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A deamidated interferon-β variant binds to integrin αvβ3

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ABSTRACT

Human type I interferons are a family of pleiotropic cytokines with antiviral, anti-proliferative and immunomodulatory activities. They signal through the same cell surface receptors IFNAR1 and IFNAR2 yet evoking markedly different physiological effects. One differentiating factor of interferon-beta (IFN-β) from other type I interferons is the presence of the Asn-Gly-Arg (NGR) sequence motif, which, upon deamidation, converts to Asp-Gly-Arg (DGR) and iso-Asp-Gly-Arg (iso-DGR) motifs. In other proteins, the NGR and iso-DGR motifs are reported as CD13- and αvβ3, αvβ5, αvβ6, αvβ8 and α5β1 integrin-binding motifs, respectively. The scope of this study was to perform exploratory surface plasmon resonance (SPR) experiments to assess the binding properties of a deamidated IFN-β variant to integrins. For this purpose, integrin αvβ3 was selected as a reference model within the iso-DGR- integrin binding members. The obtained results show that deamidated IFN-β binds integrin αvβ3 with nanomolar affinity and that the response was dependent on the deamidation extent. Based on these results, it can be expected that deamidated IFN-β also binds to other integrin family members that are able to bind to the iso-DGR binding motif. The novel binding properties could help elucidate specific IFN-β attributes that under physiological conditions may be modulated by the deamidation.

1. Introduction

Interferons are pleiotropic cytokines implicated in antiviral [1], anti-proliferative [2–4] and immunomodulatory activities [5–8]. The type I interferon family includes IFN-β, IFN-ε, IFN-κ, IFN-ω and 12 subtypes of IFN-α. All members of this family signal through the same cell-surface receptors, IFNAR1 and IFNAR2, distinctively evoking different physiological responses [9–11]. The biological effects are considered to be mediated through the interactions with the heterodimeric interferon receptor constituted by IFNAR1 and IFNAR2. Receptor binding is a sequential process with the IFNs first binding to the higher affinity IFNAR2, followed by the recruitment of IFNAR1 to form the ternary complex with subsequent receptor and cellular activation [12]. During the formation of the ternary complex, the binding of the IFNs induces a substantial IFNAR1 conformational change found to be essential for the signalling activation [12]. The current paradigm is that the different activities of type I interferons are modulated by their respective receptor recognition chemistry leading to different receptor-ligand stabilities of the ternary complex [13] in which a key role is played by the functional plasticity of the IFNAR1 [9]. Among the type I interferons, the IFN-β seems to play a unique physiological role and it is especially potent in activity requiring long-term stimulation [14]. Natural interferon-β (IFN-β) is produced by most cells in response to viral infections or the exposure to other biologics, and recombinant human Interferon-β (r-hIFN-β) preparations are also commercially available for the treatment of relapsing forms of MS [6].

It has been reported that IFN-β inhibits both angiogenesis and arteriogenesis [15]. In animal models, high doses of intratumoural IFN-β were able to ablate tumour vasculature [16,17] while low doses of IFN-β facilitate T-cell responses against tumour antigens [17]. IFN-β, but not IFN-α, has been reported to inhibit endothelial cell proliferation [11,18,19] and melanoma cell migration[20]. The way the binding interaction with IFNAR2/IFNAR1 can generate such a multitude of functional effects is still under discussion and currently topic of investigation.

Looking at the IFN-β structure some interesting properties emerge: IFN-β is a 166-amino acid glycoprotein with the primary sequence containing the motif NGR at position 25–27 which is placed in a

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structurally flexible loop region [9]. This NGR motif in IFN-β is susceptible to deamidation leading to the generation of both DGR and iso-DGR sequence motifs. The intriguing aspect here is that the iso-DGR sequence motif has been reported to act as an antagonist of αvβ3 [21], which, among all the integrins, is regarded to be the most important one, implicated in tumour angiogenesis [22]. Furthermore, peptides, which contain the iso-DGR motif, were reported to inhibit endothelial cell adhesion, as well as tumour growth and that they were able to bind other integrin family members such as αvβ5, αvβ6, αvβ8 and α5β1, although with 10–100-fold lower affinity [23]. Interestingly, integrin binding properties could be imparted by fusing cyclic iso-DGR peptides to other proteins in which they act as a tumour vasculature-targeting motif [24]. In the latter study [24], as iso-DGR cannot be prepared by standard recombinant DNA technology, the iso-DGR motif was generated artificially by exposing the fusion construct containing the NGR motif to deamidation-inducing conditions, namely incubation at a slight alkaline pH, under elevated temperature conditions. This treatment transformed the NGR motif to iso-DGR and DGR. It was shown that, out of these three motif variants, only the iso-DGR motif was able to bind to integrin αvβ3.

The scope of the present study was to investigate the potential binding properties of a deamidated IFN-β variant to integrin αvβ3. To this end, we produced a fully deamidated IFN-β-1a variant by chemical deamidation in accordance to a previously described method [25] (resulting in a mixture of the iso-DGR, DGR motifs) and compared the αvβ3 binding properties to that of IFN-β-1a which had not been subjected to the deamidation treatment.

2. Materials and methods

2.1. Materials used for the characterization study

Highly purified IFN-β-1a drug substance was supplied by Merck Serono S.A. (Switzerland). The production method used a recombinant expression of the protein in a CHO-K1-derived cell line using a commercial serum-free culture medium. Crude harvests were processed through a series of chromatographic steps, which included affinity, ion exchange, reversed-phase, and size-exclusion chromatography. The final drug substance was used as a starting material for the artificial deamidation. Highly purified integrin αvβ3, comprising the extracellular domains of the αv and the β3 subunits, was supplied by Merck KGaA (Darmstadt, Germany). The protein was expressed in a baculovirus expression system and purified by affinity chromatography and gel filtration.

2.2. Preparation of artificially deamidated IFN-β-1a

Deamidation was carried out as previously described [25]. Briefly, the drug substance was buffer-exchanged to obtain an alkaline pH and incubated at 23 °C for 20 h. After treatment, the deamidated sample was buffer-exchanged by ultrafiltration to 50 mM sodium acetate pH 3.8.

2.3. Deamidation level by peptide mapping

Peptide mapping-UPLC analysis was carried out, as previously described [25], in order to quantify the level of deamidation of the NGR sequence motif. The quantification was based on peak area ratios determined in the chromatogram for the deamidated and the native peptides respectively. Deamidation is expressed both in terms of total deamidation (the sum of peptides in which Asn25 is converted into Asp, succinimide and iso-Asp) as well as the percentage of the iso-Asp component (iso-DGR).

2.4. Surface plasmon resonance (SPR)

The binding of an artificially deamidated IFN-β variant to integrin αvβ3 was determined by SPR using the Biacore T100 instrument (GE Healthcare, Uppsala, Sweden). The running buffer was 10 mM HEPES, 500 mM NaCl, 0.005% Surfactant P20, 2 mM Ca^{2+}, pH 6.5. Artificially deamidated IFN-β and untreated IFN-β, diluted at 30 μg/mL in 10 mM sodium acetate pH 5.5, were immobilized on a CM5 sensor chip (flow cell 2 or 4) by amine coupling targeting the immobilization level at 2500 RU. Flow cell 1 or 3 was immobilized in blank conditions as a reference cell. In-house produced integrin αvβ3 was diluted, in running buffer, to obtain a concentration range from 18.75 to 300 nM (5 two-step fold serial dilutions). Five concentrations of integrin αvβ3 were injected, in single cycle mode, onto the chip surface (measurement and reference flow cells) at a flow rate of 30 μL/min with a contact time of 60 s and a dissociation time of 180 s. The chip regeneration was achieved by applying 10 mM Glycine at pH 2.0 at 30 μL/min in 30 s. Sensorgrams were evaluated in accordance to a 1:1 binding algorithm. All binding experiments were performed at 25 °C.

The association and dissociation constants were obtained by elaboration of the sensorgrams using the Biacore T100 Evaluation Software Version: 2.0.2. The K_D value, representing the affinity, is obtained from the ratio of the association and dissociation constants.

3. Results

Highly purified IFN-β-1a was subjected to deamidation-inducing conditions as described in Section 2.2. Peptide mapping analysis (as described in Section 2.3) revealed that this treatment resulted in an almost complete deamidation, > 99% total deamidation, compared with approximately 14% at baseline in the untreated control (see Table 1).

In a previous study, we have shown that this treatment did not affect the level of post-translational modifications while, on the other hand, the in vitro antiviral, antiproliferative, and immunomodulatory properties were improved [25]. Subsequently, the deamidated and the untreated material were compared with regards to their integrin αvβ3-binding properties in a SPR experiment as described in Section 2.4. To this end, a dedicated SPR-based analytical method has been developed. As described in Section 2.4 the method foresaw the immobilization of IFN-β on a sensor chip followed by the flow of integrin αvβ3 as analyte at five increasing concentrations.

Panel A of Fig. 1 shows an overlay of sensorgrams obtained by the interaction of artificially deamidated (red) and the untreated (green) IFN-β with integrin αvβ3.

The analysis has been repeated five times using different sensor chips and a standardised fresh immobilization. The method was found to be robust as could be judged from well-overlaying sensorgrams of the individual runs (not shown). Thus, the curves shown in Fig. 1 can be considered as representative. Panel B shows a control sensorgram obtained by flowing integrin αvβ3 on a sensor chip with a blank immobilization (no IFN-β). The blank does not show a signal related to the binding of integrin confirming that there is no unspecific binding to the dextran surface of the chip and that the signals observed in panel A are specific to IFN-β.

The overlay shows that integrin αvβ3 binding-related signals (in terms of RU as response to integrin addition) are present in both the

Table 1
Deamidation levels according to peptide mapping of the artificial deamidated IFN-β-1a compared with the untreated control. The result is given as the percentage of total deamidation (sum of peptides in which Asn25 is converted into Asp, succinimide and iso-Asp) and that of iso-Asp.

Sample	Peptide mapping	
	Total deamidation (%)	Iso-Asp (%)
Untreated	13.9	0.4
Deamidated	99.2	28.0