Type I interferon causes thrombotic microangiopathy by a dose-dependent toxic effect on the microvasculature

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Abstract

Many drugs have been reported to cause thrombotic microangiopathy (TMA), yet evidence supporting a direct association is often weak. In particular TMA has been reported in association with recombinant type I interferon therapies, with recent concern regarding the use of interferon in multiple sclerosis patients. However a causal association has yet to be demonstrated.

Here we adopt a combined clinical and experimental approach to provide evidence of a such an association between type I interferon and TMA. We show the clinical phenotype of cases referred to a national centre is uniformly consistent with a direct dose-dependent drug-induced TMA. We then show that dose-dependent microvascular disease is seen in a transgenic mouse model of interferon toxicity. This includes specific microvascular pathological changes seen in patient biopsies, and is dependent on transcriptional activation of the interferon response through the type I interferon receptor (IFNAR).

Together our clinical and experimental findings provide evidence of a causal link between type I interferon and thrombotic microangiopathy. As such, recombinant type I interferon therapies should be stopped at the earliest stage in patients who develop this complication, with implications for risk mitigation.

Key Points

- Type I interferon therapies can cause a dose-dependent thrombotic microangiopathy

- Recombinant type I interferon therapies should be stopped at the earliest opportunity in patients who develop thrombotic microangiopathy

Introduction

Thrombotic microangiopathy (TMA) syndromes are characterised by endothelial dysfunction, microangiopathic haemolytic anaemia and microvascular ischaemia, with diverse aetiologies which include drugs[1,2]. Clinicians evaluating TMA patients must decide whether a particular drug is likely to have caused the disease. This difficult decision requires high quality evidence, and recent work has highlighted the difficulty of attributing a causal relationship[1]. TMA is typically a rare but serious adverse event which can occur after many years of treatment. As such, an association is unlikely to be detected in randomised controlled trial data[3]. Therefore, for the large majority of drugs, causality with TMA is inferred from isolated case reports, without wider analyses of drug safety data or experimental evidence[1].

This problem is exemplified by recombinant type I interferon therapies. Recombinant interferon-α and interferon-β therapies act through the common type I interferon receptor IFNAR, and are widely used for the treatment of neoplastic, autoimmune and infectious diseases[4]. Case reports have linked TMA to both interferon-α and interferon-β therapies, the main subclasses of type I interferon[5,6]. Particular concern has been recently raised regarding interferon-β use in multiple sclerosis patients, where fatal cases of TMA have been observed[6,7]. However a causal role for interferon remains to be demonstrated, and alternative confounding aetiologies such as other drugs, complement mutations and *E.coli* exposure have been suggested[6,8].

Establishing causation in drug safety is a major challenge. Frameworks have been proposed to support evidence of a causal association between disease and environmental factors, the best known of which are the Bradford-Hill criteria[9,10]. Such frameworks include a potential role for biological and experimental studies in establishing causation. As such the demonstration of causality in drug safety benefits from a multifaceted approach to the adverse drug event, encompassing analyses of individual cases, drug safety data and experimental evidence[10]. Critically, such analyses require accurate description of the adverse drug event of interest. This is particularly relevant to the study of TMA since this is a pathophysiologically heterogeneous syndrome, with at least nine primary TMA syndromes described[2]. It is also important to establish whether an adverse event is caused by the drug's active ingredient or by other drug components[11,12]. For example, renal failure caused by intravenous immunoglobulin therapy is associated with the high sucrose content of the drug, rather then the immunoglobulin itself, with important implications for understanding the adverse event and managing risk[11].

To address these questions we performed a detailed clinical analysis of type I interferon-associated TMA cases presenting to a national TMA centre to identify important features of the clinical phenotype of this complication. We provide experimental evidence which suggests that the interferon protein itself directly causes microvascular disease, using a transgenic model of type I interferon (interferon-α1) toxicity. We subsequently consider the potential implications of these findings for the safety of patients receiving recombinant type I interferon therapies.

Methods

Patient evaluation and drug safety data

Patients with multiple sclerosis who developed thrombotic microangiopathy with interferon-β therapy were referred to the national TMA centre in Newcastle for further evaluation[13]. The study was approved by Newcastle and North Tyneside 1 Research Ethics Committee (MREC/1/3/83). Individual patient interferon dose was determined from patient records and adjusted for weight (mcg/kg). Dose-response was also further assessed through analysis of national spontaneous reporting data. Requests for safety data were submitted in the context of a registered patient safety audit (NHS Lothian DCNQIT417). Cases of interferon-associated thrombotic microangiopathy reported throughout the UK were identified through a request for spontaneous data submitted to the UK Medicines and Healthcare products Regulatory Authority (MHRA). All spontaneously reported cases of thrombotic microangiopathy associated with recombinant interferon-β from 1999 to January 2016 to the MHRA were identified, using search criteria "haemolytic uraemic syndrome", "thrombotic thrombocytopenic purpura", "thrombotic microangiopathy" and "malignant hypertension". These cases represent all TMA cases spontaneously reported using the UK "Yellow Card" spontaneous reporting scheme as well as UK cases reported in the published literature.

Immunohistochemical analysis

Paraffin-embedded renal biopsies from patients with IFN-β-associated thrombotic microangiopathy were cut into 5 μm sections, rehydrated and boiled in sodium citrate buffer (pH 6.0). Sections were stained with mouse-anti-MxA (kind gift, Otto Haller, University of Freiburg; 1:400) followed by staining with EnVision™ Gl2 System/AP Rabbit/Mouse (Dako) and counterstained with Mayer's hematoxylin (Merck).

***In vitro* effects of type I interferon on endothelial cells**

BEND.5 immortalised mouse brain endothelial cells were plated at 5 x 10^5 cells per well of a 6 well plate in 1 ml DMEM containing 10% FCS and incubated at 37°C with 5% CO_2. Recombinant mouse or human IFN-α or IFN-β (R+D Systems: Mouse interferon alpha A, 12100-1, mouse interferon beta 12400-1, human interferon beta 1a 11415-1, human interferon alpha 2 11105-1) was added to culture media at concentrations 0-10^4U/mL and incubated for 24 hours. Total RNA was isolated from each well using QIAshredder and RNeasy (QIAGEN), according to the manufacturer's instructions. Each biological replicate was assayed for expression of *Oas1A* and *Ifit1* and the housekeeping gene *Hprt* using Brilliant II SYBR master mix (Agilent technologies). Thermocycling and data acquisition were performed using ABI Prism 7900HT Real-time PCR system. A t-test was performed to compare relative gene expression at highest interferon dose to no interferon. (GraphPad Prism Version 6.0d). For human endothelial cells we accessed publically available microarray data from Interferome v 2.01[14] for human endothelial cells (HUVECs) treated with interferon-α and interferon-β (recombinant protein from Schering-Plough at 1000 IU/ml)[15]. The fold-change for genes in interferon-α and interferon-β treated HUVECs were compared.

Analysis of microvasculature in a mouse model of type 1 interferon toxicity

The generation of transgenic mice (termed GFAP-IFNα1) with astrocyte-targeted, brain-specific production of type I interferon has been described previously[16]. Animal experiments were approved by University of Sydney Animal Ethics Committee (protocol number 5374). All animal experimental procedures were carried out in compliance with local procedures and guidelines. The microvasculature of non-transgenic littermate wildtype mice (*WT*), transgenic mice with low levels of astrocyte-derived IFN-α1 production (*IFN^{Low}*) and high levels of IFN-α1 production (*IFN^{High}*) was

4

examined and quantified. Mice were perfused intracardially at 2-3 months of age, with ice-cold saline followed by 4% paraformaldehyde in PBS (n=8, each group). Brains were removed, postfixed overnight in the same fixative, dehydrated through graded alcohol solutions, and embedded in paraffin. For immunohistochemical detection of T cells and endothelial cells, sections were deparaffinized, rehydrated in graded alcohol, rinsed in phosphate-buffered saline (PBS), and blocked for 1 hour at room temperature in PBS containing 1% bovine serum albumin. The sections were then incubated overnight at 4°C with primary antibody diluted in blocking buffer (CD3, Abcam rabbit monoclonal ab16669 and CD31, Abcam rabbit polyclonal ab28364). The sections were then washed in PBS and incubated with anti-rabbit avidin-biotinylated horseradish peroxidase complex (ABC kit; Vector, Burlingame, CA) used according to the manufacturer's instructions. The total number of microvascular abnormalities was counted across three X20 magnification fields per anatomical region, with counting performed blind to mouse genotype. One-way ANOVA was performed across groups for each anatomical region (GraphPad Prism Version 6.0d). For rescue experiments *IFN*[High] mice were crossed to *IFNAR*^{-/-} mice, which lack a functional type 1 interferon receptor[17].

For RNA analysis, whole brain was removed and immediately snap-frozen in liquid nitrogen. Total RNA was extracted from the tissue samples using TriReagent (Sigma-Aldrich) performed according to the manufacturer's instructions. The RNA concentration of the samples was determined by UV spectroscopy at 260 nm. For all probe sets, a fragment of the *L32* gene was included and served as an internal loading control. The ribonuclease protection assays (RPAs) for interferon response genes were performed and analyzed as described previously[18,19], with autoradiographs quantified by densitometry using Image J software, n=3 each group.

Vascular casting of interferon transgenic mice

Vascular casting of *WT* (n=2) and *IFN*[High] mice (n=6) was performed as previously described[20], in accordance with National Institutes of Health Guidelines for Care and Use of Laboratory Animals. Briefly, deeply anesthetized animals were perfused with artificial cerebrospinal fluid (ACSF) containing Heparin, followed by 4% paraformaldehyde in PBS, and an injection of the resin Mercox (Ladd Research, Williston, VT). After resin curing, soft tissue was subsequently macerated followed by decalcification with 5% formic acid. Casts were washed, then dried by lyophilization, mounted on stubs, and sputter-coated with gold for routine scanning electron microscopy.

Results

Interferon-β causes a direct drug-induced thrombotic microangiopathy

In view of the recent regulatory concerns regarding the potential association between type I interferon therapy and TMA in multiple sclerosis patients[6], we first performed a detailed analysis of the clinical features of interferon-β associated thrombotic microangiopathy. The clinical phenotype of new and index[6] cases of interferon-β associated TMA in multiple sclerosis patients was reviewed by the UK national thrombotic microangiopathy centre[13] (n=8, Table 1, Figure 1). Microangiopathic haemolytic anaemia, renal failure and severe hypertension were universally seen and at least one other organ was affected, with prominent brain and cardiac involvement. (Figure 1A-D). No patient received any other drugs associated with TMA[1] and no other confounding TMA triggers or other autoimmune diseases were identified[1,21,22,23]. Detailed genetic evaluation, including exome sequencing, did not identify mutations in any gene associated with thrombotic microangiopathy. The final diagnostic evaluation by the national centre was uniformly consistent with a chronic drug-induced thrombotic microangiopathy (Table 1, Supplementary Table 1)[1,24,25].

Drug-induced thrombotic microangiopathies can be caused by a direct toxic effect of the drug or via indirect immune-mediated mechanisms[1]. Given that direct toxic reactions are typically dose-dependent[24], we next examined the relationship between TMA and interferon dose. Affected patients were all in the bottom weight quartile, adjusting for age and sex[26]. This observation of low weight was replicated in an independent case series[7]. Therefore patients who developed TMA received a significantly higher weight-adjusted interferon dose than those who did not develop the complication (Figure 1E P<0.001 t-test). To confirm this dose-dependence we requested national spontaneous reporting data from the UK Medicines and Healthcare Products Regulatory Agency. This showed that all cases were associated with a dose of interferon-β in excess of 50mcg per week, with 92% of cases associated with the highest available dose (Figure 1F). Evidence of interferon bioactivity (Figure 1G-H) was detected in patient renal biopsies, and anti-interferon antibodies were not detected in any patient, consistent with a direct rather than immune-mediated effect of the drug. Taken together these results suggest that interferon-β causes a toxic drug-induced thrombotic microangiopathy in multiple sclerosis patients.

Thrombotic microangiopathy is observed with all type I interferon subtypes

We next asked whether TMA was observed with all subtypes of recombinant type I interferon therapies in clinical use. The type I interferon family of proteins consists of a family of 13 alpha subtypes and a single major beta subtype[27]. These subtypes exhibit structural similarity and exert their biological activity via the common type I interferon receptor IFNAR (Figure 2A). Recombinant interferon-α therapies are used to treat a broad range of diseases such as CML, hepatitis C and melanoma. Systematic review of the literature shows that thrombotic microangiopathy has been reported as a complication of every subtype of recombinant type I interferon in clinical use; IFNα2a, IFNα2b, IFNβ1a and IFNβ1b (Figure 2B, Supplementary Figure 1).

We next compared the detailed clinical phenotype described here of interferon-β associated TMA with a comparably detailed analysis of a series of interferon-α associated TMA, analysed at a specialised centre[5] (n=8 both groups). A similar clinical phenotype is observed in both case series (Supplementary Table 2). Specifically, interferon-α and interferon-β TMA were associated with long-term therapy at high-doses[5]. Both groups displayed severe hypertension, predominant involvement of the renal microvasculature, moderate thrombocytopenia and a poor renal outcome. All published cases are summarised in the supplementary tables 3 and 4 and confirm that this clinical picture is seen in

the majority of patients. Furthermore, the transcriptomic response of human endothelial cells to both human interferon-α and interferon-β are highly correlated (Figure 2C, r=0.97)[15]. Therefore thrombotic microangiopathy can occur with all subtypes of recombinant type I interferon therapies, and these subtypes exert an almost identical effect on endothelial cells.

Thrombotic microangiopathy is associated with "interferonopathic" disease

An activated type I interferon response can be induced by recombinant protein therapies, but can also occur spontaneously in patients in whom endogenous levels of type I interferon are pathologically elevated. These "interferonopathic" diseases include monogenic disorders of interferon dysregulation, as well as more common autoimmune diseases such as SLE[28,29]. We reviewed pathological findings from patients with different diseases where transcriptomic evidence of an interferon signature has been reported, and identified an association with histopathological evidence of thrombotic microangiopathy, which is exceptionally rare as a spontaneous disease[30] (Supplementary Figure 1). Therefore thrombotic microangiopathy is observed in states of elevated endogenous and exogenous type I interferon in humans.

Type I interferon signalling is species specific, limiting interpretation of preclinical toxicity tests

Given this clinical evidence that TMA is a manifestation of interferon toxicity, we next sought to provide experimental evidence that the recombinant protein itself can cause the small vessel disease observed in the patients described here. To address whether interferon can directly cause microangiopathic disease in rodent models, we first evaluated available preclinical toxicity data used in the clinical development of both recombinant interferon -α and -β therapies[31,32]. No reports of microvascular toxicity were recorded in extensive preclinical testing of human recombinant protein in rodents. However, type I interferon signalling is highly species specific, and human interferon -α and -β do not cause upregulation of interferon response genes in mouse endothelial cells (Figure 3A,B). Therefore, preclinical rodent toxicity models cannot be used to evaluate whether interferon causes endothelial toxicity[31,32].

Transgenic overexpression of type I interferon causes a spectrum of microangiopathic disease, including changes seen in patient biopsies

To overcome the problem of species disparity of interferon action, we utilised a species-matched transgenic mouse model to evaluate the effects of chronic type I interferon exposure on small blood vessels. In our model, type I interferon (IFNα1) is transgenically produced in the brain at zero (*WT*), low (*IFN^{Low}*) or high (*IFN^{High}*) levels, leading to focal graded activation of the interferon response via IFNAR (Figure 4A)[16]. Quantitative histopathological analyses identified a dose-dependent spectrum of microvascular disease, including endothelial hypertrophy and intimal thickening, microaneuryms and perivascular inflammatory infiltrates (Fig. 4C-F, Figure 5, Figure 6). This microangiopathy was observed across anatomical regions and was dose-dependent (Figure 4G, Figure 6D P<0.001 one-way ANOVA).

The three-dimensional morphology of small blood vessels are difficult to assess using conventional histopathological techniques, therefore we performed scanning electron microscope analysis of microvascular casts. This confirmed widespread microvascular abnormalities in the brain, with prominent variations in microvascular calibre including luminal stenosis and occlusions, ectasia and microaneurysms (Fig. 5, Figure 6K-M). These abnormalities were not seen in *WT* mice. This dose-dependent type I interferon-associated microangiopathy included pathological

microvascular abnormalities seen in the biopsies of the patients described above, such as endothelial hyperplasia, luminal occlusion and microaneursym formation (Figure 7A,B).

To confirm this microvascular pathology is caused by activation of the interferon response through the type I interferon receptor IFNAR, we crossed *IFN^High* mice with mice that were null for the type I interferon receptor [17], generating *IFN^High x IFNAR1^-/-* mice. Both upregulation of interferon response genes (IRG) and microvascular disease were absent in *IFN^High x IFNAR1^-/-* mice, confirming a critical role of IFNAR in mediating disease (Figure 7C,D).

Taken together these data suggest that type I interferon therapies cause a dose-dependent thrombotic microangiopathy and the interferon protein itself can cause microangiopathic disease.

Discussion

To date, unambiguous evidence of a causal association between type I interferon therapies and thrombotic microangiopathy has been lacking. This lack of evidence, together with a limited understanding of the serious adverse event, has hampered risk mitigation measures for these drugs and makes the clinical decision as to whether to stop interferon treatment in patients with TMA difficult. Thrombotic microangiopathy is a particularly challenging adverse drug event to study since it is rare and may occur after prolonged exposure to the drug. As such, randomised clinical trials are not powered to identify a causal association[3,33]. The detection of rare or delayed adverse events which occur outside trials typically relies on analysis of case reports and signal detection through spontaneous reporting data[3]. This approach is subject to bias and confounding and therefore even if an association is identified, a causal relationship cannot be inferred. A number of conceptual frameworks have been proposed when considering how to infer causality from observational clinical data[10,34]. Some of these frameworks, such as the well-known Bradford-Hill criteria[10], suggest that experimental evidence can provide an important role in establishing causation by demonstrating biological plausibility, and direct evidence of causation in model systems. Our analyses of clinical, drug safety and experimental data satisfy the Bradford-Hill criteria and therefore support a causal link between type I interferon therapy and thrombotic microangiopathy (Supplementary Table 5).

Careful definition of the adverse event of interest is central to the study of drug side-effects[9]. This is of particular relevance here since many cases of interferon-associated TMA have been reported using diverse terms including HUS[7], TTP[35] or malignant hypertension[35,36], each implying a different pathophysiological process. It is therefore an important first step to define the clinical syndrome through a centre with expertise in the evaluation of this complex condition. Our analyses of cases suggest that interferon-β therapy is associated with a direct drug-induced thrombotic microangiopathy[2]. Direct drug-induced TMAs, such as those induced by VEGF inhibitors, are characterised by a gradual onset, exposure to high drug dose and absence of immunogenicity[1,2,24]. This phenotype is consistently reproduced in our cohort here. Three lines of evidence suggest that interferon-β associated TMA is associated with high dose therapy. Firstly, patients who developed the complication received nearly twice the weight-adjusted dose than patients who did not develop the complication. Secondly, our data shows that all cases of TMA spontaneously reported in the UK were associated with doses >50mcg/week. Thirdly, our experimental model recapitulates a striking dose-dependence of endothelial disease.

While our primary focus here has been to address recent ongoing concerns regarding recombinant interferon-β use in multiple sclerosis patients, our wider analyses suggest that thrombotic microangiopathy is associated with all subtypes of recombinant type I interferon in clinical use. Comparison of case series evaluated at centres with TMA expertise suggests that TMA with interferon-α and interferon-β therapy display a similar clinical phenotype. In particular, both are associated with prolonged exposure to high doses of interferon, with chronic changes observed on biopsy. This is characterised by panendothelial disease with prominent renal, cerebral and cardiac involvement which may evolve over months. Further review of the published literature suggests that this clinical phenotype is consistently observed in almost all reported IFN-β TMA cases and most IFN-α TMA cases. Some patients with IFN-α TMA can occasionally present with a broader phenotype more suggestive of TTP, in particular patients with underlying haematological malignancies[5]. Future deep phenotypic study of this adverse event across centres will be important to determine the spectrum and true incidence of this disease, as well as the influence of underlying morbidity on clinical presentation. We propose that the most appropriate working descriptive term for this complication is "type I interferon-associated thrombotic microangiopathy".

We have tested the hypothesis that type I interferon itself can directly cause microvascular disease. This hypothesis cannot be evaluated in preclinical rodent models due to the species-specificity of type I interferon signalling. We have therefore studied two complementary "species-matched" states of elevated type I interferon in mice and humans. Firstly, our mouse model here provides experimental advantage over preclinical rodent models, through species-matching of interferon, and allows evaluation of the chronic effects of interferon exposure. Analysis of the microvasculature of this model demonstrates that type I interferon causes a spectrum of microangiopathy, with features that overlap with microvascular disease observed in the patients we describe. Secondly we show that TMA, which is exceptionally rare as a spontaneous disease[30], is consistently observed across a broad spectrum of human "interferonopathic" diseases. In these disease states, interferon levels are spontaneously elevated in the context of activated innate immunity[27]. As such they might be considered by the Bradford-Hill criteria to represent an "analogous" system of human interferon toxicity. These diseases include rare genetic disorders of interferon dysregulation[4,27,28], as well as more common autoimmune disorders such as SLE[37]. The precise subtypes of type I interferon which are elevated in these disease states are not yet fully described, but activation of the interferon response downstream of IFNAR is typically observed[28]. Microangiopathy is a major cause of organ damage in these patients (Supplementary Figure 1)[38,39]. For example, 25% of patients with SLE affecting the kidney have evidence of TMA on renal biopsy[40,41] and microangiopathy is a dominant brain pathology in patients with SLE [31]. Therefore microangiopathic disease is consistently seen in mouse and human states of aberrantly elevated type I interferon. It is notable that in both patients and the mouse model we describe, microangiopathy is the dominant pathological feature, with microvascular thrombosis a less prominent feature. The lack of thrombosis on biopsy in many TMAs has resulted in a recent call for a change in nomenclature from TMA to microangiopathy with or without thrombosis to reflect this incongruity[42].

These findings are relevant to clinicians who must decide whether to stop recombinant type I interferon therapy in patients who develop thrombotic microangiopathy. Our data suggests a causal association and therefore the drug should be stopped at the earliest opportunity given the serious ensuing morbidity. Furthermore our findings are of relevance to risk mitigation strategies since they raise the possibility of stratification and monitoring. In particular detailed retrospective analysis of clinical features and biopsies of cases suggests that type I interferon causes a chronic TMA, which evolves over weeks to months, with a detectable prodrome (supplementary table 6). At present, renal function monitoring and blood pressure monitoring is not mandated for patients receiving type I interferon therapies and none of the patients described here were actively monitored for this complication. Therefore, despite a potential window of opportunity for early recognition, all patients presented at a late stage to emergency or critical care facilities. This late presentation is characterised by fulminant disease and organ failure, sometimes with fatal consequences.

Additional blood pressure and renal function monitoring for TMA have subsequently been introduced across Scotland following concerns about the severity and incidence of this complication in multiple sclerosis patients, where an incidence of ~1:1000 patient-years has been observed in patients treated with high-dose interferon-β [6] (supplementary figure 2). This monitoring has led to prompt cessation of interferon-β in patients who have developed severe/accelerated hypertension associated with mild renal dysfunction. Since the implementation of targeted monitoring, no patients have developed fulminant organ failure due to interferon-associated TMA (supplementary Figure 2B). We suggest that further consideration should be given to risk mitigation measures for this serious adverse drug event.

In summary we have shown that type I interferon therapies cause a direct dose-dependent thrombotic microangiopathy and the interferon protein itself can directly damage small blood vessels. The chronic and dose-dependent nature of this complication identifies an opportunity to reduce risk to the large numbers of patients exposed to these drugs. These findings should facilitate and stimulate definitive and rigorous pharmacoepidemiological study of this serious adverse event, with an emphasis on quantification of risk and the potential for mitigation across the available type I interferon therapies. In the meantime, clinicians should be aware of this association when assessing patients and stop recombinant type I interferon therapies at the earliest stage in patients who develop TMA.

Author contributions

All authors contributed to the writing and editing of the manuscript. DH, BW, JO and SC registered and analysed audit with additional input from NS, DG and DK. DH and DK collated clinical data for patients. CB, CG, YK and HB performed histopathological analyses of human renal biopsy specimens. RC performed genetic analyses and exome sequencing. OF, MB, DK were responsible for clinical care and evaluation of inpatients. IC generated transgenic mice. AJu and DH analysed neuropathology of transgenic mice. IC, TK and EM performed electron microscopic analysis of microvasculature of transgenic mice. JW analysed toxicity reports. SM performed in vitro endothelial cell culture experiments. DH, SC and SE performed pharmacoepidemiological analyses.

Potential Conflicts of Interests

DK and DH are supported by the Wellcome Trust.

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Figure 1. Interferon-β causes a direct drug-induced thrombotic microangiopathy in multiple sclerosis patients.
Recombinant interferon-β therapy causes a microangiopathy affecting multiple organs in multiple sclerosis (MS) patients: **(A,B)** MRI brain scan shows multiple small new lesions consistent with recent microvascular ischaemia, identified on diffusion-weighted sequences (white arrows). **(C)** Admission blood film showing fragmented red blood cells (black arrows). **(D)** Haematoxylin and Eosin stain of renal biopsy from patient shows pathological microvascular changes with endothelial swelling, luminal narrowing and trapped red blood cells (black arrow, scale bar = 25μm). **(E)** Patients who developed TMA, including patients from an independent cohort[7], received a higher weight-adjusted dose than unaffected MS patients treated with the same interferon-β preparation, * P < 0.001 t-test **(F)** All UK reports of thrombotic microangiopathy associated with interferon-β are associated with IFNβ1a dose >50mcg/week and 92% associated with the highest available dose (n=15 reports) **(G,H)** Evidence of activation of interferon response in renal biopsy of affected patient (MxA immunohistochemistry, red, scale bar 10μm), with biopsy from patient with TMA not associated with interferon (G: genetic atypical HUS) for comparison.

Figure 2. Thrombotic microangiopathy is associated with all type I interferon subtypes in clinical use

(A) An overview of type I interferon signalling. Interferon-α and interferon-β act via the transmembrane type I interferon receptor IFNAR, leading to widespread upregulation of interferon response genes. (3D protein models rendered using PyMOL Molecular Graphics System, Version 1.8.2.2 Schrödinger, LLC with protein data obtained from the Protein Data Bank[43] www.rcsb.org) **(B)** Dendrogram of human recombinant interferon subtypes in clinical use. Recombinant type I interferon proteins associated with TMA highlighted in red (see supplementary figure 1 for full details). **(C)** Comparison of the transcriptomic response of human endothelial cells (HUVECs) to human interferon-α and interferon-β[14]. The transcriptional response of human endothelial cells to both type I interferon subtypes is highly correlated (r=0.97, genes with fold-change >1 shown).

Figure 3. Type I interferon signaling is species specific, limiting interpretation of cross-species preclinical toxicity models Type I interferon proteins (-α and -β) act through a common receptor (IFNAR), leading to activation of downstream interferon response genes (IRGs). **(A)** Preclinical toxicity testing of human recombinant type I interferon utilises cross-species testing. Human recombinant type I interferon does not elicit a downstream interferon response in mouse endothelial cells, as measured by quantitative PCR for type I interferon response genes in bEnd.5 brain endothelial cell line. Despite extensive preclinical testing (355 mice tested with human interferon-α, 347 mice tested with human interferon-β), no reports of small vessel toxicity were observed in publically available preclinical tests for human recombinant type I interferon therapies tested in rodents[31,32]. **(B)** In contrast, dose-dependent upregulation of interferon response genes is observed in mouse endothelial cells exposed to same-species recombinant type I interferon *in vitro* (data represent mean +/-SEM * = P<0.05, t-test compared to no interferon, n=3 experiments, IFN-α shown).

Figure 4. Type I interferon causes a dose-dependent microangiopathy

(A) Overview of experimental design (see text), with structure of the fusion gene used to generate the GFAP-IFN-α transgenic mice. **(B)** Scanning electron micrograph of *IFN^{High}* vascular cast, coloured to show larger vessels (red) and microvasculature (box, yellow) **(C-E)** Haematoxylin and eosin sections of mice with maximal interferon overexpression show a spectrum of dose-dependent microangiopathic pathology, including endothelial hypertrophy and intimal thickening (C), microaneurysms (black arrows, D) and perivascular inflammatory cell infiltration and red bloods cells within narrowed lumen (blue arrows, E). Scale bars = 20μm. **(F)** Scanning electron micrograph of small vessel cast showing variation in microvessel calibre (white arrows) **(G)** Microvascular pathology in interferon-overexpressing mice is dose-dependent, *WT*= wildtype, no transgenic overexpression, *IFN^{Low}* = transgenic line with low interferon overexpression, *IFN^{High}* = transgenic line with high interferon overexpression (n=8 mice per group. Data represent mean +/-SEM one-way ANOVA p<0.001).

Figure 5. Abnormal morphology of cerebral microvascular casts of *IFN^High^* mice demonstrated by scanning electron microscopy (A,B,C) Scanning electron micrograph of cerebral microvascular casts of wildtype mice at low (x25), medium (x80) and high power (x300). **(D-F)** Abnormal microvascular morphology in *IFN^High^* mice, with variations in microvessel calibre (red arrows), ectatic vessels and microaneurysms (blue arrows). Such microvascular abnormalities were observed in all *IFN^High^* mice with highest levels of brain-restricted interferon-α1 production (n=6) but not in wildtype mice.

Figure 6. Spectrum of microvascular abnormalities observed in a focal model of type I interferon toxicity.
A spectrum of microvascular disease is observed in mice with transgenic production type I interferon in the brain, across all anatomical regions. **(A-C)** Dose-dependent microvascular pathology in the hippocampus of transgenic mice with zero, low and high levels of interferon overexpression. (Haematoxylin/eosin, abnormal small blood vessel highlighted by black arrow) **(D)** Dose-dependent microvascular pathology was observed across all major brain regions *** $p<0.01$ one-way ANOVA for each region n=8 each group. **(E-G)** Perivascular inflammatory cell infiltration: Haematoxylin/eosin (H+E), CD3 and CD31 immunohistochemistry shows small blood vessels with T-cell infiltration (CD3) and narrowing of lumen (CD31 endothelial marker). **(H)** Ectatic small vessel **(I)** Perivascular infiltration by T-cells (CD3, brown). **(J)** intravascular calcification (Scale bars E-G=20μm, H,I = 40μm). Scanning electron microscopy of microvascular cast shows **(K)** multiple small capillary microaneurysms **(L)** large microaneurysm and **(M)** ectatic microvessel

Figure 7. Specific pathological features of interferon-associated microangiopathy are seen in patient renal biopsy material and are mediated through the type I interferon receptor (A-B) Microvascular pathology identified in this model includes specific pathological abnormalities such as luminal narrowing (black arrows), endothelial hyperplasia (green arrows) and microaneurysm formation (blue arrows), which were seen in the biopsies of the patients described above. Scale bars = 20μm. **(C)** Upregulation of interferon response genes (*Isg15, Irf7, Cxcl10*) was observed in *IFN^{High}* transgenic mice and absent in mice which lack the type I interferon receptor (*IFN^{High}* x *IFNAR^{-/-}*). Total RNA was extracted from the brain of wildtype and transgenic mice and analyzed by ribonuclease protection assay and visualized by autoradiography (n=3 per group). **(D)** Rescue of microvascular pathology in *IFN^{High}* mice which lack the type I interferon receptor (*IFN^{High}* x *IFNAR^{-/-}*). Data represent mean +/-SEM *** P<0.001, t-test compared to *WT* and *IFN^{High}* x *IFNAR^{-/-}* n=8 regions.

Clinical Feature		Number of cases n=8
Underlying disease	Relapsing remitting multiple sclerosis	All
Presenting features	Microangiopathic haemolytic anaemia	All
	Emergency/critical care admission	All
	Severe/malignant hypertension	All
	Renal Failure	All
	Additional organ involvement*	All
Specialist investigation	Reduced ADAMTS13 activity***	None
	aHUS mutations** *CFH, CFI, C3, CF, CD46, THBD*	None
	Immune reaction against IFN-β (neutralising antibody titre>20 NU/ml)***	None
Chronicity	Length of treatment > 5 years	All
	Thrombotic microangiopathy with chronic changes on renal biopsy**	All
Dose dependence	Treated with >50mcg/week	All
	Weight in bottom quartile	All
Final Diagnostic Evaluation	**Direct drug-induced thrombotic microangiopathy**	**All**

Table 1. Summary of clinical features of thrombotic microangiopathy associated with interferon-β referred to the national UK thrombotic microangiopathy centre. Summary of clinical features of new and index cases[6] of interferon-associated thrombotic microangiopathy in multiple sclerosis patients referred to the national TMA centre in Newcastle. * neurological involvement 4/8 cases, cardiac involvement 6/8 cases **= data available for 7/8 cases. *** data available for 6/8 cases. Full clinical details of all cases are in Supplementary Table 1. Following diagnostic re-evaluation the clinical phenotype of the cases was uniformly consistent with a direct drug-induced thrombotic microangiopathy[2,24].

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Figure 1

No Interferon   Interferon

Figure 2

A

Type I Interferon

IFN-α   IFN-β

Type I
Interferon
Receptor
(IFNAR)

Interferon Response Gene
(IRG) Activation

B

Human
Interferon
Therapies

Type I

alpha — IFNα2a
         IFNα2b

beta — IFNβ1a
        IFNβ1b

Type II — gamma — IFNγ1a

C

IFN-β fold change

1000
100
10
1

1   10   100   1000
IFN-α fold change

Figure 3

Figure 4

A

IFN-α1 cDNA
Modified GFAP exon 1

GFAP promoter 1 2 3 4 5 6 7 8 9 3'

WT   IFN^Low   IFN^High

Analysis of microvasculature

B

Microvascular pathology   3D Morphology

Compare to patient biopsies

C

D

E

F
100μm

G

P<0.001

Microvascular abnormalities/field

WT   Low   High

Interferon dose

Figure 5

Wildtype

Transgenic type I interferon production
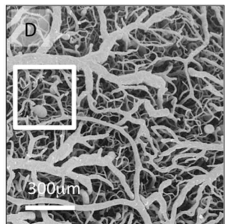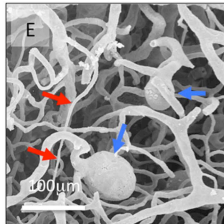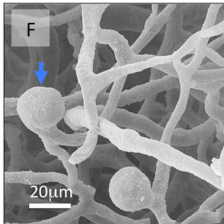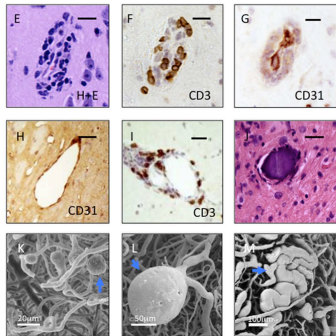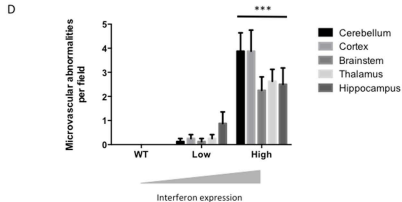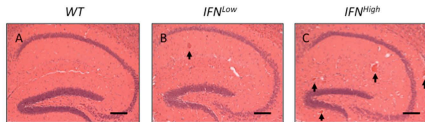
Figure 6

A — IFN-Microangiopathy —

IFN^{High} Patient Biopsy

B — IFN-Microangiopathy —

IFN^{High} Patient Biopsy

C

WT	IFN^{High}	IFN^{High} $x IFNAR^{-/-}$	
			$Isg15$
			$Irf7$
			$Cxcl10$
			$L32$

D

Microvascular abnormalities per field

IFN^{High} $IFN^{High} x IFNAR^{-/-}$

***

Figure 7