Endogenous Interleukin-18 Modulates Immune Escape of Murine Melanoma Cells by Regulating the Expression of Fas Ligand and Reactive Oxygen Intermediates

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ABSTRACT

It has been known that melanoma cells can suppress the immune system by the Fas ligand. The present study investigated whether interleukin (IL)-18, which can enhance Fas ligand expression, is produced by B16F10 melanoma cells and is involved in immune escape of tumor cells. Immunohistology, reverse transcription-PCR, intracellular fluorescence-activated cell-sorting analysis, and immunoblotting demonstrated that melanoma cells express IL-18. C57BL/6 splenocytes cultured with culture supernatants of B16F10 melanoma cells enhanced IFN-γ production, which was blocked by anti-IL-18 antibody, indicating that IL-18 in the culture supernatants is functional. In addition to IL-18, the IL-18 receptor was also detected in B16F10 melanoma cells, suggesting a role of this cytokine in regulating the functions of B16F10 melanoma cells. The functional effect of IL-18 on B16F10 melanoma cells was shown by reduction of Fas ligand expression in cells treated with anti-IL-18 antibody or transfected with IL-18 antisense cDNA. In addition, the same treatments decreased intracellular reactive oxygen intermediate levels in B16F10 melanoma cells, indicating that IL-18 regulates reactive oxygen intermediate production, which is involved in Fas ligand expression. Furthermore, transfection of IL-18 antisense cDNA into melanoma cells increased the susceptibility of tumor cells to natural killer cell lysis in vitro. When IL-18 antisense transfectedants were implanted into syngeneic mice, severe reduction of tumor cell growth was observed with concomitant infiltrated natural killer cells in the tumor area. Taken together, these results demonstrate that IL-18 has a critical role as a survival factor for B16F10 melanoma cells.

INTRODUCTION

Malignant melanoma leads to severely increased mortality, and the rate of incidence is higher than any other cancer. It has been suggested that melanoma may escape immune surveillance through several possible mechanisms, including immune suppression by soluble inhibitory factors such as IL-10, or by down-regulation of MHC molecule expression (1–3). Recently, Fas ligand-mediated immune suppression was proposed in melanoma (4). According to the authors, melanoma cells express Fas ligand to protect themselves against suppression was proposed in melanoma (4). According to the authors, melanoma cells express Fas ligand to protect themselves against suppression was proposed in melanoma (4). According to the authors, melanoma cells express Fas ligand to protect themselves against suppression was proposed in melanoma (4). According to the authors, melanoma cells express Fas ligand to protect themselves against suppression was proposed in melanoma (4). According to the authors, melanoma cells express Fas ligand to protect themselves against suppression was proposed in melanoma (4). According to the authors, melanoma cells express Fas ligand to protect themselves against

MATERIALS AND METHODS

Cells and Animals. C57BL/6 mice splenocytes were prepared using a standard protocol (19). Briefly, mice were killed by cervical dislocation, and splenocytes were prepared by mechanical disruption. The murine melanoma cell lines B16F0, B16F1, and B16F10 were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum, hereafter referred to as CM. These cell lines were used for experiments while in the log phase of growth. C57BL/6 mice were bred in the animal facility of the Korea Research Institute of Bioscience and Biotechnology and used at 6–8 weeks of age.

IL-18 Bioassay (IFN-γ Induction Assay). The induction of IFN-γ from splenocytes by IL-18 was assayed to detect IL-18 bioactivity. Briefly, C57BL/6 mice splenocytes were prepared using standard protocols. Prepared splenocytes were suspended in CM at a concentration of 5 × 10^5 cells/ml. One ml of splenocytes (5 × 10^5 cells) was plated in each well of a 24-well plate, followed by addition of 1 ml of the culture supernatants in triplicate. After 72 h of incubation, cell-free supernatants were assayed for IFN-γ production using a murine IFN-γ ELISA kit (Endogen, Inc., Cambridge, MA).
experiments, culture supernatants of melanoma cells were pre-incubated for 2 h with polyclonal antisense IL-18 antibody (20 μg/ml) or an isotope control (20 μg/ml) before the IL-18 bioassay was performed.

**RT-PCR.** Total RNA was extracted from B16F10 melanoma cells using RNAzol, according to the instructions of the manufacturer. After reverse transcription, the cDNA was incubated with IL-18 primers (sense, 5′-ACTG-TACAACCGCACAGATAATACGG-3′; antisense, 5′-AGTGAAATTACGATTATCCC-3′) or IL-18 receptor primers (sense, 5′-TCTGGAGAGAAGAAGTCATT-3′) and reverse transcriptase. The cDNA was incubated with IL-18 primers (sense, 5′-ACTG-TACAACCGCACAGATAATACGG-3′; antisense, 5′-CGCTGAAACTCCTGAAGTCC-3′) for PCR amplification. Cycling conditions for IL-18 were 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 35 cycles; conditions for IL-18 receptor were 1 min at 95°C, 0.5 min at 58°C, and 1 min at 72°C.

**Flow Cytometry Analysis.** Intracellular FACS analysis was performed to detect IL-18 or Fas ligand in melanoma cell lines. Briefly, cells were washed twice with ice-cold PBS containing 0.05% BSA and 0.02% sodium azide. After two washes, cells were fixed in 2% paraformaldehyde in PBS for 15 min on ice. Thereafter, the cells were washed once in cold PBS-BSA and resuspended in PBS containing 0.1% saponin and 0.05% sodium azide (permeabilization buffer) for 15 min, followed by incubation with rabbit anti-IL-18 polyclonal antibody or antismouse Fas ligand antibody (PharMingen, San Diego, CA) for 30 min on ice. After two washes, cells were further incubated with an appropriate FITC-conjugated secondary antibody in permeabilization buffer for 30 min on ice, followed by three washes. A FACScan (Becton Dickinson, Sunnyvale, CA) flow cytometer was used for analysis. For ROI assays, melanoma cells (1 × 10⁶ cells/ml) were incubated with 50 μM 2′,7′-dichlorofluorescein diacetate (Eastman Kodak Co., Rochester, NY) for 5 min at 37°C. After incubation, cells were analyzed using a FACScan flow cytometer.

**Transfection of IL-18 Antisense cDNA.** Murine IL-18 cDNA was cloned into pcDNA3.1 vector by blunt end ligation. The orientation of inserts was confirmed by AvaI digestion. B16F10 melanoma cells were transfected with the IL-18 antisense construct by the calcium phosphate precipitation method. The stable transfectant clones were selected in the CM containing 1.4 mg/ml

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**Table 1.** Effect of B16F10 culture supernatants on IFN-γ production from C57BL/6 splenocytes

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>IFN-γ (pg/ml)</th>
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<tbody>
<tr>
<td>B16F10 c.s.</td>
<td>153.0 ± 25.1</td>
</tr>
<tr>
<td>C57BL/6 sp.</td>
<td>1221.0 ± 68.7</td>
</tr>
<tr>
<td>rIL-18 (100 ng/ml) + C57BL/6 sp.</td>
<td>13347.0 ± 124.1</td>
</tr>
<tr>
<td>B16F10 c.s. + C57BL/6 sp.</td>
<td>11145.0 ± 432.5</td>
</tr>
<tr>
<td>B16F10 c.s. + C57BL/6 sp. + Rabbit Ig (20 μg/ml)</td>
<td>11560.0 ± 753.2</td>
</tr>
<tr>
<td>B16F10 c.s. + C57BL/6 sp. + anti-IL-18 (5 μg/ml)</td>
<td>9245.0 ± 213.5</td>
</tr>
<tr>
<td>B16F10 c.s. + C57BL/6 sp. + anti-IL-18 (10 μg/ml)</td>
<td>7755.0 ± 322.7</td>
</tr>
<tr>
<td>B16F10 c.s. + C57BL/6 sp. + anti-IL-18 (20 μg/ml)</td>
<td>4197.0 ± 195.6</td>
</tr>
</tbody>
</table>

* c.s., culture supernatant; sp., splenocytes; Ig, immunoglobulin.
neomycin (G-418, Geneticin; Life Technologies, Inc.). IL-18 expression was confirmed by intracellular staining and immunoblotting for IL-18.

**BLT Esterase Release.** After incubation of NK cell and melanoma cells, 50 μl of cell-free supernatant were collected and mixed with 150 μl of the reaction mixture [0.2 mM BLT and 0.22 mM 5,5'-dithiobis(2-nitrobenzoic acid) in PBS] in flat-bottomed 96-well plates. After a 20-min reaction at 37°C, the absorbance was read at 412 nm in an ELISA plate reader. The percentage of BLT esterase activity was calculated using the following equation: (Experimental BLT esterase release − spontaneous BLT esterase release)/(maximum BLT esterase release) × 100. Spontaneous BLT esterase release from NK cells was measured in the well without antibody, and maximum BLT esterase release was measured after cell lysis with 1% Triton X-100.

**In Vivo Tumor Model.** C57BL/6 male mice 6 weeks of age were obtained from the Laboratory Animal Division of KRIBB. The B16F10 mouse melanoma tumors were established by s.c. injection of 1 × 10^5 cells in 200 μl of PBS. Tumor size was determined by measuring with calipers every 2 days, and the values were inserted into the formula: Tumor size (cm^2) = 0.5 × (largest diameter) × (smallest diameter)^2.

**Immunohistochemistry.** The tissue samples were immersed in 25% sucrose-PBS solution for frozen sections. Each tissue was embedded in OCT compound, quick-frozen in isopentane cooled by liquid nitrogen, and sectioned into 8-μm thicknesses using a cryostat (Leica CM5060). Cryosections were dried in air and fixed in cold acetone at 4°C. These specimens were incubated with biotinylated anti-IL-18 or mouse NK cell antibody (NK1.1; PharMingen) for 1 h at 37°C in a wet chamber. Subsequently, the specimens were incubated with alkaline phosphatase-conjugated streptavidin complex. The activity was visualized by incubation with substrate solution. All specimens were counterstained with methyl green.

**RESULTS**

Detection of IL-18 mRNA and Protein in Melanoma Cells. When tissue samples from melanoma patients were immunostained with anti-IL-18 antibody, dominant IL-18 expression was observed in tumor sites (Fig. 1A). To examine whether murine melanoma cell lines were also able to express IL-18 protein, intracellular staining of IL-18 using FACSscan analysis was performed. Fig. 1B shows that B16F10 melanoma cells produced IL-18 protein. Three different subclones of the B16 melanoma cell lines, B16F0, B16F1, and B16F10, produced IL-18 protein, but there was no difference in the amount of IL-18 protein between subclones. In addition, constitutive expression of IL-18 mRNA was detected in B16F10 murine mela-
noma cells (Fig. 1C). IL-18 protein was also detected by immuno-
blotting with anti-IL-18 antibody (Fig. 1D). Collectively, these data
demonstrate that melanoma cells express IL-18 mRNA and protein.

Secretion of Functional IL-18 by Murine Melanoma Cells. To
test the production of functional IL-18 protein by murine melanoma
cell lines, culture supernatants of B16F10 murine melanoma cell lines
were harvested and examined for their ability to induce IFN-γ secre-
tion from splenocytes of C57BL/6 mice. Conditioned medium
collected from untreated C57BL/6 splenocytes induced only a slight
secretion of IFN-γ, whereas conditioned medium collected from
treated C57BL/6 splenocytes induced significant secretion of IFN-γ
(Table 1). To confirm that the observed enhancement of IFN-γ secre-
tion was specifically due to IL-18, neutralizing anti-IL-18 antibody
was used. Table 1 demonstrates that neutralizing antibody significa-
cantly abolished the enhancement of IFN-γ secretion in a dose-
dependent manner. Thus, these data demonstrate that murine mela-
noma cells secrete functional IL-18. In addition, expression of IL-18
receptor in murine melanoma cells was tested. RT-PCR revealed that
B16F10 murine melanoma cells express IL-18 receptor (Fig. 1A).
These results suggest that IL-18 produced by murine melanoma cells
can regulate the physiological functions of tumor cells.

Effects of IL-18 Antisense cDNA and Anti-IL-18 Antibody on
Expression of Fas Ligand in Melanoma Cells. It has been reported
that IL-18 up-regulates Fas ligand expression in NK cells (10) and in
myelomonocytic KG-1 cells (20). Melanoma cells express Fas ligand,
which is involved in tumor immune escape (4). To identify the
possible roles of IL-18 in regulating Fas ligand expression and sur-
vival of melanoma cells, we established a B16F10 clone that ex-
pressed a lower level of IL-18 by transfection with IL-18 antisense
plasmid (Fig. 2). FACS (Fig. 2A) and immunoblot (Fig. 2B) analysis
showed that IL-18 expression was reduced in IL-18 antisense trans-
flectants. MHC class I (Fig. 2C) and intercellular adhesion molecule-1
(Fig. 2D) expression were not altered by transfection with the IL-18
antisense construct, but interestingly CD71 (transferrin receptor),
which is involved in transferrin uptake and target cell sensitivity to
NK cells, was up-regulated (Fig. 2E).

When Fas ligand expression was analyzed by intracellular FACS
analysis, its expression was reduced in IL-18 antisense transfectants
compared with vector transfectants (Fig. 3A). In the same line, treat-
ment of B16F10 melanoma cells with anti-IL-18 antibody reduced Fas
ligand expression (Fig. 3B), indicating that IL-18 is an important
factor for regulating Fas ligand expression in B16F10 melanoma cells.
Recent reports (17, 18) indicate that Fas ligand expression is regulated
by ROIs. When B16F10 melanoma cells were treated with an anti-
oxidant, NAC, Fas ligand expression was reduced (Fig. 3C), sug-
gesting that ROIs are also involved in Fas ligand expression in melanoma
cells.

Effects of IL-18 Antisense cDNA and Anti-IL-18 Antibody on
ROI Production in Melanoma Cells. In addition to the regulatory
roles of ROIs in Fas ligand expression, it is known that some tumor
cells enhance ROI generation that is beneficial for their survival
(15) compared with their normal counterparts. ROI levels are
raised in melanoma and play a key role in resistance to Fas-
induced apoptosis in melanoma cells (16). We next asked whether
IL-18 could regulate ROI production in melanoma cells. Transfec-
tion with IL-18 antisense cDNA reduced the intracellular ROI level
compared with control cells (Fig. 4A). In addition, B16F10 mela-
noma cells were incubated with anti-IL-18 polyclonal antibody for
18 h, and ROI levels were analyzed (Fig. 4B). Anti-IL-18 antibody
reduced ROI levels in B16F10 melanoma cells, confirming that
IL-18 is critical for regulating ROI expression in B16F10 mela-
noma cells. When the cells were treated with NAC for ≈12 h, the
survival rate was prolonged in mice injected with IL-18 antisense transfectants compared with those injected with vector transfectants (Fig. 6B). In immunohistological analysis by staining with anti-NK cell antibody, infiltrated NK cells were observed in the tumor sites of mice injected with IL-18 antisense transfectants (Fig. 6C-b), but not in the mice injected with vector transfectants (Fig. 6C-a). These in vitro and in vivo data indicate that expression of IL-18 is a key element for protecting melanoma cells from host immune cells.

DISCUSSION

Malignant transformation of melanocytes is characterized by the loss of MHC class I molecules by melanoma cells, and this abnormality may account for the immune escape of malignant melanoma cells from CTL-mediated immune surveillance (3). In addition to this mechanism, Hahne et al. (4) suggested that Fas ligand-expressing melanoma cells use Fas ligand to induce apoptosis of Fas-expressing immune cells at the tumor site, and this strategy may be a general mechanism responsible for immune privilege used by tumor cells. In vivo, this may be an explanation of why malignant melanoma cells are not susceptible to NK-cell-mediated immune surveillance although they lose the expression of MHC class I molecules.

IL-18 is a recently cloned cytokine that was primarily identified by its ability to induce IFN-γ production (5). Tsutsui et al. (10) and Dao et al. (21) additionally showed that this cytokine could enhance Fas ligand expression in T-helper 1 and NK cells. We therefore examined the production of IL-18 to investigate whether this cytokine is the regulatory factor of Fas ligand expression in melanoma cells. The present study indicated that murine melanoma cell lines produce IL-18 and express IL-18 receptors (Fig. 1). Culture supernatants derived from murine melanoma cell lines induced IFN-γ secretion by C57BL/6 splenocytes (Table 1). Blocking experiments with anti-IL-18 polyclonal antibodies showed that IFN-γ production was not totally inhibited by anti-IL-18 polyclonal antibodies. It is well known that melanoma cell
lines produce IL-2 and that release of IFN-γ by splenocytes can be caused by IL-2 (22). Therefore, IL-2 might be responsible for the IFN-γ production activity found in melanoma culture supernatants after treatment with anti-IL-18 polyclonal antibodies. We performed a proliferation assay to test whether IL-18 is involved in the regulation of B16F10 cell proliferation. The data indicated that IL-18 does not affect B16F10 cell proliferation (data not shown). Because functional IL-18 protein and IL-18 receptor were expressed in B16F10 melanoma cells, we were interested in investigating whether IL-18 acts as an autocrine factor in the regulation of Fas ligand expression of these cells. Transfection with IL-18 antisense cDNA or treatment with anti-IL-18 antibody down-regulated Fas ligand expression in B16F10 melanoma cells, indicating that IL-18 commonly regulates Fas ligand expression (Fig. 3).

To our knowledge, little is known about the intracellular and molecular mechanisms that induce and regulate IL-18 production and transcription of the IL-18 gene (23, 24), whereas IL-18 signal transduction pathways are becoming clear. It has been documented that nuclear factor-κB is activated by IL-18 (25). In addition, IL-18 was able to induce activation of p56lck and mitogen-activated protein kinase, suggesting that the p56lck-mitogen-activated protein kinase pathway is involved in IL-18 signal transduction pathways (26). Recent work by Puren et al. (12) showed that IL-18 has proinflammatory properties, which are induced by IL-8 and IL-1β from CD14+ cells via direct TNF-α production by CD4+ T cells and NK cells, indicating that IL-18 may play a key role in the inflammatory cascade. In addition, it is well known that ROIs regulate the expression of inflammatory cytokines such as TNF-α through nuclear factor-κB activation and tyrosine kinase-dependent pathways (13, 14). On the
basis of these studies, we hypothesized that IL-18 may act as a regulator of ROI production by B16F10 melanoma cells. To prove this hypothesis, we performed neutralizing experiments using IL-18 anti-sense constructs made the cells more susceptible to NK cytolytic activity by up-regulating Fas ligand expression. A recent report showed that ROI levels. As mentioned above, IL-18 activates NK cytolytic activity by targeting melanoma cells, indicating that IL-18/ROI pathways may be critically important in the survival of melanoma cells (data not shown).

Suppression of IL-18 production by melanoma cells by transfection with IL-18 antisense constructs made the cells more susceptible to NK cells, probably because of reduced Fas ligand expression and intracellular ROI levels. As mentioned above, IL-18 activates NK cytolytic activity by up-regulating Fas ligand expression. A recent report demonstrated that in vivo IL-18 administration induces antitumor effects mediated by CD4+ T cells and NK cells. On the basis of our and other observations, tumor cells and immune cells seem to use IL-18 as an effector molecule to defend them. In this context, NK cells and melanoma cells also use Fas ligand as a weapon. There are some controversial reports on the expression of Fas ligand by melanoma cells, but recent studies indicate that, in humans, expression of Fas ligand seems to be dependent on the stage of melanoma (28, 29). Fas ligand expression generally is negative in primary melanomas and positive in metastatic tumors (28, 29), suggesting that the roles of Fas ligand and IL-18 in the interaction of tumor and host immune cells are variable, depending on the progression of the melanoma. CD71 was up-regulated in 18-h cultured B16F10 melanoma cells, demonstrating ROI regulator of ROI production by B16F10 melanoma cells. To prove this hypothesis, we performed neutralizing experiments using IL-18 antisense constructs.

In conclusion, the data presented in this report indicate that murine melanoma cell expression of CD71, which regulates target cell sensitivity to NK cells, is another candidate for regulating IL-18-mediated tumor susceptibility in NK cells. More extensive studies are needed to demonstrate what factors are key modulators of immune escape in melanoma cells. For either IL-18, IL-18 seems to be a key regulator of the expression of effector molecules. In this regard, the proper choice of immunotherapy using IL-18 and its related molecules should be considered on the basis of tumor progression and expression patterns of molecules. Collectively, it implies that IL-18 regulates the production of tumor survival factors, including Fas ligand and ROIs to escape from host effector cells and, in the case of melanoma, to protect themselves.

In conclusion, the data presented in this report indicate that murine melanoma cells are able to produce IL-18, which is involved in the regulation of intracellular ROI levels and Fas ligand expression, indicating that IL-18 plays a key role in the tumor activity of melanoma. The mechanisms through which factors control the regulation of IL-18 production remain to be determined.

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