Interferon-α enhances CD317 expression and the antitumor activity of anti-CD317 monoclonal antibody in renal cell carcinoma xenograft models

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A murine (mAHM) and a humanized (AHM) monoclonal antibody against CD317 (also called tetherin, BST2, or HM1.24 antigen), expressed preferentially in neoplastic B cells such as multiple myeloma, exhibited antitumor effects as a result of antibody-dependent cellular cytotoxicity (ADCC). The putative interferon (IFN) response elements IRF-1/2 and ISGF3 are present in the promoter of the CD317 gene, and IFN has been used for the treatment of not only myeloproliferative diseases but also solid tumors such as renal cell carcinoma (RCC) and melanoma. Therefore, we examined the effects of IFN on the expression of CD317 and on the antitumor activity of AHM and mAHM in RCC and melanoma. Flow cytometry and in vitro ADCC assays with human or mouse effector cells demonstrated that IFN-α markedly increased the amount of cell surface CD317 and augmented the ADCC activity of mAHM and AHM in RCC cells and to a lesser extent in melanoma cells. Administration of IFN-α to mice bearing RCC xenografts also increased the expression of CD317 in tumor cells. When coadministered with IFN-α, mAHM exhibited more profound antitumor activity in both IFN-α-sensitive and -insensitive RCC xenograft models. Thus, AHM in combination with IFN-α may be an effective therapy for the treatment of RCC. (Cancer Sci 2008; 99: 2461–2466)

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D317 (also referred to as tetherin, BST2, or HM1.24 antigen) is a type II transmembrane glycoprotein with a molecular mass of 29–33 kDa.11 It is expressed highly in terminally differentiated normal plasmacytoid dendritic cells and neoplastic B cells such as multiple myeloma (MM).15 Until recently, the physiological roles of CD317 were unknown; however, CD317 has been identified as the molecule tetherin, which inhibits the release of human immunodeficiency virus-1 particles in the presence of the accessory protein Vpu.15 In an attempt to provide a new treatment option for MM, we generated a humanized monoclonal antibody against CD317 (AHM) from a mouse monoclonal antibody (mAHM) by using complementarity determining region grafting.4 AHM and mAHM showed significant tumor growth inhibition and prolonged survival in human MM xenograft models, and the antitumor effects of AHM and mAHM were largely mediated by natural killer (NK) cell- and monocyte- and macrophage-mediated antibody-dependent cellular cytotoxicity (ADCC).5,6 In clinical trials with MM patients, administration of AHM was found to be safe with few adverse events.7

Several cis-elements for transcription factors such as AP-2, CREB, SP1, STAT3, IRF-1/2, and ISGF3 are present in the promoter region of the CD317 gene.16 Interestingly, there is a tandem repeat containing three STAT3 binding motifs between –146 and –126 from the transcription initiation site, which also contains the interferon (IFN) response elements IRF-1/2 and ISGF3, suggesting that CD317 expression is also mediated by IFN.

Interferon has been used to treat not only myeloproliferative diseases but also solid tumors such as renal cell carcinoma (RCC) and melanoma. In 2003, more than 31 000 people were diagnosed as having RCC and 12 000 died from the disease in the USA.8 Early stage RCC is treated effectively by radical nephrectomy, but the disease recurs in 30% of patients who have undergone nephrectomy.9 In addition, 30–50% of RCC patients are not diagnosed until the inoperable advanced stage,10 and so are treated with combined chemotherapy and immunotherapy with cytokines such as interleukin (IL)-2 or IFN-α. However, existing therapeutic agents have only limited efficacy with response rates between 10 and 20% and a 5-year survival of 10%.9,10 Melanoma also has a poor prognosis. Development of malignant melanoma has been reported to be approximately 1 in 75 in the USA, and the 5-year survival of advanced stage (IV) melanoma patients is only 5–10%.10,11 Dacarbazine, IFN-α, and IL-2 have been used for the treatment of advanced metastatic melanoma; however, none of them have improved survival significantly.12–14

In the present study, we examined whether mAHM or AHM is efficacious against RCC and melanoma. We show that IFN-α strongly increased the expression of CD317 and in vitro ADCC activity in RCC and, to a lesser extent, in melanoma cell lines. In addition, antitumor activity in vivo from mAHM or AHM was significantly augmented by IFN-α in RCC cells. The results support the opinion that AHM can be used for the treatment of RCC, and possibly melanoma, in combination with IFN-α.

Materials and Methods

Antibodies. mAHM (mouse IgG2ak) and AHM (human IgG1κ) were prepared as described previously:5 Mouse IgG2ak (mlgG2a; Becton Dickinson, Franklin Lakes, NJ, USA) and human IgG1κ (hlgG1; Sigma, St Louis, MO, USA) were used as control antibodies. Fluorescein isothiocyanate (FITC)-labeled AHM (AHM-FITC) and hlgG1 (hlgG1-FITC) were prepared by incubating AHM and hlgG1 with N-hydroxysuccinimide (NHS)-fluorescein (Pierce, Rockford, IL, USA) and then by gel filtration with PD-10 Sephadex G-25 (GE Healthcare Bio-Sciences, Uppsala, Sweden).16

Cells. Caki-1, A2058, and C32TG were provided by the Human Science Research Resources Bank (Osaka, Japan), ACH was

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purchased from Dainippon Sumitomo Pharma (Osaka, Japan), SEKI was purchased from Immuno-Biological Laboratories (Gunma, Japan), A498 and 769-P were gifted by Dr Y. Yokohama City University, Kanagawa, Japan), and KPK1, KPK13, SN12C, and KG2P were gifted by Dr Naito (Kyushu University, Fukuoka, Japan). Other human tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA).

Peripheral blood mononuclear cells (PBMC) were prepared from the peripheral blood of healthy volunteers using Ficoll-Paque PLUS gradient centrifugation (GE Healthcare Bio-Sciences). Then suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, the designated standard medium in the present study. Bone marrow cells obtained from nude mice (BALB/cAnNCrl-nu/nu; Charles River Laboratories, Kanagawa, Japan) were cultured for 5 days in standard medium supplemented with 50 ng/mL recombinant human IL-2 (Peprotech, Rocky Hill, NJ, USA) and 10 ng/mL recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech). Bone marrow cells treated with IL-2 and GM-CSF, which were a mixture of NK cells and macrophages with potential cell-killing activity, were designated as the bone marrow-derived lymphokine-activated killer cells (BM-LAK) in this study.

Flow cytometric analysis. Expression of CD317 on the cell surface was analyzed by flow cytometry. Cells were cultured for 3 days with or without 1000 U/mL IFN-α (pegylated IFN-α2b; Peg-Intron, Schering-plough, Osaka, Japan) and detached from the flask using Cell Dissociation Buffer (Invitrogen, Carlsbad, CA, USA). Approximately 500 000 cells were then incubated with 40 μg/mL AHM-FITC or hIgG1-FITC on ice for 30 min in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.1% sodium azide (FACS–PBS). After the cells were washed twice with FACS–PBS, they were analyzed by flow cytometry (EPICS XL; Beckman Coulter, Tokyo, Japan). The mean fluorescence intensity was calculated using CellQuest software (Becton Dickinson), and the amount of CD317 on the cell surface was quantified as described previously.(16)

Determination of antibody-dependent cellular toxicity activity. The ADCC activity of mAHM and AHM was determined by 51Cr-release assay. After cells were cultured for 3 days with or without 1000 U/mL IFN-α, 1 × 10^6 cells were labeled with 5.55 MBq 51Cr-sodium chromate (GE Healthcare Bio-Sciences) at 37°C for 1 h. The cells were then washed three times with standard medium and suspended in standard medium. The 51Cr-labeled cells were seeded on 96-well round-bottomed plates (10^4 cells/well) and incubated with mAHM, AHM, or control antibody on ice for 15 min. Human PBMC or mouse BM-LAK (5 × 10^4 cells/well) were added to the cells, and the cells were further incubated at 37°C for 4 h (A498 or SN12C) or 6 h (A2058 or SEKI). One hundred microliters of the supernatant from each well was collected and the radioactivity in the supernatant was measured using a gamma counter. Radioactivity of the wells without effector cells was considered spontaneous 51Cr release, and that of the wells incubated with detergent (1% Nonidet P-40) was considered maximum 51Cr release. Cytotoxicity (%) was determined using the formula (a – c)/(b – c) × 100, where a is the 51Cr release in each experiment, b is the maximum 51Cr release, and c is the spontaneous 51Cr release. ADCC activity was calculated by subtracting the cytotoxicity (%) of the wells without antibody from those with antibody. All experiments were done in triplicate.

Determination of in vivo antitumor activity. Five-week-old male nude and SCID mice (C.B-17/crl-nude/scid/scid; Clea Japan, Tokyo, Japan) were maintained in a specific pathogen-free facility. To eradicate NK cells, mice were injected intraperitoneally with 20 μL rabbit antiasialo GM1 antiserum (Wako Pure Chemical Industries, Osaka, Japan) 1 day before tumor inoculation. A498 cells (2 × 10^6 cells) or SN12C cells (3 × 10^6 cells) were mixed with Matrigel (Becton Dickinson) and injected subcutaneously into nude or SCID mice, respectively. When the average tumor volume reached between 160 and 170 mm^3, the mice were divided into groups consisting of seven or eight animals. Day 0 represents the day when mice were randomized and treatment was initiated. mAHM (150 μg/mouse) or AHM (400 μg/mouse) was administered intravenously twice a week for 4 weeks. Control mice received mIgG2a (150 μg/mouse) or PBS. IFN-α (30 000 U/mouse for A498 and 100 000 U/mouse for SN12C) or saline was administered intraperitoneally once a week for 4 weeks. All mice were killed on either day 29 (SN12C) or day 33 (A498).

Tumor volume was calculated using the formula \( a^{2} b/2 \) (mm^3) where \( a \) and \( b \) are the longest and shortest diameter, respectively. The animals used in the experiments were treated in accordance with the ethical guidelines promulgated by Chugai Pharmaceutical for animal care, handling, and termination.

Immunohistochemical analysis. Mice inoculated subcutaneously with A498 or SN12C were administered 30 000 U IFN-α/mouse (for A498-bearing mice) or 100 000 U IFN-α/mouse (for SN12C-bearing mice). Xenografts were resected on day 0, 1, or 3. Sections were prepared on a cryostat from frozen tumor tissues embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), fixed in 4% parafformaldehyde for 30 min, and incubated with metanol containing 0.3% H2O2 at room temperature for 30 min to quench endogenous peroxidase. The slides were incubated with 1.1 μg/mL AHM for 16 h, and incubated with 2.2 μg/mL biotinylated goat antihuman IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 30 min followed by incubation with streptavidin-horseradish peroxidase (Dako Cytomations, Carpinteria, CA, USA). CD317 was visualized by incubating the specimens in a buffer containing 50 mmol/L Tris-HCl (pH 7.6), 0.02% 3,3′-diaminobenzidine tetrahydrochloride, and 0.005% H2O2. The sections were also counterstained with hematoxylin.

Statistical analysis. The statistical significance in tumor volume was determined using Dunnett’s test. Differences with P-values < 0.05 were considered significant.

Results

Increase in the expression of CD317 in renal cell carcinoma and melanoma cells from interferon-α. IFN-α has been used for the treatment of RCC and melanoma, and therefore we explored the effect of IFN-α on CD317 expression in RCC and melanoma cells. Quantitative flow cytometric analysis revealed that without IFN-α treatment, only 6 out of 10 RCC cell lines expressed CD317, and the other four RCC cell lines and all four melanoma cell lines were negative for CD317 (Table 1). In addition, the amount of CD317 on the cell surface was mainly in the order of 10^4 molecules per cell, an amount insufficient to elicit strong ADCC by AHM.(8) The amount of CD317 on the surface of the RCC cell lines A498 and SN12C cultured without IFN-α was 3.0 × 10^4 molecules per cell and 4.9 × 10^4 molecules per cell, respectively. IFN-α increased the amount of cell surface CD317 in a dose-dependent manner. After the addition of 1000 U/mL IFN-α to the cells, the amount of CD317 on A498 and SN12C cells increased to 2.6 × 10^5 and 1.6 × 10^5 molecules per cell, respectively (Fig. 1). An increase in the amount of CD317 from IFN-α was also observed in other RCC and melanoma cell lines (Table 1). Even in the A2058 and SEKI melanoma cells that expressed only an undetectable level of CD317, 1000 U/mL IFN-α increased the cell surface amount of CD317 to a detectable level (1.6 × 10^4 for A2058 and 5.6 × 10^4 for SEKI). However, sensitivity to IFN-α was much lower than for A498 and SN12C and lower concentrations of IFN-α did not significantly increase the amount of CD317 in A2058 or SEKI melanoma cells (Fig. 1).
Augmentation of the antitumor activity of a humanized monoclonal antibody against CD317 in combination with interferon-α. AHM induced ADCC and showed antitumor activity against MM in mouse xenograft models. Because ADCC from AHM tended to correlate with the amount of cell surface CD317, IFN-α was expected to augment ADCC activity induced by mAHM and AHM. As shown in Figure 2, IFN-α treatment of A498 and SN12C cells markedly augmented the ADCC activity from AHM in the presence of human PBMC. At the same antibody concentration, ADCC activity was significantly higher against A498 and SN12C cells treated with IFN-α than against cells not treated with IFN-α (Fig. 2a,c).

Table 1. CD317 upregulation on renal cell carcinoma (RCC) and melanoma cell lines by interferon (IFN)-α

| Cell line | MFI | Medium | CD317(molecules/cell) | iFN-α | CD317(molecules/cell) |
|---|---|---|---|---|---|
| | AHM-FITC | hIgG1-FITC | | AHM-FITC | hIgG1-FITC |
| RCC | | | | | |
| KPK13 | 115 | 10 | 1.1 × 10^5 | 567 | 13 | 5.4 × 10^5 |
| 786-O | 171 | 17 | 1.6 × 10^3 | 412 | 22 | 3.9 × 10^3 |
| Caki-1 | 75 | 12 | 7.1 × 10^4 | 383 | 13 | 3.7 × 10^3 |
| A498 | 36 | 11 | 3.0 × 10^4 | 267 | 19 | 2.6 × 10^3 |
| SN12C | 46 | 3 | 4.9 × 10^4 | 150 | 3 | 1.6 × 10^3 |
| KG2F | 46 | 11 | 4.1 × 10^4 | 117 | 13 | 1.1 × 10^4 |
| 769-P | 12 | 13 | – | 95 | 13 | 9.0 × 10^3 |
| KPK1 | 11 | 11 | – | 89 | 12 | 8.6 × 10^4 |
| Caki-2 | 8 | 6 | – | 9 | 6 | – |
| ACHN | 5 | 5 | – | 11 | 7 | – |
| Melanoma | | | | | |
| A2058 | 53 | 43 | – | 528 | 58 | 1.6 × 10^5 |
| LOX IMV1 | 36 | 34 | – | 435 | 40 | 1.3 × 10^5 |
| SEKl | 77 | 60 | – | 247 | 92 | 5.6 × 10^4 |
| C32TG | 51 | 39 | – | 172 | 43 | 4.8 × 10^4 |
| G361 | 60 | 61 | – | 91 | 71 | – |

RCC and melanoma cells were cultured for 3 days with or without 1000 U/mL IFN-α, stained with AHM-FITC or hIgG1-FITC, and analyzed by flow cytometry. The amount of cell surface CD317 was calculated from the mean fluorescence intensity (MFI). *Cell surface CD317 was under a detectable level.

Fig. 1. Effect of interferon (IFN)-α on CD317 expression in A498, SN12C, A2058, and SEKI cells. (a) A498 and (b) SN12C renal cell carcinoma (RCC) cells were cultured for 3 days in the absence (black) or presence of IFN-α at 1 U/mL (blue), 10 U/mL (purple), 100 U/mL (orange), or 1000 U/mL (red). (c) A2058 and (d) SEKI melanoma cells were cultured for 3 days in the absence (black) or presence (red) of 1000 U/mL IFN-α. Thereafter, the cells were stained with AHM-FITC (solid line) or control hIgG1-FITC (dotted line) and analyzed by flow cytometry.
ADCC from AHM was also stronger when the cells were treated with IFN-α. Essentially the same results were obtained when mouse BM-LAK were used as the effector cells or with mAHM instead of AHM (Fig. 2b,d). IFN-α also enhanced the AHM-mediated ADCC activity in A2058 and SEKI melanoma cells, but the degree of enhancement by IFN-α was much less compared to that of A498 and SN12C (Fig. 2e–h).
The results that IFN-α markedly augmented the ADCC activity of both AHM and mAHM against A498 and SN12C cells even in the presence of mouse effector cells suggest that IFN-α augments the antitumor activity of AHM and mAHM in mouse xenograft models. To address this question, we first examined whether IFN-α increases the expression of CD317 in RCC cells in vivo. We used A498 and SN12C cells for the analyses because they were tumorigenic in nude and SCID mice, and also because of the higher responsiveness to IFN-α in the upregulation of CD317 and the larger degree of ADCC enhancement by IFN-α as compared to A2058 and SEKI melanoma. A498 or SN12C xenografts were taken from the mice 1 and 3 days after the administration of IFN-α, and the level of CD317 was determined by immunohistochemistry. As shown in Figure 3, the intensity of CD317 staining in the A498 and SN12C xenografts was significantly stronger even 1 day after the administration of IFN-α.

To examine the effects of IFN-α on the antitumor activity of AHM, we used mAHM because the ADCC induced by mAHM was stronger than that induced by AHM when mouse BM-LAK was used for the effector cells. A498 appeared to be an IFN-α-sensitive and mAHM-insensitive xenograft (Fig. 4a). IFN-α alone inhibited the growth of the A498 xenograft, whereas mAHM had an insignificant effect on the growth of the A498 xenograft. However, administration of IFN-α and mAHM led to more profound tumor growth inhibition than IFN-α alone. In contrast, the SN12C xenograft was IFN-α-insensitive and mAHM-sensitive (Fig. 4b). Although mAHM alone inhibited the growth of SN12C, the antitumor effect of mAHM was more profound when mAHM was administered with IFN-α. We also examined whether IFN-α augments the antitumor activity of AHM, the humanized antibody, in the SN12C xenograft model. The efficacy of AHM alone was weaker than that of mAHM, but significant antitumor activity was observed when AHM was administered in combination with IFN-α (Fig. 4c).

**Discussion**

CD317 has demonstrated higher levels of expression in MM cells than in normal tissues and solid tumors. With the exception of KP-K13 and 786-O, the RCC and melanoma cell lines examined in the present study expressed less than 10^5 molecules per cell or were negative for CD317. However, IFN-α clearly increased the expression of CD317 in 8 out of 10 RCC and melanoma cell lines examined.

The antitumor activity of mAHM and AHM is largely attributable to ADCC and tends to correlate with the amount of cell surface CD317; therefore, increased levels of CD317 would make tumor cells more susceptible to the antibodies. Indeed, IFN-α augmented the ADCC activity and antitumor activity from mAHM or AHM in both IFN-sensitive and IFN-insensitive xenograft models. AHM and mAHM caused complement-dependent cytotoxicity (CDC) in the presence of baby rabbit complement but not when human serum was used as the source of the complement. Consistent with previous results, neither AHM nor mAHM...
mediated CDC in the presence of human or mouse serum even after the target cells were treated with IFN-α, which suggests that CDC-mediated antitumor activity is unlikely in mouse xenograft models and also in humans.

In the present study, A498 and SN12C xenografts were used for the in vivo experiments because they are highly tumorigenic in nude and SCID mice and also because of the strong enhancement of ADCC by IFN-α. Augmentation of the antitumor activity induced by mAHM or AHM from IFN-α is expected to occur in other xenograft models if they are tumorigenic in immuno-deficient mice and are highly responsive to IFN-α in the upregulation of CD317. As mentioned, IFN-α has been used for the treatment of RCC and melanoma in clinical settings, but its efficacy is not satisfactory. Because the combination of IFN-α and AHM showed significant antitumor activity even in an IFN-insensitive RCC xenograft, it will be a new treatment option for RCC. ADCC enhancement by IFN-α was more pronounced in SN12C and A498 RCC cells than in A2058 melanoma cells, even though they expressed similar amounts of cell surface CD317 (1.6–2.6 × 10^4). Preliminary experiments did not clearly demonstrate the additive or synergistic effects of IFN-α or mAHM in A2058 and SEKI melanoma models. Differences in the responsiveness of RCC cells and melanoma cells to IFN-α with regard to ADCC enhancement remain to be studied. AHM was safely administered to patients with MM in phase I and II clinical trials. The level of CD317 on the surface of normal lymphocytes, monocytes, and granulocytes was less than 10^5 per cell even after the cells were treated with IFN-α. In addition, the CD317 level in normal lymphocytes and myelocytes of patients treated with IFN-α was approximately 3000 per cell, and immunohistochemistry did not detect CD317 in normal kidney and skin. Nevertheless, adverse events in combination settings have to be evaluated carefully because, although at low levels, CD317 has been detected in some normal cells such as endothelial cells.

In addition, IFN-α stimulates the immune response. A chimeric monoclonal antibody (WX-G250) that recognizes the membrane antigen present on RCC (CA-IX<sup>MNNG250</sup>) was shown to induce ADCC against RCC cell lines, and ADCC from WX-G250 was enhanced when the effector cells were pretreated with IFN-α. Thus, immunological activation of effector cells by IFN-α may also occur in RCC and melanoma patients who undergo IFN-α therapy. The activation of effector cells may also contribute to the augmentation of the antitumor activity of AHM when AHM is used in combination with IFN-α.

The mechanism underlying the upregulation of CD317 by IFN-α has not been established. The presence of STAT3 binding motifs, IRF-1/2, and ISGF3 suggests that IFN-α induces CD317 expression at a transcriptional level. Our preliminary experiments indicate that at least IRF-2 is involved in the IFN-α-induced transcriptional activation of the CD317 gene. However, Ge et al. demonstrated that GATA1 binds to and stimulates transcription from the CD317 gene promoter and that there is an increase in the expression of CD317 in leukemic cells protected from cytotoxic apoptosis (ara-C)-induced apoptosis, presumably due to the enhanced bone marrow stromal interactions. Thus, multiple factors seem to be involved in the expression of CD317 and increasing levels of CD317 may cause a chemoresistant phenotype. Nevertheless, the combination of AHM and IFN-α is still a feasible treatment option for RCC and possibly melanoma because both often become recurrent during chemotherapy.

In conclusion, IFN-α increased the expression of CD317 in RCC and melanoma cells, which resulted in an increase in the in vitro ADCC activity of both AHM and mAHM. The antitumor activities of AHM and mAHM were augmented both in IFN-sensitive and -insensitive xenograft models when combined with IFN-α. Thus, the combination of AHM and IFN-α will be an effective therapy for RCC and melanoma.

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