosion [20–24]. However, while binary therapies sometimes prove effective, more frequently the net improvement in symptoms does not justify the extra cost. This is presumably due to the fact that mono-therapies or binary therapies block only a fraction of the inflammatory processes that contribute to arthritis [25–27], leaving other mediators of inflammation unchecked.
Based on the above considerations, an ideal therapy for RA might involve administration of an optimized combination of all of the aforementioned agents. Indeed, except for the associated toxicity and cost, it is likely that such an elaborate strategy would already have been developed. A much simpler alternative, however, might be to eliminate the cell population responsible for most of the above mediators of inflammation, i.e. the activated macrophage. Importantly, two such strategies have recently been explored in clinical settings. Classical leukocytapheresis has been used to remove mononuclear phagocytes (including macrophages) from the peripheral blood of RA patients. Analysis of this experimental therapy revealed that macrophage depletion does, in fact, result in a decrease in proinflammatory cytokines, leading to an improvement in the patient’s assessment of his/her pain and other symptoms [28]. While these results are encouraging, the method unfortunately eliminates many nonpathologic mononuclear cells that can be important in fighting infectious diseases [29], and the procedure is also expensive and time consuming.

A more selective approach has involved the specific elimination of macrophages via administration of liposomally encapsulated clodronate [30]. Thus, liposomal clodronate is known to be aggressively phagocytosed by virtually all macrophages (including osteoclasts), and upon internalization the drug rapidly promotes apoptosis of the engulfing cells [30]. Several studies now show that administration of liposomal clodronate significantly reduces joint swelling, joint destruction and subchondral bone damage in rodent models of arthritis [31–33]. Other studies demonstrate that unencapsulated clodronate can also reduce bone resorption in RA and cancer patients, [34–36], presumably by blocking ATP synthesis in macrophages/osteoclasts. Taken together, these studies provide evidence that reduction in a patient’s macrophage population can constitute an effective therapy for RA.
Unfortunately, destruction of many of the body’s macrophages and their precursors can also cause adverse side effects. Thus, macrophages are involved in processes ranging from tissue remodeling and wound repair to defense against pathogens and angiogenesis [37,38]. While depletion of all cells of the monocytic lineage might not cause toxicity over the short term, sustained elimination of these cells for long periods would be expected to have serious consequences [39]. Clearly, an approach that would selectively deplete the small, but destructive subpopulation of activated macrophages, without compromising the essential functions of the much more prominent population of resting (inactivated) macrophages might achieve the desired broad scale suppression of inflammation without eliminating a nonpathologic cell lineage that performs essential functions. As will be discussed below, use of folate-linked drugs that bind and are taken up exclusively by the activated subpopulation of macrophages represents a very promising strategy to achieve this selectivity [40].

3. Folate receptor expression on leukocytes

The folate receptor (FR) is a 38 kDa glycosylphosphatidylinositol-anchored protein that binds to the vitamin folic acid with high affinity (\(K_D<1 \text{ nM}\)) [41,42]. Following binding, rapid endocytosis delivers a fraction of the receptors into a low pH compartment where dissociation of the vitamin from its receptor is promoted [43]. Interestingly, covalent conjugation of small molecules, proteins, and even liposomes to the \(\gamma\)-carboxyl moiety of folic acid does not alter its ability to bind the folate receptor and undergo endocytosis by receptor bearing cells [44–46].

As detailed in the chapter by Ratnam and elsewhere [47–51], three major forms of the folate receptor exist: FR-\(\alpha\), FR-\(\beta\), and FR-\(\gamma\). The FR-\(\alpha\) isoform is present primarily in placenta, kidney, malignant tumors and various cancer cell lines [47,48]. It is also expressed on the apical surfaces of some normal epithelia (e.g. bronchioalveolar cells of the lungs and choroid plexus of the brain), but the receptor at these epithelial sites is generally inaccessible to parenterally administered drugs [49]. FR-\(\beta\) is expressed in the placenta, hematopoietic cells of the myelomonocytic lineages and certain leukemias [47,50]. In contrast, FR-\(\gamma\), which is primarily a secreted isoform of hematopoietic origin, is difficult to detect under normal conditions [51]. As a detailed in the preface to this volume and demonstrated in the image of the normal individual shown in the left panel of Fig. 2, folate receptors in the kidneys are the only receptors that bind appreciable quantities of folate conjugates in healthy individuals. Thus, as noted in the figure, little or no uptake of folate-linked molecules is generally observed in the brain, liver, blood cells, lungs, bone marrow, heart, spleen, skeletal muscle, and intestines of healthy individuals. In contrast, uptake of folate conjugates in ovarian cancer

![Fig. 2. Scintigraphic image of both a healthy individual and an ovarian cancer patient obtained 4 h following intravenous administration of 2 mg \(^{111}\text{In-DTPA-folate}\) (courtesy of Endocyte) Note that uptake of the folate-targeted imaging agent in the healthy individual is primarily limited to the kidneys, while uptake in the cancer patient is also seen in the malignant tissue.](image-url)
patients (right panel) is also seen in the tumor masses studding the peritoneal cavity.

If FR-β is indeed expressed on the monocytic and myelocytic lineages of hematopoietic cells, the question naturally arises regarding why very little folate conjugate is detected in bone marrow and blood cells following intravenous administration. The answer probably lies in the surprising observation that while FR-β is readily detected immunologically on the surfaces of these cells, the receptor has been found to be functionally inactive [52,53]. That is, FR-β can be easily labeled on hematopoietic cells with anti-FR-β antibodies, but the receptor does not bind either tritiated folic acid or folate conjugates [52]. We have consequently concluded that FR-β is synthesized in either inactive or precursor form and that this isoform cannot bind folic acid without some type of further modification.

Curiously, it has recently been discovered that activated (but not resting) synovial macrophages, taken from patients diagnosed with rheumatoid arthritis, possess a functionally active FR-β [54]. These studies reveal that both FR-β mRNA and the expressed protein are not only more abundant in activated monocytes and RA synovial macrophages than monocytes or macrophages from healthy individuals, but they also show that the FR on the activated cell surfaces can bind folate-linked fluorophores with high affinity. Working in a different model system, we have also observed that folic acid can be utilized to target liposomes to tumor-associated macrophages in a mouse xenograft model of ovarian cancer (Turk and Low, Cancer Letters, in press).
press (2004)). Since tumor tissue generally presents an inflammatory micro-environment, these data collectively imply that expression of a functional FR-β on tissue macrophages likely occurs during macrophage activation. Although the nature of the activation process and the molecular changes within the receptor that lead to acquisition of binding affinity are still under investigation, results to date indicate that the process is both highly regulated and very complex (Hilgenbrink and Low, unpublished data). The accumulated data also strongly demonstrate that activated macrophages can be selectively targeted with folate conjugates in both cancer tissue and arthritic joints, a capability that opens new possibilities for the diagnosis and treatment of rheumatoid arthritis.

4. Diagnostic imaging of rheumatoid arthritis using folate-targeted radiopharmaceuticals

As inferred above, we have recently shown that FR expression on activated macrophages can be exploited to selectively target imaging agents to sites of inflammation in rats with adjuvant-induced arthritis [40]. Thus, EC20, a folate-conjugated radiopharmaceutical complexed with $^{99m}$Tc [55], was administered to diseased rats intraperitoneally, and its biodistribution was assessed 4 h later by nuclear scintigraphy. Resulting images revealed accumulation of EC20 in the arthritic extremities, as well as in the livers and spleens of diseased rats, but not in the joints or organs of healthy rats (Fig. 3). Importantly, the intensity of...
the images was greatly reduced in the presence of excess competing folic acid, which confirms that uptake of the conjugate was folate receptor mediated. Further, when macrophages were depleted from the arthritic animals, uptake of EC20 was abolished, suggesting that macrophages constitute the major cell type involved in conjugate retention [40]. Finally, flow cytometry of cells isolated from the livers of arthritic rats confirmed that only macrophages participate in folate conjugate uptake and that binding of folate conjugates is significantly higher in macrophages from arthritic than healthy rats. These studies thus demonstrate that folate conjugates can be targeted to activated macrophages in vivo, regardless of whether the macrophages are residents of the liver or arthritic joints.

To further test the ability of folate conjugates to deliver attached drugs to arthritic joints in vivo, dogs with naturally occurring RA were also treated with 99mTc-labeled EC20 and imaged by nuclear scintigraphy. As seen in Fig. 4, EC20 uptake was intense in the joints of arthritic dogs, but not in the limbs of healthy animals. Whole body images also demonstrated that liver and spleen from the arthritic animals accumulated significantly more EC20 than the same organs from nonarthritic animals (data not shown). Interestingly, the prominent targeting of liver and spleen in both arthritic rats and dogs suggests that the responsible macrophage population is not confined to the affected joints, but is rather distributed systemically. This observation, which has also recently been reported by others [9,56,57], may contribute to the 3–18 year shorter life expectancy for RA patients compared to healthy individuals [58].

A final demonstration that folic acid can successfully target attached drugs to inflamed joints derives from fortuitous observations that were obtained during the imaging of cancer patients with both 111In-DTPA-folate and the folate-targeted 99mTc-labeled EC20. Although the primary focus of both clinical trials was to test the ability of folate-linked radiopharmaceuticals to image malignant tissues in cancer patients, the imaging data also provided valuable insights into the distribution of folate-conjugated macrophages in healthy and diseased tissues.

Fig. 6. A schematic diagram of the proposed mechanism of macrophage elimination by folate-hapten immunotherapy (Folate-EDA-FITC) using a two-step approach. Following immunization against a potent hapten (e.g. fluorescein, dinitrophenyl or a tetanus peptide), the arthritic rat is injected intraperitoneally with a folate-conjugate of the same hapten. Folate-mediated decoration of the macrophage surface with the attached hapten then promotes recognition of the macrophage by anti-hapten antibodies. The marked macrophage is then eliminated by immune cells with Fc receptors, such as natural killer cells, B-cells and macrophages. This process is called antibody-mediated cellular cytotoxicity (ADCC). Complement activation can also promote destruction of the antibody-marked macrophage.
patients [59], an unexpected benefit that emerged was documentation that both radiopharmaceuticals could also image arthritic joints. Thus, as seen in the $^{111}$In-DTPA-folate scintigraph of Fig. 5, a patient’s arthritic knee, but not her healthy one, could readily be seen in the nuclear image [unpublished data generously provided by Endocyte]. Since the patient was later confirmed to have an inflammatory arthritis in the imaged knee, but not the adjacent knee, these data may demonstrate that folate can also mediate drug targeting specifically to arthritic joints in humans.

5. Folate-targeted therapies for rheumatoid arthritis

It has recently been demonstrated that the surfaces of folate receptor positive cancer cells can be decorated with highly immunogenic hapten molecules by targeting the cells with folate-hapten conjugates ([60], see also chapter by Lu et al.). Under conditions where the animal has recently been immunized against the hapten, the hapten-decorated cells are then rapidly bound by anti-hapten antibodies, leading to immune cell-mediated killing of the cancer cells by a process termed antibody-dependent cell cytotoxicity (ADCC). Given that such folate-targeted immunotherapies can selectively destroy FR-expressing tumor cells in cancer-bearing animals, it seemed logical to evaluate whether the same strategy might be exploited to selectively destroy FR-expressing macrophages in arthritic animals. Thus, as depicted in Fig. 6, it was hypothesized that folate-hapten conjugates might selectively decorate the surfaces of FR-expressing activated macrophages, leading to their recognition by anti-hapten antibodies and their subsequent destruction by ADCC.

To examine this hypothesis, we induced systemic arthritis in Lewis rats by foot pad injection of heat-inactivated mycoplasma [61], and then treated the rats with the aforementioned immunotherapy. For this purpose, rats were immunized against the hapten by vaccination with a KLH-hapten (keyhole limpet hemocyanin derivatized with fluorescein isothiocyanate) conjugate and then given intraperitoneal injections of folate-hapten conjugate on days 1, 4, 7, 10, 13, 16 and 19 following arthritis-induction. As seen in Figs. 7 and 8, these very early un-optimized therapeutic trials

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**Fig. 7.** Effect of administration of folate-hapten conjugate on paw swelling in adjuvant-induced arthritic rats. Rats were immunized with KLH-FITC in an equal volume of TiterMax gold adjuvant in order to generate a high anti-FITC antibody titer. On day 0, rats were induced to develop arthritis by injection of a heat-killed mycoplasma into a single foot pad. This resulted in greater swelling of the injected paw (diamonds) than the uninjected paws (triangles), as measured with digital calipers. Treatment of the rats with folate-EDA-FITC (500 nmol/kg) on days 1, 4, 7, 10, 13, 16, 19, however, resulted in a 30% reduction in swelling of the injected paw (circles) and complete reduction in swelling of the uninjected paw (squares).
yielded a reduction in paw swelling and a suppression of bone erosion in treated rats, but not in unimmunized rats or rats treated with a nontargeted hapten (data not shown). Further, since many rheumatoid arthritis patients eventually die as a consequence of organ damage, not joint inflammation, it was also important to evaluate whether the immunotherapy might reduce systemic (organ) inflammation. As seen in Fig. 9, the activated macrophage populations in both the liver and spleen were also observed to decline following folate-targeted immunotherapy. Thus, as might be anticipated from a therapy that can eliminate FR-expressing macrophages regardless of their tissue of residency, the folate-targeted immunotherapy was found to reduce the symptoms of RA systemically. Importantly, in these same studies, the un-optimized folate-targeted therapy reduced paw swelling and suppressed bone erosion with equal potency to methotrexate, the standard treatment in the clinic. Not surprisingly, another laboratory has also recently demonstrated that selective elimination of inflammatory macrophages (using an FcγR1-directed immunotoxin) can alleviate the symptoms of RA [62].

Although we are unable to elaborate further in this review, more detailed preclinical studies have subsequently demonstrated that (i) paw swelling, (ii) bone

Fig. 8. Effect of treatment of arthritic rats with folate-hapten conjugate (Folate-EDA-FITC) or methotrexate on bone erosion. Folate-EDA-FITC (500 nmole/kg-panel 3) therapy was administered on days 1, 4, 7, 10, 13, 16 and 19, and MTX (0.75 mg/kg-panel 4) therapy was administered on days 1, 7, 14 and 21 in the arthritic rats. On day 25, arthritic rats were sacrificed, and their limbs were processed for radiographic analysis. Note that Folate-EDA-FITC (panel 3) and methotrexate (panel 4) both eliminated the bone erosion characteristic of the untreated arthritic animals (panel 2). Adjuvant arthritis was induced [61] by injecting the left foot in each pair with heat inactivated Mycoplasma butyricum.
erosion, (iii) systemic inflammation, (iv) splenomegaly, (v) elevation of arthritis score, and (vi) body weight loss associated with untreated arthritis can all be essentially prevented/reversed by treatment with folate-hapten conjugates (Paulos et al., manuscripts in preparation). Since similar results have also been obtained in advanced stage collagen-induced arthritis in mice, it can be concluded that the potency of folate-targeted immunotherapy is likely independent of the arthritis model employed (Varghese and Low, unpublished data). This observation, together with the observation that arthritic joints are readily visualized with folate-targeted radiopharmaceuticals in humans (Fig. 5), augurs well for a successful response to folate-targeted immunotherapy in humans as well as in the arthritic extremities.

6. Advantages of folate-targeted drug delivery in the treatment of rheumatoid arthritis

The primary objective of drug targeting is to deliver therapeutic or imaging agents specifically to pathologic, but not healthy cells, thereby avoiding the collateral toxicity that often plagues otherwise highly effective drugs. Based on many emerging observations, it can now be concluded that the activated macrophage is a principle contributor to the induction and maintenance of rheumatoid arthritis, and consequently, would constitute a primary target for therapeutic intervention into RA. Indeed, many of the most effective anti-arthritic drugs currently in the clinic specifically inhibit major secretion products of activated macrophages (Fig. 1); however, by neutralizing only a single mediator of inflammation, these available therapies still leave many other potent secretion
products unaltered. Although both leukopheresis and clodronate liposomes attempt to completely eliminate the unwanted cell type, both methods simultaneously remove useful nonpathologic cells, leaving the patient with a compromised repertoire of immune cells. Folate-targeted therapies, in contrast, selectively attack the pathologic cell type, leaving the vast majority of macrophages unharmed. Furthermore, since no other population of white cells appears to express a functional FR, even in arthritic animals (Turk and Low, unpublished data), no other leucocytes are affected by the treatment. As a consequence, the level of toxicity associated with folate-targeted therapy appears to be very low. While long term toxicity studies will still have to be completed before the therapy can be introduced into the human population, the outlook currently appears promising.

7. Perspectives and future directions

As summarized briefly above, there is now strong evidence to indicate that activated, but not resting macrophages, express a functionally active folate receptor, and that this receptor can be exploited to deliver folate-linked imaging and therapeutic agents specifically to the arthritic appendages of mice, rats, dogs and humans (unpublished data, and Refs. [40,54]). However, because this discovery was made only recently, folate targeting has been applied to date only to the delivery of imaging agents and haptens, raising the question of whether other types of cargo might have yielded similarly promising results. As with our previous studies of folate-mediated drug targeting to FR-expressing cancers [64], we intend to also explore the delivery of low molecular weight therapeutic agents, liposomes, gene therapy vectors, radiotherapeutic agents, protein toxins, cytokines, and immunosuppressive drugs to activated macrophages in RA. Furthermore, because the activated macrophage may contribute prominently to many other autoimmune and inflammatory diseases, we also plan to explore applications of folate-mediated drug targeting in the therapy and diagnosis of such pathologies as systemic lupus erythematosus, atherosclerosis, multiple sclerosis, Crohn’s disease, psoriasis, ulcerative colitis, pulmonary fibrosis, and graft versus host disease, etc. If elimination or suppression of the activated macrophage can improve the symptoms of the above diseases also, it would not be inconceivable that a range of folate-linked drugs might someday be available for the management of multiple unwanted inflammatory processes.

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