Regulation of the Angiopoietin–Tie ligand–receptor system with a novel splice variant of Tie1 reduces the severity of murine arthritis

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Abstract

Objectives. To determine the function of the angiopoietin (Ang)–Tie ligand–receptor system, and to assess the effect of Tie1-751, a naturally occurring extracellular domain of the Tie1 receptor derived by alternative splicing, in an in vivo model of RA.

Methods. In the murine CIA model, expression of endogenous Ang1, Ang2, Tie1 and Tie2 in whole paws was analysed by quantitative RT–PCR. To assess the effect of inhibition of the Ang–Tie axis, Tie1-751 was expressed and fused to the Fc fragment of human IgG1. The effect of Tie1-751–Fc on human umbilical vein endothelial cell (HUVEC) cytoprotection and migration in response to Ang1, either alone or in combination with VEGF, was investigated. Furthermore, an in vitro angiogenesis assay was used to determine the effect of Tie1-751–Fc on Ang1-mediated angiogenesis. Finally, Tie1-751–Fc was administered in CIA, and the effects on clinical disease and joint architecture of hind foot specimens were determined.

Results. Gene expression levels of Ang1, Ang2, and receptors Tie1 and Tie2 in whole paws were significantly increased during the progression of arthritis. Tie1-751–Fc significantly inhibited HUVEC cytoprotection and migration in response to Ang1 alone, or Ang1 in combination with VEGF. Tie1-751–Fc also significantly inhibited angiogenesis induced by a combination of Ang1 plus VEGF. Finally, Tie1-751–Fc, when administered intra-peritoneally to arthritic mice, reduced clinical signs of arthritis, damage to joint architecture and infiltration of blood vessels into the synovium.

Conclusions. Our data demonstrate that the Ang–Tie ligand–receptor system is dysregulated in CIA. Tie1-751, a novel splice variant of the Tie1 receptor, inhibits Ang1/VEGF signalling, suggesting that Ang inhibition may be of therapeutic benefit in inflammatory arthritis.

Key words: Arthritis, Angiogenesis, Angiopoietin, Therapy, Endothelium.

Introduction

The tyrosine kinases with immunoglobulin-like and epidermal growth factor (EGF)-like domains (Tie) receptors, namely Tie1 and Tie2, are mainly expressed in endothelial cells and are activated by angiopoietins (Ang). The importance of the Tie receptors in angiogenesis is demonstrated by experiments where knockout of tie genes in mice resulted in defects of angiogenesis and vascular remodelling and led to embryonic lethality [1, 2]. Stimulation of Tie2 by Ang1 results in phosphorylation of the receptor and downstream signalling pathways including phosphoinositide 3-kinase/AKT, ultimately leading to endothelial migration, tube formation and survival [3–5]. Tie1 interacts with Tie2 via its extracellular domain to form a heterodimer at the cell surface, which may serve to regulate Ang1–Tie2 signalling [6–8]. Angiogenesis is significantly increased in inflammatory arthritis, including RA. This results in an increase in the...
infiltration of inflammatory cells into the synovium, which is essential for pannus formation and disease progression [9]. Targeting angiogenesis is, therefore, a rational therapeutic approach in managing inflammatory arthritis. During pathological angiogenesis, evidence suggests that the VEGF and Ang-Tie ligand–receptor system are up-regulated. For example, Shahrrara et al. [10] have shown that Ang1-positive immunostaining on synovial lining cells, macrophages and endothelial cells was significantly higher in RA samples compared with OA and normal synovial tissue. In the same study, Ang1, Ang2, Tie1 and Tie2 mRNA levels were observed to be higher in RA synovial tissue compared with normal and OA synovial tissues, suggesting dysregulation of this pathway in RA. Furthermore, in a number of human cancers, Tie1 and Tie2 expression are increased in the tumour vasculature [11–13]. The use of biologics to specifically target the Tie receptors in order to inhibit pathological angiogenesis may offer a novel therapeutic strategy in RA, particularly when combined with anti-cytokine biologics. For example, soluble Tie2 receptor was shown to be efficacious in murine CIA [14]. On the other hand, anti-Tie1 monoclonal antibody significantly inhibited pathological angiogenesis and suppressed the growth of xenograft tumours in mice [15].

In nature, homoeostatic regulation of many receptor systems generates soluble receptors, e.g. the TNF/TNF receptor and the human EGF receptor families [16, 17]. These retain ligand binding ability and may inhibit biological function of their ligands [18, 19]. Recently, in an effort to identify functional soluble splice variants of cell surface receptors, a PCR-based cloning approach was used to screen 21 cell surface receptor genes in a complex mixture of human RNA [20]. Sixty novel splice variants were identified, 10 of which were efficiently expressed and tested in the CIA model of arthritis. The i.v. administration of adenovirus encoding Tie1-751 and VEGFR1-541 demonstrated significant efficacy in CIA [20].

In the present study, we produced Tie1-751 as a secreted protein bound to Fc and investigated the mechanism of action of Tie1-751–Fc in Ang1/VEGF-mediated survival, migration and angiogenesis in human umbilical vein endothelial cells (HUVECs). The effectiveness of Tie1-751–Fc in CIA when administered intra-peritoneally was also investigated.

Materials and methods

Purification of recombinant Tie1-751–Fc

Tie1-751 splice variant cDNA was isolated from mRNAs that represent major human tissue types from healthy or diseased tissues and cell lines as previously described [20]. To construct Tie1-751–Fc, the Fc fragment of human IgG1 (from Pro100 to Lys330) was PCR amplified and fused in-frame to the 3′-end of Tie1-751 (GenBank accession number EU826590) in a pcDNA3.1 vector (Invitrogen, Renfrew, UK) via restriction digestion of the XhoI–Agel site. After sequence verification, Tie1-751–Fc was transiently expressed in human embryonic kidney (HEK) 293 cells and conditioned media were collected 72 h later. Tie1-751–Fc was purified using a Protein-A Sepharose column (GE-Amersham, Piscataway, NJ, USA), following the manufacturer’s instructions. Purity of the recombinant proteins was >95% as determined by SDS–PAGE and Coomassie blue staining.

Induction and assessment of arthritis

Male DBA/1 (H-2b) mice at 12 weeks of age (Harlan UK Limited, Oxon, UK) were injected intradermally at the base of the tail with 100 µl of an emulsion of purified bovine Type II collagen (200 µg) in complete Freund’s adjuvant (CFA; Difco Becton Dickinson, Cowley, Oxford, UK), as previously described [21]. Animal work was conducted under the Home Office project licence PPL no: 70/5446 Pathogenesis and therapy for RA under the operatives of the Animals (Scientific Procedures) Act 1986, which followed the Helsinki Declaration principles.

The first clinical signs of arthritis were considered to be present when oedema and erythema involving any one of the four paws was observed. Onset of disease was observed from 2 weeks after the collagen administration. The incidence of arthritis in each group of animals reached at least 80%. The earliest signs of disease were observed on Day 14 after immunization, with Day 26 post-immunization as the median day of arthritis onset. Treatments were administered intra-peritoneally from Day 1, with the frequency of dosing on either Days 1, 4 and 7 or Days 1, 3, 5, 7 and 9. The mice were randomized into groups, which received Tie1-751–Fc (30 mg/kg), phosphate-buffered saline (PBS; equivalent volume) or dexamethasone (0.5 mg/kg; Sigma, Poole, UK).

Paw thickness and clinical score was measured daily until Day 10. Paw swelling was measured with 0–10 mm callipers (Kroeplin, Schluchern, Germany). Each limb was assigned a clinical score as follows: 0: normal paws and no clinical features of inflammation; 1: slight oedema or erythema; 2: pervading oedema/erythema involving the entire paw; 3: pronounced oedema and erythema leading to incapacitated limb mobility.

RNA extraction and cDNA synthesis

Animals were sacrificed at different time points by a Schedule 1 method and paws were snap frozen in liquid nitrogen, disrupted using a BioPulveriser (BioSpec Products, Bartlesville, OK, USA) and transferred into RNaseasy Lysis Buffer (Qiagen GmbH, Hilden, Germany). After thawing, tissue was homogenized using a rotor–stator homogenizer (Ultra-Turrax T8; IKA Werke, Staufen, Germany). Total RNA was isolated using QIAamp RNA Blood Mini kit (Qiagen GmbH) followed by phenol/chloroform extraction and treated with DNase with RNasin (Ambion Ltd, Huntingdon, UK). cDNA was synthesized using dNTP and random hexamer primers (Invitrogen), and Moloney murine leukaemia virus reverse
transcriptase (Promega, Southampton, UK) at 42°C for an hour.

**Real-time PCR reaction**

Real-time PCR was carried out in a Rotorgene 6000 (Corbett Research, Sydney, Australia) using SYBR Green detection method. PCR reactions were performed in triplicates in a mixture of SYBR Green JumpStart Taq ReadyMix (Sigma), cDNA and appropriate primers. Primers were designed using gene-specific mouse sequences from GenBank [National Center for Biotechnology Information (NCBI)] and the Primer3 design package hosted by the Whitehead Institute for Biomedical Research. Where possible, intron-spanning primers (MWG, Ebersberg, Germany) were designed using Primer 3 Software and UCSC Genome Bioinformatics, to give a product size of 100–300 bp and primer size of 20–22 bp, to avoid self-complementarities that might lead to hairpin formation, and complementarities between primer pairs, which could give rise to primer-dimers, as follows: acidic ribosomal protein (ARP): sense primer 5’-3’ AATCTCCAGGGCACCTTT, anti-sense primer 5’-3’ ATTACACGCTGACCAGTTG (product size 101 bp), NCBI Reference Sequence NM_007475; Ang1: sense CATTCTTCGCTGCACCTTG, anti-sense GCACATTGCCCATGTGAATC (103 bp, NM_007940); Ang2: sense CAGGCAACGGAGGAAGTTATT, anti-sense AAGTGGACCGACACATGC (117 bp, NM_007946); Tie1: sense CAAGGGTCAACACACCGTGAA, anti-sense GCCAGTCTAGGTTATGAGTAGG (121 bp, NM_011587); Tie2: sense TCTGAGTGGGCCACACTAC, anti-sense TTCACACTGCAGCCACAGAC (164 bp, NM_013690). Specificity of primer sets were checked against the entire mouse genome using BLAST. Annealing temperatures for each primer were taken as 5°C lower than the melting temperature (T_m), but subsequently optimized for 60°C. To determine possible contamination and primer-dimer formation, a non-template control was included. After 2 min at 50°C and 5 min at 95°C, 40 PCR cycles were run with a denaturing step at 95°C for 15 s, annealing at 60°C for 30 s and elongation step at 70°C for 40 s. Only reactions with specific product formation were considered for further analysis. The cycle threshold (C_t) values of each reaction were determined using Corbett’s Rotor-Gene 6 software (Qiagen, West Sussex, UK). Data were analysed using the comparative C_t method.

**Isolation of HUVECs**

Human umbilical cords were collected from Chelsea and Westminster Hospital (London, UK) with informed consent after approval by the Riverside Research Ethics Committee (RREC 29/48). HUVECs were isolated by the digestion of umbilical cord veins in 0.025 mg/ml collagenase A (Roche Diagnostics, Mannheim, Germany) [22]. Cells were maintained in RPMI-1640 (Cambrex, Berkshire, UK) containing 10% (v/v) fetal calf serum (FCS; Biowest, Nuaille, France), 10% (v/v) new born calf serum (Gibco, Paisley, UK), 5 U/ml heparin (CP Pharmaceuticals, Wrexham, UK), and 15 μg/ml endothelial cell growth supplement (Marathon, London, UK) at 37°C. Endothelial cells were used between the second and fourth passage for all experiments.

**HUVEC apoptosis assays**

HUVECs were serum-starved for 24 h, in the absence or presence of Ang1 (100 ng/ml, R&D Systems, Abingdon, UK), VEGF (10 ng/ml, TCS Cell Works, Buckingham, UK) and increasing concentrations of Tie1-751–Fc. Histone-associated DNA was measured using a Cell Death Detection ELISA kit (Roche Diagnostics) according to the manufacturer’s instructions. Apoptosis was measured by staining HUVECs with FITC-conjugated Annexin V and propidium iodide (PI) according to the manufacturer’s instructions (BD Biosciences, Oxford, UK) and analysed on a BD Biosciences LSR flow cytometer using FlowJo 7.5 (Tree Star, Inc. OR, USA) software.

**HUVEC migration assay**

Cell culture inserts (8.0 μm pore size; BD Biosciences) were placed into a 24-well plate and coated with gelatin. Ang1 (100 ng/ml) and VEGF (10 ng/ml) in 0.5% FCS RPMI was added to the lower chamber. A total of 100 000 HUVECs in 100 μl of 0.5% FCS RPMI were pre-incubated with Tie1-751–Fc (1–3μM) or 0.5% FCS RPMI for 30 min and added to the upper chambers and allowed to migrate for 5 h at 37°C. After the migration period, non-migrated cells were removed from the upper side of the membrane using a cotton swab. The lower side of the membrane was fixed with ice-cold 70% EtOH for 30 min, treated with 0.5% Triton X-100 for 3 min and stained with haematoxylin for 30 min. The membrane was then rinsed with water and migrated cells were quantified by counting nuclei in three random high-power fields (100×)well.

**Angiogenesis assay**

To analyse the effect of Tie1-751–Fc on angiogenesis, a commercially available angiogenesis kit was used (AngioKit; TCS Cell Works). Cultures spontaneously developed a network of capillary-like tubules after 11 days at 37°C in 5% carbon dioxide. Wells were treated on Day 0 with Ang1 (100 ng/ml) and VEGF (10 ng/ml), alone or in the presence of Tie1-751–Fc (1 μM). Culture medium and treatments were replenished after 4, 7 and 9 days, in accordance with the manufacturer’s instructions. On Day 11, expression of CD31 was visualized by staining with mouse anti-human CD31 antibody (TCS Cell Works), followed by goat anti-mouse IgG alkaline phosphatase and p-nitrophenol phosphate, and absorbance was measured at 405 nm.

**Histological analysis and CD31 immunohistochemistry**

Hind paws were removed post-mortem and sliced along the sagittal plane before being fixed in 1%
overnight at 4°C. After washing in PBS, the paws were decalcified for 14 days, at room temperature, in 0.3 M EDTA. CD31 protein expression was examined by immunohistochemistry. Briefly, endogenous peroxidase activity was quenched by incubation in 0.3% H2O2 in methanol for 20 min. The sections were incubated overnight at 4°C with anti-mouse CD31 antibody (MEC13.3, rat anti-mouse monoclonal; Pharmingen, San Diego, CA, USA) diluted at 1:400. The sections were then incubated at room temperature for 35 min with biotinylated rabbit anti-rat antibody (1:400, Dako, Ely, UK), followed by 30 min at room temperature with avidin–biotin peroxidase complex prepared according to the manufacturer’s instructions and diaminobenzidine/H2O2 for 5 min. Immunostained sections were counterstained with Harris hematoxylin for 1 min. As a negative control, a rat IgG2a antibody was used (R35-95, Pharmingen). CD31 expression was graded in a blinded fashion and criteria assessed were: (i) proximity of positive staining to the joint; (ii) strength of immunopositive staining; (iii) degree of infiltration of synovial cells into the joint; and (iv) overall status of joint architecture. Sections were scored independently by two observers, and scores showed agreement by Bland–Altman analysis: bias (s.d.) = −50.30% (59.87%); 95% limits of agreement = −167.66, 67.06%; r² = 0.1445. Each section was also screened for changes to the joint architecture as follows: 0: normal; 1: mild (minimal synovitis, some cartilage loss, bone erosions limited to discrete foci); 2: moderate (more extensive synovial hyperplasia, destruction of large segments of the cartilage and considerable bone erosions caused by an invasive pannus front); and 3: severe (complete destruction of the joint architecture).

Statistical analyses

Data were analysed using Graph-Pad Prism 5.01 (GraphPad Software, San Diego, CA, USA). In vitro experiments were analysed using one-way ANOVA with Bonferroni’s post hoc test for multiple comparisons. In vivo data were analysed by two-way analysis of variance (ANOVA). Histology data were analysed by the Mann–Whitney test.

Results

To quantify the mRNA levels of genes of the Ang–Tie ligand–receptor system during the development and progression of arthritis, quantitative real-time PCR was performed applying an SYBR Green I detection format. Paws from naive, CFA-immunized and arthritic mice were taken at different time points covering pre-arthritic, early and late stages of acute CIA. Total RNA was extracted from whole tissue and reverse transcribed, and the resulting cDNA applied in separate real-time PCR with gene-specific primers. Results were normalized to the internal ARP levels and are presented as relative mRNA levels compared with CFA-immunized mice.

The expression of the analysed genes did not change in the paws of pre-arthritic animals (14 days after immunization with collagen but before the development of clinical signs). In contrast, expression of Ang1, Ang2, Tie1 and Tie2 increased as arthritis progressed (Fig. 1). Although the changes in gene expression were variable, reflecting the difference in disease activity in individual animals, the data suggest that most changes in gene expression occurred between Days 4 and 8 of arthritis. Transcript levels of Ang1, Ang2 and Tie2 receptor peaked on Day 8 of disease (P < 0.01 for Ang2 and Tie2, P < 0.001 for Ang1 vs pre-arthritic mice). Increases of 2.93- and 2.83-fold were observed for Ang1 and Tie2, respectively, with the mRNA levels slowly declining by Day 12. A lower 2.27-fold increase could be observed for Ang2, with mRNA levels declining by Day 12. In contrast, transcript levels of Tie1 also increased with the progression of arthritis, but showed a progressive increase from Days 4 to 12 of arthritis (Fig. 1).

To assess the functional role of Ang/Tie in vitro and in vivo, we generated proteins structurally based on Tie1. Tie1-751 is a form of the extracellular domain of Tie1 but lacks a transmembrane domain and intracellular segment. Tie1-751–Fc was produced as a dimer by genetically combining it to the Fc portion of a human IgG1 (Tie1-751–Fc). Efficient expression and secretion of Tie1-751–Fc protein from HEK 293 cells was confirmed by western blot analysis of cell culture supernatants, using anti-Myc antibody to detect the Myc tagged protein (not shown), and purity was confirmed by Coomassie blue stain (Fig. 2).

Since Ang1–Tie2 signalling is essential for endothelial cell survival [23, 24], we tested if Tie1-751–Fc could inhibit HUVEC survival. HUVEC were serum-starved for 24 h, in the absence or presence of Ang1, VEGF (as positive control) and increasing concentration of Tie1-751–Fc. Using an ELISA, which detects cytoplasmic histone-associated DNA fragments released into the cytoplasm of apoptotic cells, we confirmed that Ang1 protects against apoptosis at concentrations of 50–500 ng/ml (data not shown). We also observed that Tie1-751–Fc inhibited the cytoprotective effect of 100 ng/ml Ang1 in a concentration-dependent manner. VEGF in combination with Ang1 further promotes endothelial survival, an effect that was also inhibited by Tie1-751–Fc (Fig. 3a).

To confirm these results, we used a flow cytometry-based apoptosis assay, which can differentiate early apoptotic and late apoptotic (necrotic) cells by double staining with annexin V-FITC and PI. VEGF and Ang1 both reduced early apoptosis (annexin V-positive, PI-negative; Fig. 3b and c), although this reduction was not statistically significant. However, a combination of VEGF and Ang1 significantly reduced early apoptosis, and this was reversed by Tie1-751–Fc. Late apoptosis (annexin V-positive and PI-positive) was significantly reduced by both VEGF and Ang1 (Fig. 3b and 3d), and Tie1-751–Fc abrogated the effect of Ang1 (Fig. 3d). Interestingly, although the combination of VEGF and Ang1 also significantly reduced late apoptosis, this was not affected by Tie1-751–Fc.
An endothelial cell migration assay was performed to investigate if Tie1-751–Fc could inhibit the chemotactic response of HUVECs to Ang1 alone or Ang1 in combination with VEGF. Tie1-751–Fc significantly inhibited HUVEC transmigration across 8-μm membranes, in response to either Ang1 alone or Ang1 in combination with VEGF (Fig. 4a). Next the ability of Tie1-751–Fc to modulate angiogenesis in vitro was investigated using a co-culture system, in which capillaries develop between layers of fibroblasts and myoblasts. Tubule formation is detected by staining for anti-human CD31. Ang1 failed to stimulate angiogenesis alone; however, Ang1 enhanced \( P < 0.001 \) the pro-angiogenic affect of VEGF. This effect was inhibited by Tie1-751–Fc (Fig. 4b).

The up-regulation of the Ang/Tie axis in CIA, and the multiple inhibitory effects of Tie1-751–Fc observed in vitro, prompted us to assess the effect of Tie1-751–Fc as a potential therapeutic in vivo using our acute CIA murine model. Tie1-751–Fc at a dose of 30 mg/kg, or an equivalent volume of PBS, was administered intraperitoneally on the day of disease onset (day 1), and then on Days 4 and 7 of disease. Dexamethasone was included as a positive control. Pooled data from two independent experiments are shown (Fig. 5). Disease was scored daily, and expressed as change in clinical score from Day 1, based on all four paws (Fig. 5a). In parallel, arthritis was assessed by paw swelling, expressed as the change in paw thickness from Day 1 (Fig. 5b).

Mice treated with PBS control developed the typical signs of acute CIA, with a rapidly progressing monophasic disease. Mice that were treated with Tie1-751–Fc had significantly reduced paw swelling and clinical score as compared with mice treated with PBS control. For example, on Day 10 of established disease, the mean (s.e.m.) increase in clinical score from Day 1 of arthritis in mice treated with PBS, Tie1-751–Fc and dexamethasone...
were 2.50 (0.56), 1.29 (0.58) (P < 0.001 vs PBS) and −0.36 (0.30) (P < 0.001 vs PBS), respectively (Fig. 5a). There was also a reduction in hind paw swelling in Tie1-751–Fc-treated mice. On Day 10 of disease, the mean (s.e.m.) paw swelling (∆µm) for mice treated with PBS, Tie1-751–Fc and dexamethasone were 0.40 (0.09), 0.18 (0.07) (P < 0.001 vs PBS) and −0.21 (0.05) (P < 0.001 vs PBS), respectively (Fig. 5b). Experiments to examine the effect of five-times dosing of Tie1-751–Fc were also performed. Tie1-751–Fc or PBS was administered on Days 1, 3, 5, 7 and 9 of arthritis. On Day 10 of established disease, the mean (s.e.m.) change in clinical score from Day 1 of arthritis in mice treated with PBS, Tie1-751–Fc and dexamethasone were 2.71 (0.52), 1.5 (0.61) (P < 0.001 vs PBS) and −1.50 (0.50) (P < 0.001 vs PBS), respectively (Fig. 6a). Comparable data were obtained when paw swelling was measured (data not shown).

To investigate the mechanism of action of Tie1-751–Fc, mouse paws were taken at Day 10 of arthritis and frozen sections were cut and stained with haematoxylin and eosin (H&E) and anti-CD31 antibody. For each mouse, the metatarsal–tarsus joints of both paws were examined. Joint sections of PBS-treated mice showed high numbers of infiltrating synovial cells in the inflamed synovium, together with invasion and erosion of bone by the synovium. In contrast, joints from mice treated with Tie1-751–Fc were protected with relatively normal appearance, and well-preserved joint architecture. Representative images are illustrated in Fig. 6b and c, respectively. In addition, an overall subjective score, between 0 and 3, was used to grade CD31 immunopositive staining for anti-mouse CD31. Analysis of mean subjective scores for each treatment group showed there was a significant reduction in mice treated with Tie1-751–Fc (P < 0.05; Fig. 6d). In the same experiment, a subjective score, between 0 and 3, was used to grade joint destruction and inflammation. We observed that Tie1-751–Fc-treated mice exhibited normalization of joint architecture (P < 0.01; Fig. 6d).

Discussion

Angiogenesis is a critical event in RA pathogenesis, and is regulated by an intricate balance of pro- and anti-angiogenic regulators, which include members of the VEGF and Ang receptor–ligand families [9]. A low level of constitutive Ang1–Tie2 signalling is essential to maintain a quiescent endothelium. Endothelial activation leads to an up-regulation of Ang2 gene expression in endothelial cells and a release of Ang2 from endothelial cell Weibel–Palade bodies, that in the presence of pro-inflammatory signals sensitizes the endothelium to angiogenic stimuli [25]. Previous studies have described the expression of Angl and Ang2 [26, 27] and receptors Tie1 and Tie2 [10, 28, 29] in RA synovial tissue. Initially, we aimed to determine whether expression levels of Ang1, Ang2, Tie1 and Tie2 in whole murine paws were altered during the course of CIA, which is a well-validated mouse model of human disease. We found that the Ang–Tie ligand–receptor system is up-regulated in the active period of CIA, suggesting its importance in the angiogenesis that is critical for disease development. Our results agree with previous studies. For example, Shahrara et al. [10] demonstrated greater expression of Ang1, Ang2, Tie1 and Tie2 in RA as compared with normal or OA synovium. Furthermore, gene expression studies comparing expression of genes in CIA showed that another member of the Ang family, Ang-like 4, which is structurally related to the better characterized Ang1 and Ang2, was up-regulated in early and late stages of arthritis [30, 31].

Previously, we have identified naturally occurring splice variants of cell surface receptor genes that have a stop codon in the extracellular region coding for the extracellular domain. These result in soluble receptors lacking a transmembrane domain and intracellular segment. Functional testing of these variant proteins identified that Tie1-751, when expressed by an adenovirus, was effectual in the in vivo CIA model. These studies also demonstrated that Tie1-751 was able to interact with both Tie1 and Tie2 on HUVEC [20]. The up-regulation of the Ang/Tie system in murine CIA prompted us to investigate the effect of Tie1-751–Fc in vivo and to study its potential...
Fig. 3 Inhibition of Ang1-induced cytoprotection in endothelial cells by Tie1-751–Fc. (a) HUVECs were serum starved for 24 h, in the absence or presence of Ang1 (100 ng/ml) and VEGF (10 ng/ml), together with Tie1-751–Fc, as indicated. Endothelial apoptosis was measured by Cell Death ELISA. Dashed line represents response in serum-containing medium. Statistical analysis was carried out using one-way ANOVA with Bonferroni’s post hoc test for multiple comparisons: *P < 0.05, **P < 0.01. (b–d) HUVECs were serum-starved for 24 h, in the absence or presence of Ang1 (100 ng/ml) and VEGF (10 ng/ml) plus Tie1-751–Fc. Endothelial apoptosis was measured by flow cytometry using annexin V-FITC plus PI. (b) Representative data are illustrated. (c and d) Data are mean (S.E.M.) of three independent experiments. Dashed line represents response in serum-containing medium: (c) early apoptosis (annexin V-positive, PI negative); (d) late apoptosis (annexin V-positive, PI positive). Statistical analysis was carried out using one-way ANOVA with Bonferroni’s post hoc test for multiple comparisons: *P < 0.05, **P < 0.01, ***P < 0.001. ns: not significant.
mechanism of action using in vitro endothelial cell-based assays. In this study, we expressed Tie1-751 as a dimer (Tie1-751–Fc), to remove any confounding effects of adenovirus-mediated gene delivery.

Our results confirm previous studies which show that Ang1 is a survival signal for endothelial cells, and that Ang1 in combination with VEGF produces further cytoprotection [23, 24]. Ang1 has also been shown to be chemotactic for endothelial cells [32]. Crucially, we observed that Tie1-751–Fc inhibited Ang1-mediated protection of HUVEC from apoptosis induced by serum withdrawal, and reduced HUVEC chemotaxis mediated by either Ang1 alone or by Ang1 in combination with VEGF. In addition, we found that Ang1 alone did not induce angiogenesis, but enhanced the angiogenic response induced by VEGF, and that this effect was also markedly inhibited by Tie1-751–Fc. The lack of effect of Ang1 alone contrasts with previous studies which have shown that Ang1 promotes formation of endothelial sprouts, an important step in angiogenesis [33] or the formation of capillary-like tubules in adult bovine aortic endothelial cells grown on collagen gels [34]. However, in these

**Fig. 4** Tie1-751–Fc inhibits Ang1-induced endothelial migration and angiogenesis. (a) Endothelial cells were stimulated for 5 h at 37°C with Ang1 (100 ng/ml) and VEGF (10 ng/ml), alone or in the presence of increasing concentrations of Tie1-751–Fc. HUVEC migration across an 8-µm membrane was measured. Data are mean (s.e.m.) of five independent experiments. Statistical analysis was carried out using one-way ANOVA with Bonferroni’s post hoc test for multiple comparisons: *P < 0.05, **P < 0.01, ***P < 0.001. (b) Endothelial cells were stimulated for 11 days, in the absence or presence of Ang1 (100 ng/ml) and VEGF (2 ng/ml), alone or in the presence of 1 µM Tie1-751–Fc. Data are mean (s.e.m.), representative of two independent experiments, and were analysed using one-way ANOVA with Bonferroni’s post hoc test for multiple comparisons: ***P < 0.001. ns: not significant.
studies, Ang1 was over-expressed [33] or the medium was supplemented with fibroblast growth factor-2 [34]. In fact, our results agree with other studies. For example, experiments using an HUVEC and fibroblast co-culture system showed that tube formation by Ang1 is dependent on VEGF since anti-VEGF and anti-VEGFR2 antibodies blocked the formation of HUVEC tubes [35]. Furthermore, in vivo evidence that Ang1 only promotes vascular network maturation in combination with VEGF was provided by a micropocket assay of neovascularization [36].

The exact mechanism of action of Tie1-751–Fc is currently unknown. The inhibition of Ang1-induced cytoprotection by Tie1-751–Fc would suggest that it is selectively counteracting the activity of Ang1. Interestingly, Kontos et al. [37] have shown that Tie1 inhibits UV irradiation-induced apoptosis. Our results also contrast with a recent paper which showed that knockdown of Tie1 did not inhibit the effects of Ang1 on apoptosis [38]. It is possible that Tie1-751–Fc inhibits activation of the Ang1–Tie2 signalling by sequestering ligand and inhibiting Tie1–Tie2 co-operation by forming non-signalling heterodimers. In support of this, we have previously reported that Tie1-751 directly binds to both Tie1 and Tie2 on the surface of endothelial cells [20]. Taken together, our observations of enhanced effects of Ang1 in combination with VEGF on survival and angiogenesis agree with...
previous studies that propose cross-talk of signalling between these factors in endothelial cells.

Finally, we examined the effect of Tie1-751–Fc fusion proteins on established CIA. This model has been widely used for in vivo testing of candidate therapeutics for RA, such as TNF-α inhibitors [39–41]. Moreover, using this model we and others have shown that inhibition of angiogenesis improves disease [21, 42, 43]. In our experiments, administration of Tie1-751–Fc fusion protein produced a modest but significant reduction in disease severity in CIA. This improvement in the severity of disease was observed for both clinical score and paw swelling. In comparison with controls, there was an improvement of joint architecture in the hind feet of arthritic mice treated with Tie1-751–Fc. There was also a significant reduction of CD31 immunopositive staining, reflecting a reduction of synovial blood vessels. Tie1-751–Fc was less effective at reducing disease severity compared with the corticosteroid, dexamethasone. However, we propose that Tie1-751–Fc mediates its actions in CIA by inhibiting angiogenesis in the synovium of the inflamed joints. Therefore, Tie1-751–Fc is unlikely to have the same efficacy as dexamethasone, which inhibits a number of key inflammatory pathways. Increasing administration of Tie1-751–Fc from three-times to five-times dosing did not increase therapeutic activity, suggesting daily dosing and a preventative regime of treatment should also be tested. It would also be interesting to test if there is a synergistic effect of combining anti-TNF-α and Tie1-751–Fc. Moreover, Tie1-751–Fc has the advantage that it is derived from a naturally occurring protein, so is expected to be significantly less immunogenic than other biological therapies.

In summary, we tested Tie1-751–Fc fusion protein, a naturally occurring splice variant that binds to Tie1 and Tie2 directly on the endothelial cell surface. We show that Tie1-751–Fc modulates Ang1/VEGF-mediated HUVEC survival, migration and angiogenesis. In addition, Tie1-751–Fc appears to be efficacious in murine CIA, and we postulate that this is due to the inhibition of angiogenesis in the synovium of inflamed joints. The present work provides proof of principle on the Ang/Tie inhibitiors in in-esis in the synovium of inflamed joints. The present work we postulate that this is due to the inhibition of angiogen-

Rheumatology key messages

- Tie1-751–Fc, a variant of Ang receptor Tie1, modulates Ang1-mediated endothelial responses and is efficacious in murine CIA.
- This work provides proof-of-principle for use of Tie1-751–Fc in RA.

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H.M.S. and E.M.P. designed the study. N.M.M., Y.R. and P.F.S. designed and carried out the in vivo arthritis studies. S.K. assisted in the design and analysis of the in vitro studies. P.J. performed the protein expression and purification, and oversaw the project data analysis. E.M.P. and M.F. assisted in the study design and coordination, and oversaw the data analysis and drafting of the manuscript. All authors have read and approved the final manuscript.

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