Treatment with anti-NAP monoclonal antibody reduces disease severity in murine model of novel angiogenic protein-induced or ovalbumin-induced arthritis

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Summary
Rheumatoid arthritis (RA) is a polyarticular inflammatory, angiogenic disease. Synovial angiogenesis contributes to inflammation in RA. In this study we have developed an arthritic model in rats using a novel angiogenic protein (NAP), isolated from human synovial fluid of RA patients. We produced anti-NAP monoclonal antibodies (mAbs) and investigated the therapeutic efficacy of the same in adjuvant-induced or NAP-induced arthritis as a model of human RA. The treatment of arthritic rats with anti-NAP mAbs resulted in effective amelioration of paw oedema, radiological arthritic characteristics, serum levels of vascular endothelial growth factor (VEGF) and NAP, compared to that of untreated arthritic animals. Further, profiling of angiogenic markers such as synovial microvessel density, angiogenesis, CD31, VEGF and fms-like tyrosine kinase (Flt1) by immunohistochemistry both in arthritic and anti-NAP mAb-treated animals revealed the efficacy of mAb as an anti-angiogenic functional antibody. Therefore, NAP may be an attractive target to design anti-angiogenic and anti-arthritic therapies to control the pathogenesis of arthritis.

Keywords: angiogenesis, monoclonal antibodies, novel angiogenic protein, rheumatoid arthritis, VEGF

Introduction
Rheumatoid arthritis (RA) is a chronic inflammatory and systemic autoimmune disease characterized by hyperplasia of synovial cells and angiogenesis [1]. The progression of synovitis in both adjuvant-induced arthritis (AIA) and RA is characterized by a pronounced tumour-like expansion of the synovium [2]. Consequently, neovascularization may play a pivotal step during disease progression. Several polypeptide growth factors and angiogenic factors contribute to neovascularization found in RA joints [3]. An important mediator of angiogenesis is endothelial selective vascular endothelial growth factor (VEGF), which also induces vascular permeability. It has been shown by several groups, VEGF is important in the development of RA joint destruction by the significant correlation between serum VEGF at presentation and the magnitude of radiological deterioration [4]. The intensive search for markers of prediction and prognosis in RA has been the subject of a large number of studies, and a huge variety of possible markers have been reported. Several lines of evidence support that calcium and membrane binding protein (CaMBP) is one of the critical cytokines in the proinflammatory and proangiogenic cascade [5,6]. They are involved in numerous functions, ranging from control of cell cycle progression, cell differentiation and enzyme activation to regulation of muscle accumulation at the sites of inflammatory joints, and diseased conditions in RA are responsible for the pathogenesis of diseases by promoting angiogenesis [7,8]. During arthritic conditions, expression of VEGF and CaMBP are shown to increase angiogenesis and inflammation [9]. The availability of markers that could help to identify patients with more aggressive, rapidly progressive RA with poorer prognosis would offer a rational basis for early and aggressive treatment. In this way it may be possible to avoid many irreversible clinical complications [10]. The number of disease modifying anti-rheumatic drugs (DMARDs) available has increased in recent years. While the majority of these DMARDs act as immunomodulatory drugs in RA, some also act by inhibiting the angiogenic process [11]. However, the mechanism of the inhibitory effects of DMARDs on angiogenesis remains obscure [12]. The effectiveness, cost and toxicity of the new agents vary widely. The use of monoclonal antibodies (mAbs) in RA has
been valuable in assessing the role of various inflammatory mediators and cell-bound molecules in disease pathogenesis [13]. mAbs bind to their targets with high specificity, and therefore have excellent potential as therapeutic agents. Biotechnological advances have allowed the production of large quantities of engineered mAbs for therapeutic use [14]. Recent research in RA has identified important mediators of synovitis. In an in-vitro assay, RA synovial fluids (SF) have been shown to induce morphological changes in human endothelial cells, with the formation of a tube-like structure and induction of angiogenesis [15]. During the last decade, monoclonal antibodies targeting these have been tested in clinical trials. Specific therapy targeted against tumour necrosis factor (TNF)-α alone using anti-TNF-α mAbs or soluble TNF-α receptors has been effective in murine collagen-induced arthritis (CIA) by reducing the incidence and severity of disease [16]. Recent studies have shown that therapy with rituximab is one of the treatment options for optimizing RA therapy [17]. Furthermore, mAbs directed against this CaMBP gives a promising result in the AIA model, which is a reliable model for RA because it mimics exactly RA of the human joint [18].

In the present study, our data indicate that 67 kDa protein isolated from SF of RA patients is rheumatoid factor (RF), which is calcium-binding in nature and mediates the inflammatory and destructive process in RA. Monoclonal antibody for novel angiogenic protein (NAP) was produced and the same was used to explore the synergistic role of VEGF and NAP to evaluate the relationship of these proteins in RA. We also studied the correlation of important angiogenic markers CD31, an endothelial cell proliferation indicator, and fms-like tyrosine kinase (Flt1), the receptor for VEGF in AIA and the NAP-induced arthritis (NIA) model. Using enzyme-linked immunosorbent assay (ELISA) and immunohistochemical studies we found that a high level of VEGF is expressed with increased microvessel density (MVD) in RA. Monoclonal antibodies directed against NAP ameliorate the disease incidence in NIA and an established AIA rat model. Our studies indicated that anti-NAP mAbs have a potent anti-arthritis effect which targets angiogenesis and can be useful for individualization of therapeutic strategies in treatment of RA.

Materials and methods

Recruitment of patients

Patients who fulfilled the American College of Rheumatology criteria for RA [19] were recruited from the out-patient Department of Pathology, JSS Hospital, Mysore, with the approval of the medical college ethics committee and as per the guidelines of the Institutional Review Board. Informed consent was obtained from all the patients. The patient group comprised seven women and three men, with an age range of 38–67 years. Patients had active disease and disease duration of ≤ 2 years. All knee joints demonstrated signs of active synovitis at the time of aspiration.

Animals

Wistar rats (aged 4–5 months) were obtained from the central animal facility of the Department of Zoology, University of Mysore, Mysore, India. All the animal experiments were approved by the Institutional Animal Ethics Committee, University of Mysore, Mysore and studies were conducted according to the guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India.

Generation of monoclonal antibodies for NAP

Novel angiogenic protein was isolated and purified from human SF of patients with RA, as per the method described previously by us [20]. In brief, inside-out vesicles prepared from red blood cells (RBC) (3–4 mg vesicle protein/ml of SF) were mixed with SF and incubated at 37°C for 20 min in the presence of 1 mM calcium-containing buffer [2 mM NaCl/5 mM KCl/0·5 mM ethyleneglycol tetraacetic acid (EGTA)/2 mM Tris pH 7·4]. Membrane vesicles, bound to SF proteins in a calcium-dependent manner, were washed twice using this buffer in order to eliminate unspecifically bound proteins. The specifically bound proteins were released from membrane by including 1 mM EGTA minus calcium-containing buffer by centrifugation at 28 000 g for 30 min at 4°C. The supernatant containing NAP was dialysed and purified further by size exclusion chromatography using Sephadex G-100, after which its identity was determined by peptide mass fingerprinting and N-terminal protein sequencing. The purified fraction was assayed for proangiogenic activity using human umbilical vein endothelial cells (HUVECs) for tube formation [21]. Purified NAP was used to produce monoclonal antibody. Briefly, BALB/c mice were immunized four times over a 2-month period with 50 μg of purified NAP with Freund’s adjuvant. Serum samples were collected 2 weeks after the second, third and fourth immunizations and screened for anti-NAP antibody using indirect ELISA. Spleen from mice that displayed high antibody titres were used subsequently to generate hybridomas using standard spleen cell/myeloma fusion. Briefly, NAP-primer B cell 1 × 10⁸ (splenocytes) from mouse producing high-titre neutralizing antibodies were fused with logarithmically growing Sp2/0 myeloma cells (1 × 10⁸), using polyethylene glycol-1500. Hybridoma selection was carried out in hypoxanthine–aminopterin–thymidine (HAT) medium. The resulting monoclonal hybridomas were grown to confluency and the cell supernatant from a single clone was collected as a source of anti-NAP mAb, verified using ELISA in which NAP was used for capture of the anti-NAP mAb, and purified by protein-A agarose affinity column chromatography. Further
immunodetection of anti-NAP mAb was carried out by Western blot analysis.

Animal model

Arthritis was induced in Wistar rats by subcutaneous (s.c.) injection of NAP or ovalbumin (OVA; Sigma, St Louis, MO, USA), as described previously [22]. There were five groups containing six animals, each in duplicate, as follows: group 1, controls; group 2, positive control (OVA-induced arthritis (AIA; untreated)); group 3, NIA untreated; groups 4 and 5 served as test (AIA DMRD-treated and NIA mAb-treated), respectively. All rats except controls were sensitized twice during a 6-week period with 2 mg/ml of OVA or 50 µg/ml NAP emulsified in complete Freund’s adjuvant (CFA) (Sigma) and administered s.c. At the end of 6 weeks, animals received an intra-articular injection of 2 mg/ml of OVA or 50 µg/ml NAP in CFA in order to induce arthritis. The control rats were injected only with Freund’s adjuvant.

Treatment protocol for AIA or NAP-induced arthritis

Arthritis was achieved in 6–7 days post-IA injections and was considered as day ‘0’. The treatment commenced after the onset of arthritis. Rats were randomized and grouped based on paw swelling and clinical score before treatment. Animals were treated with anti-NAP mAb intraperitoneally at a dose of 0.3 mg/kg body weight, twice weekly for 4 weeks. Simultaneously, another test group of animals received DMRD-sulphasalazine (0.4 mg/kg body weight). Negative and positive control groups of animals received 100 µl saline.

Evaluation of arthritis

After arthritis induction, rats were monitored periodically before and after treatment for clinical parameters such as paw thickness, oedema, degree of redness and flexibility of joints, and arthritis score was assigned from 1 to 4, based on the severity of paw inflammation (Table 1). The paw volume was measured daily. Radiographs of inflamed joints were taken after the induction of arthritis and at the end of the study using the Meditronics X-ray analyser (Mumbai, India). Zero to three subjective grading systems were then used to evaluate different parameters, including degree of soft tissue swelling and bone erosion. The radiological score referred to the sum of the subjective scores for each of the above parameters.

ELISA

Concentration of VEGF and NAP were quantified as described earlier by us [23]. Serum samples collected from rats were coated on an ELISA plate using coating buffer at 4°C overnight. Subsequently, wells were incubated with the chosen antibodies using either anti-VEGF antibody or NAP antibody. Wells were washed, followed by incubation with secondary antibodies tagged to alkaline phosphatase (Genei, Bangalore, India) and developed with 100 µl of p-nitrophenyl phosphate solution. The optical density at 405 nm was measured in a Medispec ELISA reader (Winooski, VT, USA). The VEGF or NAP concentration in the synovial fluid was calculated based on the standard curve.

Histopathology and immunohistochemical analysis

Synovium tissue from rats was processed as reported elsewhere [24]. In brief, tissues were paraffin-blocked and 3-µm-thick sections were prepared, fixed and stained using haematoxylin and eosin (H&E). All sections were randomized and evaluated by a trained blinded observer unaware of the clinical status of the animals or the treatment received in order to evaluate the arthritis severity. Sections were immunostained with anti-VEGF, anti-CD31 and anti-Flt1 antibodies. An ImmunoCruz staining system was used for dianaminobenzide (DAB) staining, according to the manufacturer’s recommendations (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Coverslips were mounted on slides and sealed for microscopy. Labelled cells were imaged on a Carl Zeiss fluorescence microscope, (AX10. Imager.A2, Berlin, Germany) with an attached charged coupled device (CCD) camera.

Statistical analysis

Data expressed as mean ± standard deviation (s.d.) were analysed by one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) to compare control and treated groups; P < 0.05 were considered to be statistically significant. All statistical analysis was performed using SPSS statistical software version 13.0.

Results

Isolation of NAP and generation of anti-NAP mAb

NAP was isolated from synovial fluid using a binding-release assay to inside-out vesicles of RBCs. Partially purified NAP upon gel filtration column chromatography

Table 1. Assessment of arthritic score in adjuvant-induced arthritic rats.

<table>
<thead>
<tr>
<th>Increased in paw thickness (mm)</th>
<th>Arthritic score</th>
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<tbody>
<tr>
<td>0.2–1.0</td>
<td>1</td>
</tr>
<tr>
<td>1.1–2.0</td>
<td>2</td>
</tr>
<tr>
<td>2.1–3.0</td>
<td>3</td>
</tr>
<tr>
<td>3.1–4.0</td>
<td>4</td>
</tr>
<tr>
<td>4.1 and above</td>
<td>5</td>
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yielded one major peak with tube formation activity in human umbilical vein endothelial cells. NAP showed a molecular weight of 67 kDa (Fig. 1a). A high titre (1:50,000) antibody against NAP protein was obtained after repeated booster doses of NAP upon fusion of splenocytes from these mice with Sp2/0 myeloma cells. The cell supernatants were screened for NAP-specific antibodies by indirect ELISA. Of the resulting 92 hybridomas, 56 positive hybridomas were identified, 18 of which showed significant titres. Each of the 18 hybridomas was screened further to obtain seven stable, high-titre hybridomas. After a further two rounds of rescreening, one lead hybridoma (P1H2.S1C4.S2G3) was isolated that represents the first murine anti-NAP mAb. The generated mAb clearly showed specificity towards the purified NAP, as verified by Western blot (Fig. 1b).

Validation of anti-arthritic effect of anti-NAP mAb in AIA and NIA rat models

AIA and NIA rat models have been developed for preclinical studies as standard animal models of RA in humans. A massive increase in the joint inflammation, paw oedema, bone erosion and degree of redness was observed both in the AIA and NIA rat models when compared to the normal group. The treatment protocols, which included administration of anti-NAP mAbs, was started after the onset of the arthritis, i.e. from day 6, where an arthritic score of AIA or NIA rats was 4 (3.2 mm). Significant reduction in the arthritic score [2 (1.6 mm)] was evident in rats treated with anti-NAP mAb, validating the functional efficacy of the anti-NAP mAb (Fig. 2c).

Anti-NAP mAb as potential anti-arthritic agent

Treatment of the anti-NAP mAb (0.3 mg/kg body weight) resulted in inhibition of joint inflammation, paw thickness and redness, as evident in Fig. 2a. The final arthritic score of AIA and NIA rats was 4 (3.2 mm), while in the anti-NAP mAb-treated rats was found to be 2 (1.6 mm). After 4 weeks of treatment the joints of anti-NAP mAb-treated and untreated rats were exposed to X-ray for radiological evaluation and radiographs indicated decreased soft tissue swelling and bone erosion compared to the untreated rats (Fig. 2b). These results revealed that the anti-NAP mAb shows a good ameliorating effect on both AIA and NIA rat models.

Anti-NAP mAb treatment inhibits VEGF production

To determine whether anti-NAP mAb inhibits VEGF mediated angiogenesis, we tested the effect of anti-NAP mAb on the production of VEGF in AIA or NIA rats. Data on ELISA indicated that anti-NAP mAb had an effect on the secretion of VEGF165 under in-vivo conditions. The quantity of VEGF165 in serum increased in untreated rats during the experimental growth period. In contrast, there was inhibition of VEGF165 secretion in treated animals (Fig. 3a). The results indicated that animals treated with DMRD also showed inhibition of VEGF165 secretion.

Effect of anti-NAP mAb treatment on NAP production

The inhibitory effect of anti-NAP mAb on the production of NAP in AIA or NIA rat models was determined by ELISA. The level of NAP in serum increased in untreated AIA or NIA rats with an increasing lapse of time. Anti-NAP mAb-treated rats showed a decreased level of NAP. As shown in Fig. 3b, anti-NAP mAb treatment resulted in inhibition of NAP secretion, indicating a possible role for NAP in inflammation and the use of anti-NAP mAb for clinical diagnosis and as a therapeutic agent.
Anti-NAP mAb inhibits neovascularization

In order to verify the anti-angiogenic effect of anti-NAP mAb in arthritic conditions, the synovium tissue from the anti-NAP mAb-treated and untreated rats was stained with H&E. Synovium sections from the AIA or NIA rats appeared well vascularized [24 vessels/high-powered field (v/HPF)]; in contrast, anti-NAP mAb-treated synovium sections were characterized by a pronounced decrease in vascular density (12 v/HPF) showing 50% less vascularization compared to untreated rats (Fig. 4). Immunohistochemistry data revealed that when compared to the untreated group, the synovium from anti-NAP mAb-treated animals showed a decreased expression of angiogenic markers CD31, Flt1 and VEGF (Fig. 5 and Table 2). The results indicated that anti-NAP mAb targets vascularization in AIA and NIA rats.

Discussion

Angiogenesis is an important phenomenon of synovial inflammation in RA [25]. Following chronic inflammation, up-regulation of VEGF increases pathogenesis of RA, such as vascular permeability resulting in oedema, protein leakage, bone erosion and progressive destruction of the joints [26,27]. More recent studies have addressed the role in arthritis of another important family of molecules involved in angiogenesis, namely the angiopoietins. These molecules, together with their cell-surface receptors Tie-1 and Tie-2, play a key role in the development of the vasculature. In RA, Ang-1 is expressed in human RA synovium in lining cells, macrophages, fibroblasts and endothelium [28,29]. Like Tie-1, Tie-2 is also expressed on a variety of cells in the synovium and up-regulated in RA [28]. Hence, the delicate balance between members of the Ang and Tie families may contribute to vascular formation in RA [30]. Several other angiogenic growth factors, such as platelet-derived growth factor (PDGF), fibroblast growth factor

Table 2. Effect of anti-NAP mAb on expression of angiogenic markers CD31, VEGF and Flt1.

<table>
<thead>
<tr>
<th>Rats synovial section</th>
<th>CD31</th>
<th>VEGF</th>
<th>Flt1</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>AIA</td>
<td>27</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>NIA</td>
<td>24</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>AIA + DMRD</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>NIA + mAb</td>
<td>10</td>
<td>9</td>
<td>9</td>
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AIA: adjuvant-induced arthritis; NIA: NAP-induced arthritis; DMRD: disease-modifying rheumatoid drug.
(FGF)-2, epidermal growth factor (EGF), insulin-like growth factor (IGF-I), hepatocyte growth factor (HGF), TNF-α, transforming growth factor (TGF)-β, interleukin (IL)-1, IL-6, IL-8, IL-13, IL-15, IL-18, angiogenin, platelet-activating factor (PAF), angiopoietin, soluble adhesion molecules and endothelial mediator (endoglin), play an important role in angiogenesis in rheumatoid arthritis [31]. The synovium of RA patients and joints from rats with adjuvant-induced arthritis contain increased amounts of FGF-2 [32]. Rodent models have been used extensively to study the mechanisms underlying the VEGF-mediated angiogenic process in arthritic diseases and to develop new therapeutic interventions, including those based on inhibition of angiogenesis by targeting VEGF [15,33,34]. In our previous study we purified a CaMBP, NAP, which had angiogenic properties. The importance of calcium-binding proteins in angiogenesis and inflammation has also been reported earlier, proving that calcium-binding proteins are

Fig. 3. Effect of anti-NAP mAb treatment on in-vivo production of NAP or VEGF165. NIA or AIA in rats were treated with anti-NAP mAb or DMRD; serum samples were collected from the rats every week of treatment and were subjected to ELISA to measure (a) VEGF165 or (b) NAP. ELISA data show inhibition of VEGF or NAP production compared to untreated rats. Results are mean ± standard deviation of six animals in each control or test group. (a) and (ab) Statistically significant at \( P < 0.05 \) when compared with control. (c) Statistically significant at \( P < 0.05 \) when compared with (a). (d) Statistically significant at \( P < 0.05 \) when compared with (ab) and (c).

Fig. 4. Histopathological findings. Synovium tissue was excised from control, AIA or NIA of both anti-NAP mAb-treated or -untreated rats. Paraffin-block and 3 μm sections were prepared. The sections were stained with haematoxylin and eosin and microvessel density was verified. The arrows indicate blood vessels in the synovium sections. (a) and (ab) Statistically significant at \( P < 0.05 \) when compared with control. (c) Statistically significant at \( P < 0.05 \) when compared with (a). (d) Statistically significant at \( P < 0.05 \) when compared with (ab) and (c).
also potent angiogenic mediators [7,35]. Earlier, our laboratory reported the proinflammatory role of CaMBPs isolated from ascites fluid from mouse mammary carcinoma cell lines that could activate respiratory burst [20]. Consistent with previous reports, NAP isolated from SF of RA induces oedema in the footpad, revealing proinflammatory activity. Reports showing that the presence of CaMBPs at sites of acute and chronic inflammation have long been noted. Indeed, assessment of serum levels of CaMBP molecules have been suggested to track disease activity in patients with inflammatory disorders such as ulcerative colitis, chronic inflammatory bowel disease, psoriatic arthritis (sPA) and RA [35], and is also a valuable marker [36–38]. We have developed a model using NAP similar to the AIA model of RA in Wistar rats to examine the role of NAP in the development of this disease. Our results show that the levels of NAP and VEGF in AIA and NIA animals were found to increase in serum. Similar to other reports [36,39,40], NAP levels in the serum elevated gradually after the onset of arthritis, with the highest level at 21 days after induction. Treatment with antibodies such as anti-TNF-α antibody has influenced the expression of other proinflammatory cytokines involved in RA [41]. Antibodies against calcium- and membrane-binding protein have reduced the accumulation of neutrophils in air pouch models of acute gouty arthritis [42]. Annexins are another class of CaMBPs which induce angiogenesis via stimulation of VEGF production. S100A4 induce angiogenesis through interaction with annexin II on the surface of endothelial cells [36]. Treatment with anti-S100A12 antibodies, anti-renal cell carcinoma antigen (RAGE) antibodies and soluble-RAGE (sRAGE) and CaMBPs have reduced inflammation effectively in animal models of arthritis [7]. Consistent with previous reports, our data demonstrate that treatment with anti-NAP mAb of AIA or NIA rat models effectively reduces paw swelling, degree of redness and flexibility of the rear ankle joints, indicating the neutralization and potential therapeutic effect of these antibodies. Quantification of growth factor VEGF and NAP by ELISA indicated an increased amount of VEGF or NAP correlating with the progression of the disease, whereas in the case of anti-NAP mAb-treated animals, a decrease in the amount of NAP or VEGF levels in sera was evident. The effect of anti-NAP mAb on proliferation of endothelial cells is especially visible when observing blood vessel formation in synovium. Histopathological studies showed clearly the inhibition of blood vessel formation in H&E staining. Consistent with the above results, immunohistochemical staining for CD31, Flt1 or VEGF revealed reduced synovial vessels, thus suggesting that anti-NAP mAb also influences angiogenesis in synovium. Our results have shown that there was extensive neovascularization in synovium of NIA or AIA rats due to VEGF or NAP. As there is inhibition of revascularization and reduction in VEGF or NAP levels in serum, anti-NAP mAb is affecting the angiogenesis either directly or indirectly. Additionally, these results confirm that NAP is a proinflammatory/pro-arthritic factor, as well as being a pro-angiogenic factor.

Fig. 5. Immunohistochemical staining. The sections were stained with anti-CD31, anti-VEGF or anti-Flt1 antibodies and processed as described in Material and methods, and photographs were taken using a charge-coupled device (CCD) camera attached to a Leitz–Diplan microscope.
In conclusion, the present data indicate that NAP is a potent proinflammatory and pro-angiogenic factor in NIA or AIA rat models. Anti-NAP mAb treatment decreased significantly the severity of arthritis and improved the histological findings in established NIA or AIA rat models. Anti-NAP mAb also reduced the neovascularization and proinflammatory proteins, resulting in a decrease in MVD and thereby an anti-arthritic effect. Anti-inflammatory and anti-angiogenic effects are likely to be interdependent mechanisms, resulting in a profound anti-angiogenic effect in NIA or AIA rat models. Anti-NAP mAb can also be used as a diagnostic tool for detection of NAP in sera and effusions of patients with inflammatory disorders. These findings, showing that in-vivo administration of anti-NAP mAb suppressed arthritis on established AIA or NIA rats, suggest that anti-NAP mAb treatment may serve as a new and additional therapeutic modality for RA. However, research needs to be continued to understand the importance of NAP, and further clinical trials using anti-NAP mAb may prove to be much more effective and cost-effective, and with fewer side effects.

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Disclosure
The authors declare no conflict of interests.

References
27 Steinbrech DS, Longaker MT, Mehrara BJ et al. Fibroblast