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Research Article

Attempts at suppression of amyloidogenesis in a mouse model by a variety of anti-inflammatory agents

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ABSTRACT

Objective: The mainstay of AA amyloidosis prevention and treatment is suppression of inflammation. In the present study we have tried to determine the efficacy of a variety of anti-inflammatory agents at suppressing AA amyloidosis in a mouse model of the disease.
Methods: AA amyloidosis was induced in Swiss male mice using amyloid enhancing factor and $AgNO_3$. Suppression of amyloid formation was studied in comparison to saline, using i.p. injections of several non-steroidal anti-inflammatory agents, TNF-α inhibitors, interferon-α, leflunomide and a variety of chemotherapeutic agents, commonly used in the treatment of inflammatory illnesses such as methotrexate, azathioprine, chlorambucil and cyclophosphamide. The degree of splenic amyloid deposition was determined using Congo red staining of smears and a 5 grade scale.
Results: The alkylating agents, chlorambucil and cyclophosphamide, each resulted in a significant 88% reduction in amyloid deposition, yielded the most striking effect on amyloidogenesis suppression in the enhanced mouse model ($p<0.0002$). The non-steroidal anti-inflammatory agents tested varied widely in their ability to suppress amyloid formation in our mouse model, but only diflunisal was significantly effective, inducing a suppression of 57% ($p=0.04$). Other chemotherapeutic agents tested, methotrexate and azathioprine, yielded 32% and 27% suppression, which fell short of statistical significance. Surprisingly, the immunomodulatory agents etanercept, infliximab, leflunomide and interferon-α had insignificant effects on amyloid formation in this model.
Conclusion: Our findings suggest that alkylating agents may have a role in the prevention of amyloidogenesis. Further testing of these agents in animal models and in the clinical setting is needed.

1. Introduction

Amyloid A (AA) amyloidosis is a systemic disorder, characterized by the extra-cellular deposition of a fibrillar protein, originating from serum amyloid A (SAA). SAA is an apolipoprotein, primarily produced in the liver, levels of which are increased immensely in response to inflammatory stimuli, mediated by a myriad of cytokines, including tumor necrosis factor-α [1]. Chronic inflammatory states, the most notable of which are rheumatoid arthritis (RA) [2], familial Mediterranean fever (FMF) [3] and inflammatory bowel diseases (IBD), as well as chronic infections, may be complicated by continuous SAA elevation, culminating in secondary, or reactive AA amyloidosis. Over time, the tissue deposition of AA may result in organ malfunction, typically presenting initially as proteinuria, which invariably progresses to nephrotic syndrome and renal failure. Curbing the underlying inflammatory disorder, and consequently, SAA levels, forms the cornerstone of therapy in patients with AA amyloidosis. Notwithstanding, new therapeutic approaches are still required.

AA amyloidosis is being studied extensively in animal models, in which a variety of anti-inflammatory agents have been found to successfully inhibit amyloidogenesis. To this end, we have previously shown the beneficial effects of colchicine, steroids and high dose pravastatin in an enhanced model of mouse amyloidosis [4–6]. However, in clinical practice, the dramatic effect of colchicine on the prevention of amyloidosis is mostly evident in FMF patients, whereas the benefit of steroids evident in mouse amyloidogenesis, is questionable in man, particularly when the significant morbidity, associated with their long-term use, is taken into account. Hence, the need for additional, safe and efficacious anti-amyloidogenic agents is obvious.

The efficacy of other anti-inflammatory and immunosuppressive medications, including NSAIDs, methotrexate, leflunomide, chlorambucil and cyclophosphamide as well as the biologic agents, infliximab, etanercept and interferon-α, commonly given to patients with rheumatoid arthritis and autoinflammatory states [7], has not been fully

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evaluated in animal models of AA amyloidosis. Their well-known value in suppressing inflammatory states in addition to sporadic reports of their ability to attenuate the deterioration of clinical amyloidosis, makes them logical therapeutic candidates. However, efficacy should be substantiated, in view of the enhanced carcinogenesis associated with the long term use of immunosuppressive therapy [8].

In the present study, the efficacy of the above mentioned anti-inflammatory agents, all of potential benefit in suppression of AA amyloidosis, is examined in a mouse model of enhanced disease, in an attempt to corroborate clinical impression with pathological evidence.

2. Materials and methods

2.1. Animals

All experiments were performed in male Swiss mice, 2 to 6 months old, weighing 40–50 g, originating from Survey's Veterinary Institute (Beth Dagan, Israel), and were in accordance with the guidelines of the animal experimentation committee of our medical center.

2.2. Induction of amyloidogenesis

The production of amyloid enhancing factor (AEF) and the induction of enhanced amyloidogenesis were performed as described previously [5,9,10]. Briefly, AEF was derived from spleens of pre-amyloidotic mice, processed by acetone extraction, suspended in 0.5 ml PBS and injected intravenously into the studied mice (1 μg/animal). $AgNO_3$, in capacity of an inflammation inducing agent, was administered subsequently over 3 successive days (2% in saline, 0.5 ml/day, s.c.). The study drug, in each experiment was administered by a daily, intra-peritoneal injection (i.p.), as demonstrated in Table 1. Initial drug dose was 20-fold of the daily human dose taking into account increased drug metabolism in the mouse. If this initial dose proved to be lethal (based on preliminary experiments, data not shown), it was reduced in half, and in half again, if necessary, until no lethality was caused. Control groups received the same amyloid induction regimen, consisting of AEF and $AgNO_3$, but i.p. injections of saline alone, without a study agent. Animals were sacrificed on days 3 to 4 of the experiment, and their spleens were examined for the presence and amount of amyloid deposition, using the "crush and smear" (C&S) technique, and graded on a 1 to 5 scale, as described previously [9,10]. Experiments were repeated 2 to 5 times. Results shown are the mean of all examined mice of the group.

2.3. Pharmaceutical agents and administration

Pharmaceutical agents, suspended in 0.5 ml saline, were administered once daily, on each day, from day 0 to the end of the experiment, by an i.p. injection, unless otherwise noted. The following agents were used: sodium diclofenac (Voltaren, Novartis, USA), indomethacin (I 7378, SIGMA-ALDRICH, Inc., St. Louis, MO, USA), diflunisal (D 3281, SIGMA-ALDRICH, Inc.), ibuprofen (I 4883, SIGMA-ALDRICH, Inc.), ketolarc (K1136, SIGMA-ALDRICH, Inc.), methotrexate (06563, SIGMA-ALDRICH, Inc.), azathioprine (A 4638, SIGMA-ALDRICH, Inc.), cyclophosphamide (C 0768, SIGMA-ALDRICH, Inc.), chlorambucil (C0253, SIGMA-ALDRICH, Inc.), etanercept (Enbrel, Immunex, Thousand-Oaks, CA, USA), infliximab (Remicade, Schering-Plough, Kenilworth, NJ, USA), leflunomide (Arava, Hoechst Marion Roussel, Germany) and interferon-α (Intron A, Schering-Plough, Belgium).

2.4. Statistical analysis

Student's *t* test (2 tailed) was used to determine significant differences ($p<0.05$).

3. Results

The efficiency of various anti-inflammatory agents at suppression of amyloidogenesis in our enhanced mouse model is summarized in Table 1. The efficacy of the different NSAID preparations was variable. Diclofenac, 1.3 mg/day, given over 3 days by i.p. injection, yielded a 29% reduction in amyloid content compared to saline injections, falling short of statistical significance ($p=0.06$). Indomethacin, 1 mg/day, yielded results of a similar range (24% reduction in amyloid content) yet failed to reach statistical significance as well. Diflunisal, 4.2 mg/day, was far more effective, yielding a 57% suppression of amyloidogenesis ($p=0.04$). Both ibuprofen (30 mg/day) and ketolarc (0.2 mg/day) failed to suppress amyloidogenesis by any means. Similarly, acetylsalicylic acid, at 3.3–6.7 mg/day given over 3 and 4 days, did not result in any significant reduction in amyloid content (data not shown).

The immunomodulatory agents, methotrexate and azathioprine, both resulted in a statistically insignificant 30% suppression in amyloidogenesis, which was of similar magnitude to that yielded by diclofenac and indomethacin. Another immunomodulator, leflunomide, in a 4 day experiment, was of no benefit as well (10 mice overall, data not shown).

TNF-α inhibition with neither etanercept, 0.3 mg/day nor infliximab (5 mg/day and 10 mg/day) was efficacious at inhibiting amyloidogenesis in the enhanced mouse model. Similarly, interferon α, conferred insignificant suppression in 3 day experiments, at 2 different doses of 0.05 and 0.035 million units (data not shown).

By far, the most significant suppression of amyloidogenesis in the enhanced mouse model was achieved with the alkylating agents, cyclophosphamide and chlorambucil, each yielding an 88% reduction in amyloid deposition ($p<0.0002$) in both 3 and 4 day experiments.

4. Discussion

In this study we have shown that some NSAIDs, namely diflunisal and perhaps diclofenac are variably efficacious at inhibiting amyloidogenesis in a mouse model of AA amyloidosis, while other NSAID compounds, such as ibuprofen and ketolarc, are completely ineffective. Although when achieved, the degree of amyloidogenesis suppression with NSAIDs was comparable to that attained by immunomodulators such as methotrexate and azathioprine, it was far less impressive than the 88% reduction of amyloid content observed following prophylaxis with the alkylating agents, cyclophosphamide and chlorambucil.

Common practice advocates inhibition of the underlying inflammatory process as the most successful approach for the prevention of AA amyloidosis. Accordingly, it may be presumed that suppression of amyloidosis in our mouse model was the result of reduced production of inflammatory cytokines, such as IL-1β, IL-6 and transforming growth factor-β (TGF-β), as was previously shown in other models [11,12], leading to decreased formation of the acute phase protein SAA, the precursor of AA. Whether the tested preparations also interfere with the process of fibril deposition or precursor degradation in tissues, is still unknown. Interestingly, it has been suggested that in Alzheimer's, the innate immune system participates in neurodegeneration. Here, augmentation of the low-grade, chronic up-regulation of pro-inflammatory cytokines, which is associated with aging, leads to neuronal toxicity by way of an increased concentration of amyloidogenic peptides in neuronal cells and astrocytes [13]. The observation that NSAIDS decrease β amyloid deposition both in humans inflicted with Alzheimer's disease as well as in the transgenic mouse model [14,15], suggests that, at least part of the effect here shown, may be mediated in a similar manner, by a combined anti-inflammatory and anti-amyloidogenic effects.

Moreover, the discrepancy that was revealed between the different NSAID agents, given in a comparable anti-inflammatory dose, indeed supports the idea that the efficacious compounds do not necessarily exert their anti-amyloidogenic effect only via inhibition of inflammation. It is particularly so with diflunisal, a NSAID that was