Type I and II IFNs Inhibit Merkel Cell Carcinoma via Modulation of the Merkel Cell Polyomavirus T Antigens

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Abstract

Merkel cell carcinoma (MCC) is a rare and highly aggressive skin cancer associated with the Merkel cell polyomavirus (MCV). As MCC cell lines show oncogene addiction to the MCV T antigens, pharmacologic interference of the large T antigen (LTA) may represent an effective therapeutic approach for this deadly cancer. In this study, we investigated the effects of IFNs on MCC cell lines, especially on MCV-positive (MCV⁺) lines. Type I IFNs (i.e., Multiferon, a mix of different IFN-α subtypes, and IFN-β) strongly inhibited the cellular viability. Cell-cycle analysis showed increased sub-G fractions for these cells upon IFN treatment indicating apoptotic cell death; these effects were less pronounced for IFN-γ. Notably, this inhibitory effect of type I IFNs on MCV⁺ MCC cell lines was associated with a reduced expression of the MCV LTA as well as an increased expression of promyelocytic leukemia (PML) protein, which is known to interfere with the function of the LTA. In addition, the intratumoral application of Multiferon resulted in a regression of MCV⁺ but not MCV⁻ MCCs in vivo. Together, our findings show that type I IFNs have a strong antitumor effect, which is at least in part explained by modulation of the virally encoded LTA. Cancer Res. 72(8); 2120–8. ©2012 AACR.

Introduction

Merkel cell carcinoma (MCC) which is also known as neuroendocrine carcinoma of the skin is a rare, highly aggressive skin cancer, with a strong and continuous increase in incidence over the past years (1). UV exposure and immune suppression are known risk factors for MCC (2). Indeed, MCC is much more frequent in severely immunosuppressed populations caused by immune suppressive drugs in organ transplant patients, lymphoma, or HIV infection (3). In accordance with the notion that many cancers with infectious etiologies are more prevalent in the context of immunosuppression, the presence of MCV was identified clonally integrated in the genome of most MCC cells (4). Meanwhile, many studies confirmed the association of MCC with MCV (5, 6). MCC encodes the potential oncoproteins small and large T-antigens (LTA; ref. 7). Notably, it has been shown that the maintenance of MCV-positive (MCV⁺) MCC cell lines critically depends on the presence of MCV LTA sustaining the role of MCV in the pathogenesis of MCC (8, 9).

IFN has been first described as secreted macromolecule produced by cells after treatment with heat-inactivated influenza virus (10). Indeed, IFNs are a large family of multifunctional, secreted proteins which have antiviral, antitumoral, and immune modulating effects mediated through IFN-stimulated gene (ISG) expression (11). Three types of IFNs have been described in mammals. Type I IFNs (α, β, ε, κ, λ, and ω) as well as type III IFNs (IFN-λ1–3; ref. 12) are produced ubiquitously in response to viral infection, double-stranded RNA, or other stimuli. In contrast, type II IFN-γ is only induced in activated T lymphocytes and natural killer cells (11, 13). The biologic activities of IFNs are initiated by binding to their cognate receptors, that is, predominantly the IFN-α/β receptor for type I, the IFN-γ receptor for type II, and the IL10R2/IFNLR1 for type III IFN. Upon binding to the respective receptors different signal cascades are activated. The classical Janus—activated kinase (Jak)/STAT pathway leads to the transcription of a distinct set of genes which mediate the biologic effects of these cytokines, that is, antiproliferative effects and antiviral activity for type I and III IFNs and immune modulatory effects for type II IFNs (11). It is important to note, however, that the cellular responses to engagement of the IFN receptors are subject to several modulating factors, for example, the activation status of the cells, binding of other cytokines/chemokines, or environmental factors such as hypoxia.

The therapeutic use is currently largely restricted to type I IFNs. For example, recombinant IFN-α2 has been U.S. Food and Drug Administration approved for the therapy of hairy cell leukemia or adjuvant therapy of melanoma (14, 15). In addition, IFN-ω is used for the treatment of hepatitis B and C and HIV-associated Kaposi sarcoma whereas IFN-β is used for therapy of multiple sclerosis (16–18).

The antiviral activity of type I IFNs has also been investigated for polyomaviruses. In this regard, type I IFNs can both limit the replication of John Cunningham (JC) virus and...
interfere with expression of virally encoded genes (19). Similarly, IFN-γ is able to suppress BK virus gene expression (20). Indeed, both publications show a downregulation of polyomavirus LTA expression upon IFN treatment. Moreover, IFNs have been shown to induce the expression of promyelocytic leukemia (PML) protein; PML is known to modulate infection of cells by JC virus via interaction with LTA encoded by the polyomavirus (21). Given the recently showed oncogenic addiction of MCV+ MCC cell lines toward LTA expression, IFNs appear as a promising therapeutic option for MCC. Indeed, Krasagakis and colleagues have already shown sensitivity of a MCC cell line toward IFN-α (22). Furthermore, the clinical activity of IFN in MCC is described in anecdotal reports (23, 24). Here, we studied the impact of different types of IFN, that is, type I Multiferon (a mix of 5 IFN-α subtypes), IFN-β-1a as well as type II IFN-γ, on MCV+ and MCV+ MCC cell lines both in vitro and in vivo, revealing a striking effect of type I IFNs on the viability of MCV+ MCC cells.

Materials and Methods

Ethics statement

The presented work was conducted according to the principles expressed in the Declaration of Helsinki. The generation and characterization of MCC cell lines was approved by the Institutional Review Board of University Hospital Würzburg (sequential study number 124/05). All the animal experiments were approved by the local authorities (Regierung von Unterfranken; animal experiment request Az. 55.2-2531.01-59/06) according to the legal requirements.

Cell culture

The MCV+ cell lines WaGa, BroLi, MKL-1, and MKL-2 as well as the MCV− MCC cell lines UISO, MCC13, and MCC26 (25–27) have been described previously. For doxycycline inducible T-antigen knockdown, retroviral-infected MKL-1 p53 TA tet, MKL-2 pH pTA tet, and WaGa pH pTA tet cell lines (9) were used. All cell lines were grown in RPMI-1640 medium (PAN Biotech) supplemented with 10% FBS (Biochrom AG), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Sigma Aldrich).

Animal experiments

Five-week-old female NOD.CB17/Prkdc<sup>scid</sup> mice were obtained from Harlan Winkelmann and housed under specific pathogen-free conditions. Tumors were induced by s.c. injection of 5 × 10<sup>6</sup> cells/100 μL mixed 1:1 with Matrigel Matrix (Becton Dickinson) into the lateral flank of the mice. Multiferon (Swedish Orphan) treatment was started 10 days after cellular injection. A total of 10,000 units of Multiferon in 50 μL PBS (PAN Biotech) or 50 μL PBS were injected intratumorally every day (n = 6 for each group) as previously described (28).

Immunoblotting

Cell lysates of MCV+ MCC cell lines cultured for 4 days in 24-well plate at 1 × 10<sup>5</sup> cells per well with 50,000 units/mL Multiferon; or IFN-β (PeproTech) or 10,000 units/mL IFN-γ (PeproTech) were generated as previously described (9). After SDS-PAGE, samples were transferred to nitrocellulose membranes (GE Healthcare), blocked 1 hour with PBS (Sigma Aldrich), containing 0.05% PBS-Tween 20 (PBS-T) supplemented with 5% powdered skim milk and then incubated overnight with a primary antibody. Following 3 washing steps with PBS-T, membranes were incubated with a peroxidase-coupled secondary antibody (DAKO) followed by use of the Plus-ECL Chemiluminescence Detection Kit (Thermo Scientific). Antibodies used were CM2B4 (1:1,000) for MCV LTA protein, H-238 rabbit polyclonal antibody (1:200) for PML (both Santa Cruz Biotechnologies), and TUB 2.1 (1:250; Sigma Aldrich) for β-tubulin.

MTS assay

Cell proliferation, metabolism, and viability were measured with the MTS cell assay (Promega) according to the manufacturer's instructions. MCC cell lines were cultured in triplicates at 1,000 (MCC13, MCC26), 3,000 (UISO), 10,000 (WaGa), or 80,000 (BroLi, MKL-1, MKL-2) cells per well with 0.781, 3,125, 12,500, or 50,000 units/mL of type I or 0, 156, 625, 2,500, and 10,000 units/mL of type II IFN for 7 days. Cell proliferation of MCV+ cells upon T-antigen knockdown was determined by culture of the respective cells for 5 days in the presence of 1 μg/mL doxycycline (Sigma Aldrich).

Cell-cycle analysis

For cell-cycle analysis, 5 × 10<sup>4</sup> (MCC13, MCC 26, and UISO) or 1 × 10<sup>4</sup> (MKL-1, MKL-2 WaGa, and BroLi) cells per well were cultured for 7 days with 50,000 units/mL Multiferon or IFN-β or 10,000 units/mL IFN-γ. Single-cell suspensions were fixed with 5 mL of ice-cold ethanol (100%) overnight at 4°C; cell pellets were resuspended in 1 mL PBS supplemented with 1% fetal calf serum (FCS), 0.05 mg/mL propidium iodide (Pt; Sigma Aldrich), and 0.1 mg/mL RNase A (Fermentas) and incubated for 1 hour at 37°C. Flow cytometry was conducted on a FACSCan flow cytometer and analysis made with FlowJo analysis software (Tree Star Inc.).

Immunohistochemistry and immunofluorescence

Immunohistochemistry on formalin-fixed and paraffin-embedded tissue was carried out as previously described (9). For antigen recovery, deparaffinized sections were incubated with DAKO Target Retrieval Solution (DAKO), pH 9.0 for 40 minutes at 90°C and rinsed twice with bidistilled water and once with PBS, incubated with blocking solution (DAKO) at room temperature and after 2 additional washes stained overnight with a rabbit monoclonal antibody specific for cleaved caspase-3 (D178; 1:2,000; Cell Signaling). Detection of the antibody was conducted with Dako EnVision-HRP (DAKO) and Nova Red Substrate Kit (Vector Laboratories) following the manufacturer's protocol. To show possible interaction of PML and LTA the Duolink system was used according to the manufacturer's protocol (Olink Bioscience). For microscopy, a Leica DM750 microscope with ICC50 digital microscope camera (Leica) was used.

For immunofluorescence, cytopsins of 6 × 10<sup>4</sup> WaGa cells cultured with 3,500 units of Multiferon per mL for 3 days or of untreated cells, respectively, were fixed in acetone for 10 minutes, rinsed with PBS, and incubated with blocking solution (DAKO) for another 10 minutes. After another washing step,
cells were stained first with PML H-238 rabbit polyclonal antibody (1:100; Santa Cruz Biotechnologies) and with Cy3-labeled goat anti-rabbit IgG (H+L) secondary antibody (1:200; Dianova) each for 45 minutes.Slides were mounted with Vectashield with DAPI (Vector Laboratories) and analyzed with TCS SP2 confocal fluorescence microscope (Leica).

Statistical analysis

Statistical analysis was conducted with Prism 5.03 (GraphPad Software, Inc.). The Wilcoxon test was applied to test sensitivity of MCV\(^+\) and MCV\(^-\) cell lines to treatment with IFNs, the antiproliferative effect of type I and type II IFNs between MCV\(^+\) and MCV\(^-\) cell lines and differences in the viability of MCV\(^+\) cells with T-antigen knockdown compared with untreated controls. All analyses of IFN effect were conducted for the highest IFN doses used. Furthermore, \(t\) tests were carried out to compare tumor volumes of WaGa xenografts in the \textit{in vivo} experiments after the Kolmogorov–Smirnov test confirmed Gaussian distribution of tumor volumes.

Results

IFNs inhibit proliferation and viability of MCC cells

In a first series of experiments we tested the effect of Multiferon, IFN-\(\beta\), and IFN-\(\gamma\) on the proliferation, metabolism, and viability of 4 MCV\(^+\) and 3 MCV\(^-\) MCC cell lines by the MTS cell proliferation assay. These assays revealed on one hand that the MCV\(^+\) MCC cell lines are much more sensitive to IFN treatment (Wilcoxon test, \(P = 0.0039\)) and on the other hand that type I IFNs have a more pronounced effect (Wilcoxon test, \(P = 0.0313\)) than IFN-\(\gamma\) (\(P = 0.25\); Fig. 1). The strongest inhibition was...
observed for Multiferon and IFN-β on MCV+ cell lines; notably, 2 of the MCV+/C0 MCC cell lines appeared insensitive to all IFNs. The effect of IFN-γ was at best very weak and independent of the viral status of the cell line (Wilcoxon test, $P = 0.25$; Fig. 1).

**Type I and II IFNs variably induce apoptosis in MCC cells**

To define the mechanisms of the inhibitory effects of the IFNs on MCC cell lines and to explore possible differences in MCV+ and MCV- cell lines, cell-cycle analyses for all 7 MCC cell lines treated with the different IFNs in comparison to untreated controls were conducted (Fig. 2). These analyses revealed that most of the cells lines, which displayed impaired proliferation and viability upon type I IFNs in the previous series of experiments, are characterized by an increase of the fraction of cells in the sub-G0 phase suggesting apoptotic cells death. However, this notion did not hold true for the MCV+ MCC cell line MKL-1. It should be further noticed that already in the absence of IFNs BroLi is characterized by a high frequency of cells in sub-G0 phase. More important, however, IFN-γ did not affect the cell-cycle distribution of any of the MCC cell lines tested.

**Downregulation of T antigens by IFNs**

Expression of MCV LTA is necessary for the maintenance of MCV+ MCC cell lines (8, 9). We confirmed this notion by use of a doxycycline inducible expression of short hairpin RNA (shRNA) against MCV T antigens in MKL-1, MKL-2, and WaGa cell lines. Silencing of T-antigen expression results in a clear reduction of cellular viability in MCV+ MCC cells (Fig. 3).
reduction of cellular viability after 5 days compared with untreated control group ($P = 0.031$; Fig. 3). Consequently, we next tested whether the observed effects of type I IFNs on MCV$^+$ MCC cell lines were mediated by a downregulation of LTA expression. Determination of protein expression of MCV LTA in MCC cell lines after 7 days of incubation with type I and type II IFNs revealed a decrease in LTA expression in response to treatment with type I IFNs for all cell lines analyzed; this downregulation is particularly pronounced for Multiferon (Fig. 4A). In contrast, incubation with IFN-$\gamma$ leads only in WaGa cells to a reduced expression level of MCV LTA.

Induction of the PML protein by IFNs

In addition to the negative regulation of the LTA expression, a number of proteins that interfere with the oncogenic proteins of viruses are regulated particularly by type I IFNs. The most prominent of these with respect to an impaired LTA function is the PML protein. Indeed, we could show by Western blot analysis that PML is highly upregulated in MCC cells upon treatment with IFNs (Fig. 4A). With the exception of WaGa cells, type I IFNs cause a stronger PML induction than IFN-$\gamma$. This induction of PML by type I IFN was further showed by immunofluorescence of untreated or IFN-treated WaGa cells. Here, a marked increase in the number of PML nuclear bodies was obvious (Fig. 4B).

Antitumor activity of Multiferon against MCC in vivo

To translate these observations into the in vivo situation, we took advantage of recently established xenotransplantation
mouse models for the MCV⁺ WaGa and the MCV⁺ UISO cell lines. Therapy by intratumoral Multiferon injection was initiated 10 days after inoculation of tumor cells. Subsequently, Multiferon was injected intratumorally every day during the period of therapy. Upon Multiferon treatment the MCV⁺ WaGa-derived xenografts not only stalled growth but actually regressed, whereas the controls injected with PBS alone progressed (Fig. 5A). Indeed, tumor volumes of Multiferon and PBS-treated mice were significantly different at day 20 and at day 23, respectively (P = 0.0004 and P < 0.0001; unpaired t test). In contrast, MCV⁻ UISO xenografts did not alter their growth pattern as compared with those tumors injected with PBS alone. To elucidate the mechanism of impaired tumor growth in the Multiferon-treated animals, we stained sections of the respective tumors for cleaved caspase-3 as a marker of apoptosis. Notably, Multiferon treatment results in a higher expression of cleaved caspase-3 in treated WaGa tumors (Fig. 5B); thus, the observed in vivo effects of Multiferon are not only due to an impaired proliferation but also to an increased rate of apoptosis. This notion is in line with both with the in vitro findings as well as the active regression of the established tumors subsequent to Multiferon treatment. Our in vitro data suggests that PML might contribute to the regression of the tumors. In order to further expand this observation we conducted Duolink analysis to determine whether PML would interact with LTA; this technique generates positive signals only if the proteins are in close proximity (29). Analysis of MCV⁺ WaGa xenografts did indeed reveal positive signals primarily in the nucleus showing a colocalization of PML and LTA in WaGa cells (Fig. 5C). As a control, we could not observe such an interaction in MCV⁻ UISO xenografts.

Discussion

Since their discovery in 1957 by Isaacs and Linderman (10), IFNs are regarded as drugs with a potential to treat cancer. Because of their ability to directly or indirectly interfere with the expression or function of oncogenic viral proteins, IFNs also seem to be particularly suitable to treat virally induced cancers. In the present report, we scrutinized the effects of type I and II IFNs on MCC, a highly aggressive skin cancer for which the viral oncogenesis has recently been indicated.

The impact of IFNs on MCC cells is characterized by an impaired proliferation, metabolism, and viability particularly after type I IFN treatment; these effects were much more pronounced in MCV⁺ cell lines and largely associated with the induction of apoptosis. Notably, however, in the case of the MCV⁺ cell line MKL-1 the strong inhibiting effect of type I IFNs was not associated with the induction of apoptosis. Interestingly, a similar observation has been reported for human lung carcinoma cells (30). It should be further noted that the BroLi cell line harbors a significant proportion of apoptotic cells already in the untreated control group. This observation suggests that MCV infection itself may render the host cell prone to apoptosis. Indeed, MCC since its initial description by Toker (31) has been known for its high rate of apoptosis (32). For several DNA viruses, including polyomaviruses, induction of apoptosis of the infected cell is part of the viral replication cycle ensuring the release of the virions. This host cell apoptosis is initiated by expression of the very late viral protein (33), which, however, has not been elucidated for MCV yet. It should be further noted, that specific
miRNAs have been suggested to be involved in MCV virion release (34).

As mentioned earlier, recent reports described that IFN-γ treatment interferes with LTA expression in BK virus–infected cells (20) and that type I IFNs interfere with LTA expression in JC virus–infected cells (19). These facts together with the profound effects of type I IFNs on the survival of MCV+ MCC cell lines and the recent demonstration that MCV+ MCC lines critically depend on the expression of LTA (8, 9) prompted us to scrutinize the modulation of LTA expression of MCV+ MCC cells upon IFN treatment. This analysis revealed that type I IFNs, that is, Multiferon and IFN-β, strongly reduced the expression of MCV LTA. Interestingly, the strength of the effect of the different IFNs is not uniform for all MCV+ MCC cell lines, but rather individual, showing the complexity of IFN signaling in general as well as in MCC. Beside the direct inhibition of LTA expression, IFNs may also interfere indirectly with the function of the LTA. For example, the impact of a JC virus infection on human glial cells is reduced by an IFN-β-dependent induction of PML expression (21). This effect is based on a functional inhibition of LTA due to trapping of this protein within PML nuclear bodies via interaction the conserved LXCXE amino acid motif common to all viral oncoproteins that bind pRB (9, 35). In general, the antiviral activity of PML and PML nuclear bodies are well established (21, 36–38). As shown here, IFN treatment strongly induces PML expression in MCV+ MCC cells; moreover, Duolink immunohistochemistry analysis of MCV+ WaGa xenotransplants revealed a colocalization of PML and LTA. Thus, based on similar recent reports for other viruses (21, 35–38), upregulation of PML expression upon IFN treatment results in an increased interaction of both proteins and thus a reduction of free LTA (21). These facts indicating that PML mediates the antiviral effect by sequestering viral and host proteins, that are indispensable for transcription of viral proteins. The effect of IFNs both on the expression as well as the functional activity of LTA is likely to explain both the robustness and the speed of the anti-MCC effect of IFNs that is actually faster and more pronounced than the genetic knockdown of LTA (8). Importantly, the antitumor effect of the type I IFN Multiferon observed in vitro was also translated into the in vivo setting taking advantage of a newly established xenotransplantation model for the MCV+ WaGa cell line but not the MCV+ UISO cell line. Although we could detect an antiproliferative effect of Multiferon on UISO cells in vitro, albeit to a lesser extent, there was no reduction in tumor growth of the in vivo xenotransplants. This observation might be due to the fact, that the UISO xenografts grew very rapidly and therefore the Multiferon dosage used might not be sufficient for a successful treatment. It should be noted, however, as this xenotransplantation model is based on severely immune deficient mice, immune modulating effects of IFNs could not be addressed.

The complexity of IFN signaling is reflected by the observation that the direct in vitro antiproliferative effect of IFNs—albeit less pronounced—is not restricted to MCV+ MCC cells. The fact that the MCV+ UISO cell line is also affected by IFN suggests the involvement of additional mechanisms. For example, IFN-α induces apoptosis in many kinds of cells by upregulation of TRAIL. Interaction of TRAIL with its receptors results in a signal cascade which activates effector caspases such as cleaved caspase-3 (39). To this end, we observed an increased presence of activated caspase-3 in tumors of Multiferon-treated mice harboring WaGa xenotransplants. Moreover, it has been shown for myeloma cell lines that IFN-α–induced apoptosis is at least in part mediated via PML by TRAIL induction (40). Thus, further experiments are warranted to elucidate the precise role of PML in the reduced viability of MCC cells after treatment with type I and II IFNs. Moreover, beside PML and TRAIL, there are a multitude of ISGs, which are supposed to be involved in apoptotic cellular pathways that may explain the sensitivity of MCV+ MCC cell lines to IFN treatment (reviewed in ref. 41), for example, ISG54 (42) and USP18 (43) have recently been described as a mediator or regulator of IFN-induced apoptotic cell death. Still, MCC 13 and MCC 26 stayed nearly unaffected upon IFN treatment; a notion readily explained by the ability of cancer cells to become resistant to IFNs, that is, by overexpression of STAT5 as it has been previously shown for melanoma (44) or epigenetic silencing of genes involved in IFN signaling (45, 46). Another report showed the suppression of insulin-like growth factor–binding protein 7 (IGFBP7) in IFN-α resistant hepatocellular carcinoma (HCC) cells (47). However, the crucial mechanism remains unknown. Further possible mechanisms for IFN resistance could be mediated by miRNAs. Actually, it was recently described that sensitivity of HCC cells toward IFN-α is regulated by miRNA-146a targeting the SMAD4 protein (48). All these reports illustrate the diversity of mechanisms underlying IFN resistance. Which of these multiple mechanisms mediates insensitivity in the 2 MCC cell lines has yet to be determined.

In summary, the present work provides several lines of evidence that IFNs, particular type I IFNs, exert direct inhibitory effects on MCC cell lines in vitro and in vivo. Mechanistically, this effect seems largely due to an induction of apoptotic cell death. Treatment of MCC cell lines with the different IFNs inhibited the expression of virally encoded LTA and induced the expression of PML, which has been previously showed to interfere with the functional activity of the LTA. Consequently, based on the here presented data as well as the well established immunomodulating effects of IFNs such as reinduction of MHC class I molecules or the activation of immune competent cells, treatment of MCC with type I IFNs appears as a promising therapeutic option for patients with MCC: a notion substantiated by several case reports on successful therapy of metastatic MCC lesions by localized type I IFN therapy; thus, these observations are advocating the investigation of especially type I IFNs for therapy of MCC in clinical trials.

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