Tumor-suppressive effects of natural-type interferon-β through CXCL10 in melanoma

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1. Introduction

Melanoma is a malignant tumor that develops by transformation of melanocytes, which are present in various organs or tissues including the skin, retina, cranial pia mater, and intestine [1]. Melanoma is curable in the early stages; however, its prognosis is sometimes poor when the tumor progresses to advanced stages [2]. Melanoma often metastasizes through the lymphatic system, resulting in progression from early stages to advanced stages.

Recently, therapy for melanoma has greatly improved with the use of immune checkpoint modulators such as anti-programmed death receptor-1 antibodies and anti–cytotoxic T-lymphocyte-associated protein 4 antibodies. Although BRAF inhibitors and MEK inhibitors are now available for unresectable and distant metastatic melanomas, the advanced melanomas often show dismal outcomes (the median overall survival is 15.9 months) [3]; therefore, adjuvant therapies to inhibit recurrence and metastases are still needed.

Type 1 interferon (IFN) is in widespread use as an adjuvant therapy, mainly for stage II and resectable stage III melanoma (according to the staging system of American Joint Committee on Cancer) [4]. Prospective, randomized, multicenter treatment trials have demonstrated that high-dose IFN-α-2b and pegylated IFN can improve relapse-free survival [5]. Some reports have indicated that local administration of natural-type IFN-β may improve the prognosis including the 5-year survival rate [6] and the recurrence rate [7,8]. On the other hand, Yamamoto et al. reported that high-dose IFN-β (310–4012 IU/g) accumulates in the regional lymph nodes 2 h after local administration of 3 × 10^6 IU IFN-β but only a low dose (0–17.5 IU/ml) is found in peripheral blood [9].

Considering the tumor-suppressive effects of local administration of natural-type IFN-β on lymphatic metastasis of melanoma

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and high-dose accumulation in regional lymph nodes after local administration of IFN-β, we hypothesized that natural-type IFN-β induces expression of molecules in lymphatic endothelial cells (LECs) in the tumor microenvironment and contributes to inhibition of lymphatic metastasis including regional lymph node metastasis and in-transit metastasis.

The present study was conducted to identify melanoma-suppressive molecules that are up-regulated in lymphatic vessels following treatment with natural-type IFN-β. As a result, we identified chemokine (C-X-C motif) ligand 10 (CXCL10), which was significantly induced by treatment of LECs with IFN-β.

2. Materials and methods

2.1. Cells and cell culture

Human melanoma cell lines SK-MEL-1, SK-MEL-5, A375, and RPMI-7951 were purchased from the American Type Culture Collection (Manassas, VA). The human melanoma cell line G-361 was purchased from the Riken BioResources Center (Tsukuba, Japan). Human dermal LECs were purchased from PromoCell (Heidelberg, Germany), and human skin fibroblasts (FBs), NB I-RGB, were purchased from Riken Gene Bank (Tsukuba, Japan). All cells were cultured in a humidified atmosphere (5% CO2) at 37 °C according to the manufacturer’s instructions.

2.2. Treatment with IFN-β and CXCL10

Natural-type IFN-β protein with sugar chains produced by human fibroblasts (Feron) was kindly provided by Toray Medical (Tokyo, Japan). LECs, FBs, and melanoma cells were treated with IFN-β at a final concentration of 10^3 IU/ml in complete culture medium. Melanoma cells (RPMI-7951 cells and G-361 cells) were treated with CXCL10 recombinant protein (R&D Systems, Minneapolis, MN) at a final concentration of 0, 50, or 250 µg/ml in complete culture medium.

2.3. Oligonucleotide microarray analysis

Using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany), total RNA was extracted from LECs that were treated or not treated with 10^3 IU/ml IFN-β for 48 h. For expression profiling, oligonucleotide microarray analysis was performed at Hokkaido System Science (Sapporo, Japan) using Agilent Human Genomic microarray 8 × 60 K Ver.2.0 (Agilent Technologies, Santa Clara, CA). The raw data were normalized with a locally weighted scatterplot smoothing linear algorithm using the software Feature Extraction 10.7.3.1 (Agilent Technologies).

2.4. Quantitative real-time reverse transcription-PCR (RT-PCR)

Total RNA was extracted from cultured cells using the RNeasy Plus Mini Kit (QIAGEN), cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies, Rockville, MD). Quantitative RT-PCR was performed with the SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) and a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The mRNA quantity of each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and PCR conditions are shown in Supplementary Table 1.

2.5. Enzyme-linked immunosorbent assay (ELISA)

LECs, FBs, and melanoma cells were treated with IFN-β, which was added at a final concentration of 10^3 IU/ml in the complete culture medium on day 1 after the medium was changed. The concentration of CXCL10 in the culture supernatant at 24 h after addition of IFN-β was determined with an ELISA using the Human CXCL10/IP-10 Quantikine ELISA kit (R&D Systems) according to the manufacturer’s instructions. The luminescence intensity was quantified with the DTX 800 Multimode Detector plate reader (Beckman Coulter, Fullerton, CA) at an optical density of 450 nm.

2.6. Plasmid transfection and generation of stable clones

To generate stable clones of A375 cells in which CXCL10 was knocked down, HuSh shRNA plasmids against CXCL10 (5’-AACTGGATTGTGGTCTGACTTCTCT-3’) along with the gene encoding puromycin N-acetyl-transferase (OriGene, Rockville, MD) were used. A non-effective 29-mer scrambled shRNA cassette was used as a control. Transfections were performed with TurboFect 8.0 reagent (OriGene) according to the manufacturer’s instructions. Stable control and CXCL10-knocked down clones were selected and maintained with 1.0 µg/ml puromycin.

2.7. Invasion assay

The invasion assay was performed using the CytoSelect 96-Well Cell Invasion Assay Kit (Cell Biolabs, San Diego, CA) according to the manufacturer’s instructions. Invasive melanoma cells were stained, extracted, and quantified with a plate reader (Wallac ARVO MX, Perkin Elmer, Waltham, MA) at an optical density of 560 nm.

2.8. Statistical analysis

Statistical analysis was performed with commercially available software, SPSS version 18 (SPSS Japan, Tokyo, Japan). Significant differences were analyzed with the Kruskal–Wallis test, one-way analysis of variance, and multiple comparisons test (Bonferroni method). P < 0.05 was considered statistically significant.

3. Results

3.1. Genome-wide microarray indicates up-regulation of CXCL10

To detect genes up-regulated by IFN-β in LECs, genome-wide oligonucleotide microarray analyses that can examine expression of 56,792 genes were performed using LECs that were treated or not treated with IFN-β. The microarray analyses identified 893 up-regulated genes and 665 down-regulated genes in treated LECs compared to non-treated LECs. Of the 893 up-regulated genes, 20 genes were up-regulated >30-fold (Table 1). Among these 20 genes, three genes encode secretory molecules that may affect melanoma cells adjacent to lymphatic systems. The product of one of these three genes, CXCL10, is closely involved in the inhibition of melanoma proliferation and invasiveness [10]. Consequently, we further examined the gene product.

3.2. CXCL10 is significantly induced by IFN-β in LECs compared to FBs

To confirm the induction of CXCL10 in LECs following treatment with IFN-β, mRNA expression at 0, 3, 6, 12, 24, and 48 h after treatment with IFN-β in LECs and FBs was analyzed with RT-PCR. CXCL10 mRNA was significantly induced in LECs compared to FBs (Fig. 1a). We next performed ELISA tests using the culture supernatant 24 h after treatment with IFN-β to confirm the induction of the secretory protein following IFN-β treatment. We confirmed that the secretory protein was significantly induced by IFN-β treatment in LECs compared to FBs (Fig. 1b).
3.3. The melanoma cell lines exhibit a wide spectrum of sensitivity to IFN-β

To evaluate the sensitivity of melanoma cells to IFN-β, the sensitivity of each melanoma cell line to IFN-β was examined using the proliferation assay following treatment with IFN-β. The melanoma cell lines exhibited a wide spectrum of sensitivity in the order of RPMI-7951, A375, G-361, SK-MEL-5, and SK-MEL-1 (high to low sensitivity; Suppl. Fig. 1a); the cells could be classified into two groups: cell lines with high sensitivity to IFN-β including RPMI-7951 and A375 cells, and cell lines with medium to low sensitivity to IFN-β including G-361, SK-MEL-5, and SK-MEL-1 cells.

3.4. CXCL10 is significantly induced by IFN-β in cells with high sensitivity compared to medium to low sensitivity

To elucidate the association between CXCL10 expression levels and sensitivities of melanoma cells to IFN-β, the mRNA expression at 0, 3, 6, 12, 24, and 48 h after treatment with 0 IU/ml IFN-β was analyzed with RT-PCR. The peak CXCL10 expression levels in each melanoma cell line (RPMI-7951 > A375 > G-361 > SK-MEL-5 > SK-MEL-1 cells) were completely consistent with the order of sensitivity to IFN-β (Fig. 2a). We next performed ELISA tests using the culture supernatant 24 h after treatment with IFN-β to confirm induction of the secretory protein by IFN-β treatment. We confirmed that the concentration levels of the secretory protein in each melanoma cell line (RPMI-7951 > A375 > SK-MEL-5 > G-361 > SK-MEL-1 cells) were nearly consistent with the order of sensitivity to IFN-β (Fig. 2b).

3.5. CXCL10 reduces proliferation of IFN-β-sensitive cells as well as resistant cells

To elucidate the role of CXCL10 in melanoma proliferation, a cell proliferation assay was performed after treatment of RPMI-7951 and G-361 cells (as representatives of relatively high and medium to low sensitivity to IFN-β, respectively) with 0, 50, or 250 μg/ml CXCL10 recombinant protein. Cell proliferation of RPMI-7951 and G-361 cells at day 5 was similarly suppressed by treatment with CXCL10 recombinant protein in a dose-dependent manner. We noted a significant difference in each graph (Kruskal–Wallis test, P = 0.01 and < 0.001 in the graph of RPMI-7951 and G-361 cells, respectively) and a significant difference between the cell numbers following treatment with 0 and 250 μg/ml CXCL10 recombinant protein in both cell lines (non-parametric multiple comparison test, P = 0.007 in RPMI-7951 cells and P < 0.001 in G-361 cells) (Fig. 3a).

3.6. CXCL10 knockdown renders melanoma cells less sensitive to IFN-β

To confirm the tumor-suppressive effects of CXCL10 in melanoma, we performed the cell proliferation assay using A375 cells in the presence (þ) or absence (−) of 250 μg/ml CXCL10 recombinant protein in a dose-dependent manner. We noted a significant difference in the graph of RPMI-7951 cells (non-parametric multiple comparison test, P = 0.007 in RPMI-7951 cells and P < 0.001 in G-361 cells) (Fig. 3a).
which CXCL10 were knocked down as representative of relatively high sensitivity to IFN-β. A375 cells in which CXCL10 was knocked down were less sensitive to IFN-β than A375 cells transduced with non-effective shRNA at both 48 and 96 h after treatment (Fig. 3b).

3.7. CXCL10 reduces the invasive capacity of melanoma cells

To evaluate the effects of CXCL10 on the invasive capacity of melanoma cells, a Matrigel invasion assay was performed using A375 cells treated with 0, 50, or 250 μg/ml CXCL10 recombinant protein. The invasive capacity was reduced in treated A375 cells in a dose-dependent manner compared to non-treated A375 cells (Fig. 3c).

3.8. CXCR3-B is up-regulated in cells with high sensitivity to IFN-β and down-regulated in cells with medium to low sensitivity

To elucidate the association between the expression of CXCR3-B, which encodes the CXCL10 receptor, in melanoma cells and the sensitivity to IFN-β, we used RT-PCR to examine CXCR3-B mRNA expression in melanoma cells after treatment with IFN-β. The expression levels in RPMI-7951 and A375 cells, which are highly sensitive to IFN-β, were up-regulated at both 6 and 24 h after treatment compared to non-treated cells. Conversely, the levels in G-361 and SK-MEL-1 cells, which have medium to low sensitivity, were down-regulated at both 6 and 24 h (Fig. 4).

4. Discussion

The present study demonstrated the tumor-suppressive effects of natural-type IFN-β on melanoma cells via CXCL10, which is derived from melanoma cells and the local lymphatic system. Previous reports described that IFN-β treatment induces CXCL10 in metastatic melanoma cells and FBs [11,12] and that CXCL10 reduces melanoma proliferation and invasiveness [10]. The present study demonstrated that (i) IFN-β induces a significantly higher amount of CXCL10 in LECs; (ii) The sensitivity to IFN-β was significantly correlated with the level of CXCL10 induction following IFN-β treatment in melanoma cells; (iii) The sensitivity to CXCL10 was independent of the sensitivity to IFN-β in melanoma cells; (iv) The sensitivity to IFN-β was correlated with the expression of CXCR3-B in melanoma cells. These previously known and novel findings potentially suggested that (i) the sensitivity of melanoma cells to IFN-β is closely correlated with the level of CXCL10 induction by
IFN-β and the CXCR3-B response to IFN-β in melanoma cells, and (ii) IFN-β suppresses lymphatic metastasis by inducing a high amount of CXCL10 from the local lymphatic system.

CXCL10 is a member of the CXC chemokine family and binds to the chemokine (C-X-C motif) receptor (CXCR3) to exert its biological effects [13]. CXCR3 is classified into three splice variants: CXCR3-A, CXCR3-B, and CXCR3-alt. Activation of CXCR3-A induces chemotaxis and proliferation in various cell types, whereas activation of CXCR3-B inhibits cell proliferation and migration and induces apoptosis [13]. Based on these data, CXCR3-B may play critical roles in the tumor-suppressive effects of CXCL10 on melanoma cells. This encouraged us to focus on CXCR3-B expression in melanoma cells treated with IFN-β.

Interestingly, CXCR3-B expression was down-regulated in melanoma cells with medium to low sensitivity to IFN-β. This indicates that a certain type of melanoma is not suppressed by IFN-β treatment, even though IFN-β induces a high amount of CXCL10 from the local lymphatic system and melanoma cells. IFN treatment may have a poor effect on melanoma in some cases, suggesting that CXCR3-B on melanoma cells in such cases may be down-regulated by IFN treatment as shown in the present study. On the other hand, the present study demonstrated that CXCL10 suppressed the proliferation of G-361 cells with medium to low sensitivity to IFN-β as well as RPMI-7951 cells with high sensitivity, suggesting that CXCL10 exerts tumor-suppressive effects on melanoma regardless of the sensitivity to IFN-β. This fact suggests the possibility of choosing local administration of CXCL10 recombinant protein for IFN-β treatment-resistant melanoma cases.

The present study demonstrated that CXCL10 is significantly induced by IFN-β treatment in LECs compared to FBs, although a previous report showed “strong and sustained” CXCL10 induction from FBs following IFN-β treatment [12]. These facts suggest that CXCL10 is induced from LECs by IFN-β treatment in remarkably high amounts. This high amount of CXCL10 from the local lymphatic system following IFN-β treatment may suppress melanoma progression in combination with CXCL10 from melanoma cells when CXCR3-B is up-regulated on melanoma cells. Conversely, CXCL10 secreted from the local lymphatic system and melanoma cells following IFN-β treatment only minimally suppresses melanoma progression when CXCR3-B is down-regulated by IFN-β treatment of melanoma cells.

Our data showed two melanoma cell lines with low sensitivity to IFN-β treatment that were derived from lymphatic system-metastasized lesions (Suppl. Fig. 2), possibly because IFN-β-resistant melanoma cells after IFN treatment may be selected as melanoma cells derived from lymphatic system-metastasized lesions. Marder et al. reported that p53 mutation attenuates the tumor-suppressive effects of IFN-β [14]. Conversely, our data showed that all the melanoma cell lines with high sensitivity to IFN-β treatment have p53 mutation (Suppl. Fig. 2). Although the reason for this discrepancy is not clear, IFN-β may exhibit p53-independent tumor-suppressive effects on melanoma cells, similarly as IFN-α and IFN-γ stimulate a p53-independent apoptotic pathway [15,16].

In the present study, IFN-β treatment was performed with a final concentration of 10^3 IU/ml for LECs, FBs, and melanoma cells. This IFN-β dose was determined based on the fact that 310–4,012 IU/g IFN-β accumulates in regional lymph nodes after local administration of a clinically used dose of IFN-β [9]. Based on these observations, the in vitro conditions of this study are comparable to the in vivo tumor microenvironment of the local lesion treated with IFN-β.

A limitation of the present study is the in vitro conditions. CXCL10 is involved in various biological functions including chemotaxis, apoptosis, and angiostatic effects [17], in addition to regulation of tumor proliferation and invasion. This indicates the need for in vivo research to confirm the roles of CXCL10 in melanoma.

In conclusion, our data suggest that IFN-β may suppress melanoma progression by inducing CXCL10 from the local lymphatic system and melanoma cells. Down-regulation of CXCR3-B by IFN-β may be associated with resistance to IFN-β treatment.

Conflicts of interests

We obtained a grant for clinical research from Toray Medical Co., Ltd.

Transparency document

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Appendix A. Supplementary data

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References

dependent and p53-independent apoptosis, respectively, Oncogene 24 (2005) 605–615.
