TNF receptor gene therapy results in suppression of IgG2a anticolonagen antibody in collagen induced arthritis

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Background: Therapeutic strategies to block tumour necrosis factor alpha (TNFα) activity in experimental autoimmune arthritis models and rheumatoid arthritis (RA) have proved highly successful, and provide sustained beneficial effects. Objective: To examine whether TNFα inhibition has immunological activity beyond the reduction of inflammation in collagen induced arthritis (CIA), an established experimental model of RA. Methods: Arthritic DBA/1 mice received single periarticular injections of retroviral constructs encoding human TNF receptor (TNF-R) into the affected articular paw, at the onset of arthritis. Severity of arthritis, antibodies to collagen type II (CII), and extent of pathological joint damage of arthritis paws were compared between TNF-R and media treated (control) animals 3, 7, 14, 21, and 49 days after disease onset. Results: Severity of CIA was significantly decreased in TNF-R treated animals compared with controls, 14–34 days after disease onset. Joint destruction was reduced in TNF-R injected joints and in the uninjected contralateral and ipsilateral paws of TNF-R treated animals. Seven days after disease onset, TNF-R treated mice had lower levels of inflammatory Th1 driven IgG2a antibodies to CII (p<0.05) than controls. This altered the anticolonagen IgG2a:IgG1 ratio towards Th2 driven IgG1. Conclusions: Local TNF-R gene therapy in CIA appears to have systemic effects on the anti-CII antibodies. The overall influence of TNF-R gene therapy is that it inhibits the progression of CIA mainly by suppressing the inflammatory Th1 response rather than by stimulating a Th2 response. Therefore, periarticular TNF-R gene therapy may have excellent therapeutic potential in RA.

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease manifested by progressive synovial joint inflammation and erosion of the subchondral bone. Interactions between synovial hyperplasia, inflammation, and an altered humoral and cellular immune system characterise the pathology of RA. Collagen induced arthritis (CIA) induced in DBA/1 mice by intradermal injection of collagen type II (CII), is an animal model of RA. Histological appearance of the erosions of cartilage and bone, inflammatory and immune cytokines, genetic and clinical features associated with both RA and CIA are similar. Although the aetiopathogenesis of RA is still unclear, several studies suggest that activated CD4+ T cells have an important role in initiating and perpetuating the chronic inflammatory characteristic of this disease. It has been suggested that in RA, pathophysiologically processes influencing the immune response may be driven by activated Th1 cells with insufficient Th2 cell differentiation to down modulate the ongoing inflammation. Skapenko et al showed that CD4+ memory T cells from patients with untreated RA demonstrate an intrinsic abnormality towards differentiating into specific cytokine producing effector cells that might lead to the typical Th1 dominated chronic inflammation in RA. Furthermore, high levels of circulating autoantibodies to CII are always associated with CIA, and seem to be essential for development and propagation of disease. Autoantibodies to CII belonging to the IgG2 subclass have been shown to be most efficient in binding complement, another crucial factor for initiation of joint inflammation in CIA. Also, IgG2a production is associated with a Th1 response, whereas IgG1 production is associated with a Th2 response.

Tumour necrosis factor alpha (TNFα) is a macrophage derived cytokine with multiple inflammatory and immunoregulatory properties. Raised levels of TNFα have been found in the sera and synovial fluid of patients with RA, suggesting it has an important role in the pathogenesis of RA. Tissue expression of TNFα and its two receptors (p55 and p75 TNF-R) is seen at several sites within the synovial membrane and the cartilage/bone junction, indicating that a wide variety of cells may be targets for TNF activity in RA. TNFα produced by synovial cells in RA seems to contribute to a cytokine cascade that subsequently leads to increases in interleukin (IL)1β and granulocyte macrophage-colony stimulating factor (GM-CSF) levels. Studies suggest that neutralisation of TNFα down regulates the production of IL1, IL6, IL8 and GM-CSF, thereby improving the arthritic disease process. Gene therapy, requiring direct intraarticular or periarticular administration of vectors encoding for anti-inflammatory cytokines, avoids the delivery problems associated with a Th2 response. Therefore, periarticular TNF-R gene therapy may have excellent therapeutic potential in RA.

Abbreviations: CIA, collagen induced arthritis; CII, collagen type II; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme linked immunosorbent assay; GM-CSF, granulocyte macrophage-colony stimulating factor; II, interleukin; PBS, phosphate buffered saline; RA, rheumatoid arthritis; SEM, standard error of mean; stTNF-R, soluble tumour necrosis factor receptor I; TNFα, tumour necrosis factor alpha; TNF-R, tumour necrosis factor receptor

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associated with protein administration. In this study, retroviral vectors encoding for human p55 TNF-R were given peritaurically to paws of CII immunised mice on the day of arthritis onset to elucidate whether anti-inflammatory TNF-R gene therapy could ultimately influence the reactivity of autoimmune lymphocytes in CIA.

MATERIALS AND METHODS

Retroviral vector production

Retroviral construct—namely, MOIN-sTNF-Rc-Ig, encoded a fusion protein consisting of the extracellular domain of human 55 kDa TNF-R covalently linked to the C2 through C3 domains of mouse IgG1 heavy chain. The soluble TNF receptor (sTNF-Rc-Ig) was amplified from sTNF-Rc-Ig plasmid using TNFαsR5 and Ig3 oligomers. The sTNF-Rc-Ig gene was inserted into the BamHI site of the retroviral construct MOIN, resulting in MOIN-sTNF-Rc-Ig. To produce high titre virus, the vector was transfected into Phoenix cells, a 293-based amphotropic packaging cell line, the supernatant harvested 48 hours after transfection, and titres determined by a standard plaque assay.

Induction and assessment of CIA

Female DBA/1 mice 6–8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). Native bovine CII (provided by Dr Marie Griffiths, University of Utah) was solubilised at 2 mg/ml in 0.01 M acetic acid at 4°C overnight and emulsified with an equal volume of CFA (Difco Laboratories, Detroit, MI). Mice were given 100 µl of emulsion containing 30 µg of CII by intradermal injections at the base of the tails. Mice were weighed weekly and overall health status was noted. They were monitored daily for onset and progression of disease. Onset of CIA was determined upon observation of appearance of the first signs of definitive redness, oedema, and touch to the bone in the wrist or ankle joints of the mice. In this manner both the synovial joint and the surrounding muscle cells would be transfected. Arthritic paws of the mice were given periarticular injections of either 100 µl of Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, NY) (group 1) or 1.6×105 pfu/ml of MOIN-sTNF-Rc-Ig in 100 µl of DMEM (group 2) at arthritis onset. Mice were assigned at random to one of the two groups and sacrificed at the onset date. Periarticular injections were done aseptically by guiding a Hamilton syringe with a 27 gauge needle at a 45 degree angle into the affected joint. The retroviral vector encoding for TNF-R was injected into the joint once resistance was encountered due to the needle touching the bone in the wrist or ankle joints of the mice. In this manner both the synovial joint and the surrounding muscle cells would be transfected. Arthritic animals were clinically assessed five times a week and paw measurements were recorded three times a week for seven weeks after disease onset and at the start of treatment. An established arthritis scoring system was used to evaluate disease: 0, normal appearance and flexion; 1, erythema and oedema; 2, visible joint distortion; 3, ankylosis detectable on flexion. Each limb was measured with a constant tension caliper (Dyer, Lancaster, PA) and graded, giving a maximum possible score of 12 for each mouse.

Histology

Histological analysis was conducted to determine the extent of joint damage. Front and rear paws were removed post mortem and stored in 10% neutral buffered formalin. Paws were decalcified for 18 days in 10% formic acid, dehydrated, and embedded in paraffin blocks. Sections were cut along a longitudinal axis, mounted, and stained with haematoxylain and eosin. Specimens were cut approximately to the mid-line, and then sagittal central samples mounted for evaluation. This allowed a consistent geographic evaluation. Five to 10 samples were mounted (usually 4–6 samples for each slide), and after staining the slides were permanently bonded with coverslips. A minimum of three separate sections for each specimen was evaluated in a blinded fashion. On the front paws, wrist and metacarpal joints were scored and on the rear paws, ankle and metatarsal joints were scored. Slides were evaluated for the presence of synovitis, panus formation, marginal erosions, architectural changes (mostly subluxation), and destruction. Synovitis was judged by thickness of the synovial membrane and scored from 0 (<3 cells thick) to 5 (>30 cells thick); panus was scored as 0 (no panus formation) to 5 (joint space filled by panus and extensive panus proliferation); erosions scored as 0 (no erosions visible) to 5 (loss of visible cartilage and major bone loss due to erosion); architectural changes scored from 0 (normal joint architecture) to 5 (complete fibrosis and collagen bridging). An overall score based on these collective points ranging from 0 (classic normal joint appearance) to 5 (destructive erosive arthritis with major bone remodelling) was then assigned to each section.

Measurement of serum human sTNF-R levels

All mice were pre-bled before CII immunisation and then bled at onset of arthritis, and at 3, 7, 14, 21, and 49 days after disease onset and administration of treatment. Sera were separated from all samples and stored at −80°C. The levels of human sTNF-R in mouse sera were determined by a quantitative sandwich enzyme immunoassay technique using human TNF-R Quantikine ELISA Kit (R&D systems, Minneapolis, MN), according to the manufacturer’s protocol. Briefly, 200 µl of sera and serial dilutions of standards were pipetted into wells precoated with murine monoclonal antibody against sTNF-R and incubated for two hours at room temperature. Plates were washed three times and 200 µl sTNF-R conjugate was added to each well and incubated for another two hours at room temperature. Unbound antibody-enzyme reagent was removed by washing the plates, and 200 µl of conjugate was added and incubated for 30 minutes. Thereafter, 50 µl of stop solution was dispensed into each well and the plates were read at 450 nm by an ultraviolet max spectrophotometer (Molecular Devices, CA). Negative pre-bled control sera and human sera with a 1000-fold dilution were titred on each plate to ensure uniformity of the assay.

Measurement of serum anti-type II collagen antibody levels

All mice were pre-bled before CII immunisation and then bled at onset of arthritis, and at 3, 7, 14, 21, and 49 days after disease onset. Sera were separated from all samples and stored at −80°C, and the levels of serum antibovine CII antibodies were determined by enzyme linked immunosorbent assays (ELISAs) as described previously. Briefly, ELISA plates (Nunc-Immuno plates, Denmark) were coated with 100 µl of coating buffer (0.4 M phosphate buffer pH 7.6) containing 3 µg of bovine collagen II, at 4°C overnight. Plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (Sigma, St Louis, MO), and non-specific binding was blocked by PBS containing 5% non-fat milk overnight at 4°C. One hundred microlitres of mouse sera diluted at 1/1000 in 5% milk/PBS was added to each well, except blank wells, and incubated overnight at 4°C. Subsequently, the plates were washed six times in PBS containing 0.05% Tween 20 and incubated with alkaline phosphatase conjugated goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Birmingham, AL) at 37°C for one hour. In assays to determine the isotype of the bound antibody, alkaline phosphatase conjugated goat anti-mouse antibodies specific for IgG, IgG1, or IgG2a were used. Plates were washed six times again and developed for 5–20 minutes in the dark by the addition of
IgG2a anticonnectan antibody suppression due to TNF-R gene therapy

Results

Effect of retroviral vector mediated periarticular delivery of TNF-R on collagen arthritis

Onset of arthritic disease was defined as the appearance of definitive signs of oedema and erythema in the paw. Onset of arthritis occurred in a single joint in all animals, and subsequently progressed to other joints. The joint exhibiting the first signs of clinical arthritis received a single periarticular injection of 100 µl of MOIN-sTNF-Rc-Ig containing a viral titre of 1.6×10^7 pfu/ml, at arthritis onset (day 1), while control animals received equal volumes of media into the first affected paw. Progression of arthritis was evaluated until 49 days after disease onset. Data were analysed using the SPSS-PC statistical software (SPSS, Chicago, IL). Group comparisons between TNF-R treated and control mice were performed by the two tailed independent t test. Comparisons of more than two means were conducted using the one way analysis of variance, and p<0.05 was considered to be significant.

Histological assessment of paws of control and MOIN-sTNF-Rc-Ig treated animals

Examination of the joint pathology demonstrated that the degree of synovitis, erosion, architectural changes of the bone, and overall joint destruction were significantly reduced in the arthritic paws of the MOIN-sTNF-Rc-Ig treated mice compared with the control animals (figs 2 and 3). The MOIN-sTNF-Rc-Ig injected paws showed a considerable reduction (p<0.05) in disease pathology compared with media injected control paws. The histological scores also revealed a significant reduction in the degree of joint destruction in the contralateral (p<0.05) and ipsilateral (p<0.01) paws compared with the control paws. These findings suggest that local periarticular delivery of MOIN-sTNF-Rc-Ig significantly reduces the ongoing disease process in the affected paw, and has marked systemic effects that block the progression of the inflammatory process to the uninjected joints, thereby preventing end stage destructive bone damage in these arthritic joints. This was consistent with levels of IL1β and...
TNFα mRNA measured in the paws. The contralateral and ipsilateral paws of the MOIN-sTNF-Rc-Ig treated animals had lower levels of both these cytokines than the injected and the similar paws of the control animals (data not shown).

Effect of retroviral gene therapy delivering TNF-R on anti-CII antibody levels
Serum samples were obtained from both control and treated animals at 3, 7, 14, 21, and 49 days after disease onset. The effect of MOIN-sTNF-Rc-Ig treatment on the humoral response to CII at these different times was determined by assaying the sera for antibodies to bovine CII (fig 4). Total immunoglobulin levels were lower in MOIN-sTNF-Rc-Ig treated animals than in controls (p<0.05) three weeks after onset of arthritis and administration of treatment (fig 4A). Subsequently, the isotype of the anti-CII antibody response was assessed at different times. Lower anti-CII IgG levels were seen in MOIN-sTNF-Rc-Ig treated animals than in controls (p<0.05) seven days after treatment (fig 4B). No significant differences were seen in the anti-CII IgG1 levels between the two groups (fig 4C); however, the anti-CII IgG2a titres were significantly lower (p<0.05) in sera from MOIN-sTNF-Rc-Ig treated mice than in controls seven days after arthritis onset (fig 4D). At that time, because anti-CII IgG1 levels were similar in the two groups but anti-CII IgG2a titres were markedly decreased in the TNF-R treated mice, a significant shift (p<0.005) in the anti-CII IgG2a:IgG1 ratio towards IgG1 was seen (fig 5). These observations suggested that although periarticular anti-inflammatory TNF-R gene therapy does not cause an isotype switch of the anti-CII autoantibody, it leads to an alteration in the anti-CII IgG2a:IgG1 ratio towards IgG1 by predominantly down regulating the Th1 mediated IgG2a response rather than up regulating the Th2 driven IgG1 response. The suppressive effect on IgG2a antibodies is consistent with an influence on the Th1 cells rather than Th2 cells. We have demonstrated that periarticular administration...
of retroviral construct MOIN encoding for marker gene LacZ into the arthritic paw had no effect on the clinical disease in CIA or the anti-CII antibody profile, suggesting that the above observations were a result of TNF-R and not an effect of the retroviral vectors (data not shown).

Measurement of local TNFα expression in control and MOIN-sTNF-Rc-Ig treated animals
Local expression of TNFα in the joints of MOIN-sTNF-Rc-Ig treated and control animals was measured by ELISA at 3, 7, 14, 21, and 49 days after treatment and disease onset. It was noted that the TNFα levels in the MOIN-sTNF-Rc-Ig injected paws were slightly higher than in media injected control joints, although these differences were not statistically significant (fig 6A). Paradoxically, it was observed that local TNFα levels in the uninjected arthritic ipsilateral joints of the MOIN-sTNF-Rc-Ig were lower than the ipsilateral joints of the control animals at all times, achieving statistically significant values 14 days after disease onset (fig 6B). Similar results were also seen in the arthritic contralateral joints (data not shown). Consequently, when the local TNFα expression in all the

Figure 3  Histological scoring of arthritic joints. Arthritic joints were analysed by histology using haematoxylin and eosin staining for extent of joint damage and assigned a histological score (as described in “Materials and methods”). Each column represents the mean scores (SEM) for the total number of mice in each group. *p < 0.05; **p<0.01.

Figure 4  Effect of periarticular delivery of MOIN-sTNF-Rc-Ig on the antibody profile to bovine CII. At arthritis onset mice received single periarticular injections of MOIN-sTNF-Rc-Ig or media into the affected arthritic paw. Serum samples were collected 3, 7, 14, 21, and 49 days after arthritis onset. Titres of antibodies to bovine CII were measured by ELISA. Each column represents the mean ratios (SEM) for the total number of mice in each group for each time. n=5 for both the media and MOIN-sTNF-Rc-Ig treated mice. **p<0.005.

Figure 5  Effect of a single periarticular delivery of MOIN-sTNF-Rc-Ig on the antibovine CII IgG2a:IgG1 ratio. Titres of anti-bovine CII IgG1 and IgG2a were measured by ELISA. The anti-CII IgG2a:IgG1 ratio 3, 7, 14, 21, and 49 days after disease onset is represented. Each column represents the mean ratios (SEM) for the total number of mice in each group for each time. n=5 for both the media and MOIN-sTNF-Rc-Ig treated mice. **p<0.005.
injected, contralateral, and ipsilateral joints was cumulatively analysed, an overall reduction in the TNFα was observed in MOIN-sTNF-Rc-Ig treated animals compared with controls from 7 to 49 days after disease onset (fig 6C). These results showed that MOIN-sTNF-Rc-Ig was not completely successful in lowering the TNFα levels in the first joints to be affected. This can be explained by the markedly raised levels of proinflammatory cytokines 26 27 associated with the onset of clinical disease and inflammation in CIA. It appears that our therapeutic treatment given at the onset of arthritis cannot effectively lower the already raised levels of TNFα present in the joint at disease onset but can significantly reduce TNFα levels in uninjected joints as they become affected, leading to overall disease amelioration.

Because local MOIN-sTNF-Rc-Ig treatment had beneficial systemic effects we assayed the expression of human TNF-R levels in sera over the time course of the experiment. Serum human sTNF-R levels from both control and MOIN-sTNF-Rc-Ig treated mice obtained at different times were measured by ELISA. Three days after disease onset and MOIN-sTNF-Rc-Ig administration, the human sTNF-R level in the circulation was 16.7 pg/ml. This value fell to 12.7 pg/ml seven days after disease onset and treatment and thereafter was undetectable at 14, 21, and 49 days after onset of disease. Therefore, the beneficial systemic effects of periarticular TNF-R gene therapy resulted in a generalised reduction in the local TNFα levels in the treated animals.

DISCUSSION
CIA and RA have several common immunological and pathological features,28 including the involvement of inflammatory cytokines in the arthritic aetiology. IL1 levels are increased in mice with CIA. Whereas the administration of recombinant IL1 to mice with CIA aggravates the disease process,22 9 treatment with IL1 receptor antagonist protein inhibits both RA and CIA,30–32 thus implicating IL1 as one of the mediators of the inflammatory process in this disease.33 IL2 and IL6 are also associated with the immunopathogenesis of CIA.34 35 These results provide compelling evidence that many cytokines participate in an immune network regulating the pathogenesis of RA. The onset of clinical symptoms and inflammation in CIA is highly dependent upon a predominantly Th1 response, characterised by the presence of anticollagen IgG2a antibodies and the proinflammatory cytokines TNFα and interferon γ.26 27 TNFα participates in the cytokine cascade by upregulating IL1β and GM-CSF levels, leading to synovial inflammation and joint erosion.1 15 17 20 Anti-TNFα monoclonal antibody therapy has been shown to ameliorate disease significantly both in clinical trials with patients having longstanding RA and in experimental arthritis.10 Our results support previous findings17–20 and indicated that TNF-R delivered locally at the arthritic paw using a retroviral vector inhibited the disease by reducing the number of paws affected and the severity of arthritis. Histology revealed that while TNF-R injected paws showed significant local effects, including reduced bone damage and a marked improvement in disease, more pronounced protective effects occurred in the uninjected paws. This suggests that the therapeutic treatment given at the onset of arthritis, resulting in a generalised reduction in the local TNFα levels in the treated animals.
IgG2a anticolonagen antibody suppression due to TNF-R gene therapy

IgG2a anticolonagen antibody suppression due to TNF-R gene therapy was effective in concentrating TNF-R within the periarticular route of delivery of the retroviral vectors in our study. In unpublished studies we have shown that retrovectors encoding a marker LacZ gene, injected periarticularly into the affected joints at arthritis onset showed good LacZ transduction into the injected joint, but had no effect on the clinical disease or on the anti-CII antibody profile in CIA. Thus, these findings indicate that the outcome of MOIN-sTNF-Rc-Ig treatment on CIA is an effect of the anti-inflammatory TNF-R rather than the retroviral vector. A previous study showed paradoxical effects after adenovirus mediated delivery of TNF-R, with amelioration of arthritis 10 days after treatment followed by a rebound in disease despite the presence of bioactive TNF-R fusion protein. This discrepancy with our findings may be due to different vectors and routes of delivery of the anti-inflammatory cytokines. The retroviral vectors in our study were given percutaneously, whereas the previous study used an intravenous route. Additionally, the delayed rebound inflammation observed in the latter study might possibly be attributed to the immunogenic effects associated with the use of adenoviruses. A previous report indicated that percutaneous delivery of viral IL10 by adenoviral vectors inhibited CIA development in both the injected and uninjected paws, and significantly reduced the antibodies to autologous CII while having no effect on the circulating antibodies to heterologous CII. These differences suggest that cytokines other than TNF are critical to the development of autoantibodies in this model.

In conclusion, these findings suggest that local TNF-R treatment has systemic effects that inhibit the production of the proinflammatory cytokine TNFα in the uninjected arthritic joints and suppress Th1 cells without up regulating the Th2 cells, eventually leading to an overall improvement in CIA. Future studies will be done to evaluate whether repeated periarticular administrations of retrovirus mediated TNF-R might have a more sustained effect on the humoral response and clinical progression of disease in CIA. Thus, retrovector mediated percutaneous delivery of the anti-inflammatory cytokine TNF-R may be a suitable strategy for alleviating disease in patients with RA.

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