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Full Length Article

Endothelial cell functions impaired by interferon in vitro: Insights into the molecular mechanism of thrombotic microangiopathy associated with interferon therapy

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ABSTRACT

Introduction: Interferon (IFN)-α and IFN-β approved for treatment of chronic hepatitis C viral infection and multiple sclerosis respectively have been linked to thrombotic microangiopathy (TMA) affecting renal function. Since the molecular mechanisms underlying this severe complication remain largely unclear, we aimed to investigate whether IFN affects directly in vitro endothelial cell functions associated with angiogenesis and blood haemostasis, as well as endothelial cell-derived vasodilators of nitric oxide (NO) and prostacyclin.

Methods: Proliferation and survival of human umbilical vein endothelial cells (HUVECs) were measured by BrdU incorporation and alamarBlue assays. Angiogenesis was evaluated in co-cultures of HUVECs and human dermal fibroblasts. Fibrinolysis molecules were measured with ELISA. NO and prostacyclin were measured using a fluorescent NO-specific probe and a competitive enzyme immunoassay, respectively.

Results: HUVEC proliferation was dose-dependently inhibited by IFN-β1a and IFN-β1b, but not by IFN-α2a and IFN-α2b. Consistently, IFN-β1a and IFN-β1b also reduced survival of HUVECs, but this again was not observed with IFN-α. However, both IFN subtypes inhibited VEGF-induced development of capillary-like structures, but the effect of IFN-α was less potent than IFN-β. In addition, both IFN subtypes upregulated interferon inducible protein 10 production from treated co-cultures while suppressing angiogenesis. Furthermore, intracellular NO generation was reduced by IFN-α2a and IFN-β1a, whereas prostacyclin release from HUVECs was not affected by IFN. Importantly, both IFN-β1a- and IFN-β1b-treated HUVECs showed a marked reduction in urokinase-type plasminogen activator release and a much greater secretion of plasminogen activator inhibitor-1 than tissue-type plasminogen activator compared with untreated cells, suggesting decreased fibrinolytic activity. IFN-α, however was less effective in modulating the fibrinolysis system.

Conclusions: We demonstrate the detrimental effects of IFN on endothelial cell functions mediated with angiogenesis and fibrinolysis, which could potentially cause the loss of physiological endothelium thromboresistance and facilitate the development of vascular complications in a clinical setting. Mechanistically, our findings have implications for understanding how IFN therapy can foster the development of TMA.

1. Introduction

Type I interferons (IFNs), IFN-α and IFN-β, are pleiotropic cytokines with an important role in innate immunity against viral infection, immunomodulation and cancer surveillance [1,2]. Type I IFNs exert their effects through binding to a shared receptor complex composed of the two subunits, IFN-α/β receptor 1 (IFNAR1) and IFNAR2 to form the ternary complex. IFN-induced receptor dimerization drives the activation of Janus kinases (JAKs), which in turn initiate downstream signaling cascades mainly via the signal transducer and activator of transcription (STAT) proteins. Because of their biological activities, these two cytokines are indicated for the treatment of several medical

Abbreviations: IFN, interferon; TMA, thrombotic microangiopathy; HUVECs, human umbilical vein endothelial cells; IFNAR, IFN-α/β receptor; JAKs, Janus kinases; STAT, signal transducer and activator of transcription; mAb, monoclonal antibody; VEGF, vascular endothelial growth factor; PMA, phorbol 12-myristate 13-acetate; BrdU, 5-bromo-2′-deoxy-uridine; LDH, lactate dehydrogenase; HDFs, human dermal fibroblasts; IP-10, interferon inducible protein 10; NO, nitric oxide; PGI_2, prostacyclin; vWF, von Willebrand factor; TF, tissue factor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; VEGFR2, VEGF receptor-2; TTP, thrombotic thrombocytopenic purpura; HUS, haemolytic uremic syndrome; SLE, systemic lupus erythematosus

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conditions. Human IFN-α2 is approved for treating chronic hepatitis B and C viral infection and various cancers, while IFN-β1 is extensively used in the therapy of relapsing-remitting multiple sclerosis [3,4].

Treatment with IFN can impact adversely on small blood vessels causing thrombotic microangiopathy (TMA), a serious microvascular occlusive disorder [5]. Initial reports implicated IFN-α therapy in the development of lesions of TMA in patients with chronic myeloid leukemia [6,7]. However, more recently, TMA has increasingly been reported in IFN-β treated multiple sclerosis patients given high dose and long-term IFN therapy, which in some cases was fatal and associated with failure of renal function [8–10]. The mechanism underlying this severe complication however remains unresolved.

Endothelial cell injury is considered the central and probably inciting factor in the sequence of events leading to TMA [11]. Pathologic findings in TMA include endothelial swelling and necrosis, and glomerular and vascular thrombosis [12]. Notably, TMA also occurs in cancer patients on therapy with bevacizumab [13,14], a humanised monoclonal antibody (mAb) targeting vascular endothelial growth factor (VEGF), which is a pivotal angiogenic factor involved in both physiological and pathological angiogenesis [15]. While VEGF is fundamental in maintaining endothelial cell homeostasis, treatment with bevacizumab is associated with a disruption in the endothelial equilibrium state. This suggests that like VEGF antagonism, there may be a causal link between IFN treatment and vascular endothelial dysfunction.

Endothelial cells constituting the vasculature's inner lining help to maintain blood flow and synthesize many molecules involved in the regulation of vascular tone, and blood coagulation and fibrinolysis processes. Since functional endothelial cells are essential for maintaining vascular homeostasis and preventing thrombosis, we investigated systematically the direct effects of different type I IFN subtypes on human endothelial cell functions in vitro to assess their ability in regulating (1) cell proliferation and survival; (2) the development of endothelial cell-derived capillary-like structures; and (3) endothelial cell-produced molecules contributing to vasodilation, coagulation and fibrinolysis. In this study, we used human umbilical vein endothelial cells (HUVECs), a well established and most commonly used human primary cellular model generally representing relevant cell type characteristics of vascular endothelial cells. Although endothelial cells originating from different vascular beds display heterogeneity of function and phenotype, HUVECs share common endothelial markers and adhesion molecules with glomerular microvascular endothelial cells.

2. Materials and methods

2.1. Reagents

Since recombinant human IFN-α2a, IFN-α2b, IFN-β1a, IFN-β1b and VEGF-A165 (WHO International Standard, NIBSC Working Standard or WHO Reference Reagent: NIBSC code 95/650, 95/566, 00/572, 00/574 and 02/286) were available in house, they were used in experiments. Mouse anti-human IP-10 mAb, mouse anti-human VEGFR2-PE-conjugated mAb and relevant isotype control Abs were purchased from R&D Systems. Medium 199 and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. All other reagents used were of the purest grade available.

2.2. Cell culture

HUVECs purchased from Cellworks were cultured in endothelial cell growth medium-2 supplemented with defined growth factors, 2% FBS and gentamicin/amphotericin-B (Lonza Biologics Plc) in an incubator at 37°C with 5% CO_2 and passaged by trypsinization (trypsin/EDTA solution, Lonza Biologics Plc). HUVECs at passages from 3 to 9 were used for experiments.

2.3. Flow cytometric analysis of expression of IFNAR and CXCR3

HUVECs were harvested using trypsin-EDTA solution and collected by centrifugation. Following initial treatment with a human Fc receptor blocking reagent (R&D Systems), cell surface expression of the heterodimeric IFNAR complex was detected using APC-conjugated anti-IFNAR1 mAb (R&D Systems) or PE-conjugated anti-IFNAR2 mAb (PBL Assay Science). Surface expression of CXCR3 on HUVECs was detected using PE-conjugated anti-CXCR3 mAb (R&D Systems). In parallel, the cells were stained with the respective isotype-matched control Ab. The stained cells were examined by flow cytometry using FACSCanto II (BD Biosciences) by counting 30,000 cells. Data were analysed using FACSDiva (version 6) and FlowJo (version 10) softwares and expressed as median fluorescence intensity (MFI).

2.4. Cell proliferation

HUVECs were seeded in 96-well plates at a density of 8×10^3 cells per well in the assay medium supplemented with 10% FBS in the absence or presence of increasing concentrations of IFN. Following stimulation for 2 days, 10 μM 5-bromo-2′-deoxy-uridine (BrdU) was added to the cultures, and following incubation for a further 16 h, DNA synthesis was measured using the BrdU detection kit (Roche) according to the manufacturer's instructions. BrdU incorporation was measured at A_{370} nm with A_{492} nm as a reference wavelength using a plate reader (Spectra Max, Molecular Devices). The absorbance intensity is directly proportional to the number of proliferating cells.

2.5. Cell viability

HUVECs were seeded in 96-well plates at a density of 1×10^4 cells per well in assay medium supplemented with 0.5% FBS in the absence or presence of increasing concentrations of IFN. After 48 h incubation, the alamarBlue reagent resazurin (oxidized form in blue colour), an oxidation-reduction indicator (Serotec), was added to the cultures and the cells were incubated for a further 24 h. The resorufin products (reduced form in pink colour) converted from resazurin by metabolically active cells were then measured at A_{570} nm with A_{600} nm as a reference wavelength using the Spectra Max plate reader. The absorbance intensity is directly proportional to the number of viable cells.

2.6. Cell cycle distribution

HUVECS were incubated in assay medium supplemented with 10% FBS without or with IFN for 24 h. After treatment, the cells were harvested using trypsin-EDTA solution, rinsed with PBS and fixed with ice-cold 70% ethanol at 4 °C for 1 h. After a washing step, the fixed cells were treated with the RNase/propidium iodide staining solution (BD Biosciences) for 15 min at room temperature and the fluorescence intensity of the stained DNA was analysed by flow cytometry. For each measurement, 30,000 cells were counted.

2.7. Apoptosis

Cell apoptosis was measured using the annexin V binding assay as described previously [16]. Briefly, HUVECs were incubated in assay medium free of serum without or with IFN for 24 h followed by trypsinization. The cells were then washed and stained with fluorescein-conjugated annexin V and propidium iodide from the apoptosis detection kit (Invitrogen) according to the manufacturer's instructions and analysed by flow cytometry. The annexin V-positive (annexin V +) cells were counted as apoptotic cells.

2.8. Cytotoxicity

Cytotoxicity was determined by measuring the activity of