**In Vivo Interferon Regulatory Factor 3 Tumor Suppressor Activity in B16 Melanoma Tumors**

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Abstract

Delivery of transcription factors to cancer cells to reprogram gene expression may represent a novel strategy to augment the production of immune stimulatory cytokines and trigger a more potent antitumor response. In the present study, a bicistronic retroviral vector (AP2) was used to transduce B16-F0 melanoma cells with IFN regulatory factor (IRF)-3, which has been shown to activate type 1 IFN genes (IFN-β and IFN-α) as well as other cytokines. Gene-modified B16 melanoma cells were inoculated s.c. into C57BL/6 syngeneic mice. In animals receiving IRF-3 B16 melanoma cells, tumors grew at a 4- to 5-fold reduced rate, and tumors that developed from these mice had a moderate-to-dense infiltration of inflammatory cells, whereas only low levels of lymphocyte infiltration were observed in mock-transduced B16 tumors. Furthermore, tumor growth was not inhibited in severe-combined immunodeficient mice after inoculation of IRF-3-expressing B16 cells, which suggested that IRF-3-mediated antitumor responses were dependent on a functional adaptive lymphocyte response. Interestingly, these in vivo effects on tumor growth correlated with higher mRNA expression of chemokines such as MIP-1β, RANTES, and IP-10, as well as dramatic increases in vitro in the inducibility of cytokine mRNA such as IFN-β, TNF-α, and interleukin 6. Our results demonstrate that with weakly antigenic tumors such as B16 melanoma, IRF-3 gene transfer can mediate important antitumor responses. These findings suggest a novel role for IRF-3 as a potential molecular target for gene therapy of cancer.

Materials and Methods

Cell Lines and Culture Medium. B16.F0 is a murine melanoma cell line of C57BL/6 origin and was maintained in DMEM (Wisent) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics.

Retroviral Transduction. Construction and characterization of the bicistronic AP2 to GFP retroviral vector has been described previously (16). cDNA of human IRF-3 was inserted into multiple cloning site of the retrovector AP2 (Ecorl/blunt/XhoI). Pantrropic retroviral supernatant was generated by transfection of the retrovirus vector pAP2-GFP or pAP2 IRF-3-GFP into 293 GPG packaging cells using LipofectAMINE (Life Technologies, Inc.) and sorted by FACS for GFP expression. For retroviral transduction, 10^6 B16 melanoma cells were seeded in 10-cm plates and incubated with retroviral supernatants filtered from virus-producing cultures in the presence of LipofectAMINE (6 μg/ml). The stable transfectants, IRF-3-GFP B16 and AP2-GFP B16 (AP2-B16) cells were selected by FACS and tested for IRF-3 expression by immunoblot analysis.

Tumor Formation. Young (6–8-week-old) female C57BL/6 or immunodeficient mice (SCID) were purchased from Charles River (Montreal, Quebec, Canada) and handled under the guidelines of the Canadian Council on Animal Care. Mice were shaved in the right flank area and were given injections s.c. with either 1 × 10^6 mock-transfected AP2-B16 cells or IRF-3 B16 in a total volume of 100 μl of PBS. Tumor growth was followed by Vernier caliper measurement every other day from day 7 after injection. All of the experiments included seven mice/group. Tumor volume was calculated according to the following formula:

\[ V = \frac{4}{3} \pi \left( \frac{d}{2} \right)^2 h \]

where V is the volume, d is the diameter of the tumor perpendicular to the longest diameter, and h is the height of the tumor perpendicular to the longest diameter.

IFNs are cytokines endowed with potent antiviral and immunomodulatory activities, capable of exerting marked antitumor effects in both animal models and patients (6), and have served as an important treatment modality for a number of solid tumors and hematological malignancies (7). IRF-3 is a member of the IRF transcription factor family that plays an essential role in the regulated expression of type 1 IFN genes, RANTES and ISGs (8–12). Production of IFN-α and IFN-β in turn can stimulate both innate and acquired arms of the immune response (13). Substitution of serine and threonine residues in the amino acid 396–405 region of IRF-3 with aspartic acid (IRF-3-SD) creates a constitutively active form of IRF-3 that is able to stimulate the transcription of target genes in the absence of virus induction (9). In addition, the constitutive expression of IRF-3-5D alone is sufficient to induce apoptosis in human embryonic kidney 293 and human Jurkat T cells (14, 15). Because of its critical role in the activation of the IFN cascade, IRF-3 was selected as a prototype gene to modify B16 melanoma cells to determine whether ectopic IRF-3 expression could enhance cytokine production and trigger an antitumor response in syngeneic mice. We found that a gene transfer of IRF-3 into B16 melanoma cells modulated the cytokine profile of the tumors and inhibited tumor development by recruiting inflammatory cells to the site of the B16 tumor.
IRF-3 TUMOR SUPPRESSOR ACTIVITY

SCID mice were injected with IRF-3-transduced (AP2-B16 and IRF-3-B16) and mock-transduced AP2 B16 melanoma cells (10^6) and mock-transduced AP2 (40HAU) by using Trizol reagent (Life Technologies, Inc.) RNA protection assays were performed using two Riboquant multiprobe template sets from PharMingen. The mCK-3b and mCK-5 template was used for the T7 polymerase-directed synthesis of high specific activity [32 P]UTP-labeled antisense RNA probes. The probe set contained 12 probes for mCK-3b and 11 probes for mCK-5, including two housekeeping genes, GAPDH and L32. Probe (3 × 10^6 cpm) was hybridized with each RNA (10 μg) sample overnight at 56°C. RNA samples were digested with RNase A and T1, purified, and resolved on 5% denaturing polyacrylamide gels. Internal housekeeping genes were analyzed to confirm equal RNA loading.

**Results and Discussion**

Injection of IRF-3-transduced B16 Cells Leads to Suppression of Tumor Growth in Vivo. Because B16 is a well-characterized, poorly immunogenic s.c. tumor (17, 18), we focused our studies on this model to evaluate whether ectopic expression of IRF-3 could be used as an immunomodulatory transcription factor. Retrovirally transduced B16 cells (AP2-B16 and IRF-3-B16 melanoma cells) after Sendai virus infection (AP2-B16 and IRF-3-B16) at 19 days after injection (AP2-B16 and IRF-3-B16) at 19 days after injection (17, 18). If the lack of growth in AP2-B16 versus IRF-3 B16 melanoma in the syngeneic model was related to changes in the growth pattern of the tumor cell line, slower growth would have been expected in SCID mice. Because growth suppression was observed only in immune competent animals, this observation provides evidence that growth inhibition is not attributable to growth pattern changes of the B16 IRF-3. Together, these results suggest that IRF-3 overexpression in B16 melanoma cells results in up-regulation of RANTES, IP-10, and MIP-1β mRNA expression in vivo. In A, total RNA (10 μg) was isolated from various transduced B16 tumors (mock-transduced AP2 B16, n = 7; IRF-3 B16, n = 10) and hybridized with the mCK-5 multiple probe before digestion with RNase. Separation of protected fragments was performed by 5% SDS gel electrophoresis. Fragment assignment was determined by migration of protected fragments relative to internal standards. Induction of RANTES, IP-10, and MIP-1β mRNA expression was confirmed by performing two additional RPA analyses. In B, cytokine mRNA expression levels were quantified relative to the internal control GAPDH.

**Fig. 1.** Expression of IRF-3 inhibits B16 tumor growth in vivo. In A, IRF-3-B16 melanoma cells (□) and mock-transduced AP2 B16 (●) cells (1 × 10^6) were injected with s.c. into female C57BL/6 (syngeneic mice) or SCID mice (nonimmunocompetent). Tumor growth was followed by perpendicular caliper measurement every other day from day 7 after injection. In B, SCID mice were injected with IRF-3-transduced (□) and mock-transduced AP2 B16 melanoma cells (●; 1 × 10^6). Tumor growth was followed by perpendicular caliper measurement every other day from day 7 after injection.

**Fig. 2.** IRF-3 overexpression in B16 melanoma cells results in up-regulation of RANTES, IP-10, and MIP-1β mRNA expression in vivo. In A, total RNA (10 μg) was isolated from various transduced B16 tumors (mock-transduced AP2 B16, n = 7; IRF-3 B16, n = 10) and hybridized with the mCK-5 multiple probe before digestion with RNase. Separation of protected fragments was performed by 5% SDS gel electrophoresis. Fragment assignment was determined by migration of protected fragments relative to internal standards. Induction of RANTES, IP-10, and MIP-1β mRNA expression was confirmed by performing two additional RPA analyses. In B, cytokine mRNA expression levels were quantified relative to the internal control GAPDH.
sacrifice). H&E stain: magnification
jected mice (C57BL/6) excised at day 19 (day of
jected AP2 and IRF-3 B16 in-
the tumors, compared with mock-transduced AP2 B16 tumors (16%
lymphocyte infiltration was detected in 25 and 12%, respectively, of
of the tumor. Within the IRF-3 B16 tumor mass, moderate or dense
ated by histology within the tumor cell mass, as well as at the margin
IP-10 are specialized to recruit effector cells, including monocytes,
tissue (2123), which suggests that IRF-3 transduction into B16
creased Expression of Chemokines.
progression has been
were examined in tumor cells derived from mock-transduced AP2
( n = 7) or IRF-3-transduced B16 tumors ( n = 10). Cytokine mRNA
expression from solid tumor was determined by RNase protection
assay. Interestingly, the IRF-3 B16 tumors displayed a 2- to 3-fold
increase in basal mRNA levels of RANTES, IP-10, and MIP-1β
compared with AP2 B16 tumors (Fig. 2). Chemoattractants such as
IP-10, RANTES, and MIP-1β recruit lymphocytes into malignant
tissue (21-23), which suggests that IRF-3 transduction into B16
melanoma enhanced cytokine release in the tumor microenvironment.
Whether IRF-3 overexpression is directly involved in cytokine secre-
tion or additional inflammatory cells recruited to the microenviron-
ment are required is currently under investigation.
Because inflammatory chemokines such as RANTES, MIP-1β and
IP-10 are specialized to recruit effector cells, including monocytes,
granulocytes, and effector T cells, tumor infiltration was next evalu-
ated by histology within the tumor cell mass, as well as at the margin
of the tumor. Within the IRF-3 B16 tumor mass, moderate or dense
lymphocyte infiltration was detected in 25 and 12%, respectively, of
the tumors, compared with mock-transduced AP2 B16 tumors (16%
moderate infiltration; Fig. 3, A and B). Interestingly, dense lympho-
cyte infiltration was clearly visible at the margins of the IRF-3 B16
tumors (75%) when compared with mock-transduced AP2 B16 tu-
mors (12%; Fig. 3, C and D). Although ectopic expression of IRF-3
had no direct inhibitory effect on B16 melanoma cells growth in vivo,
a striking effect on local tumor infiltration in vivo was observed.
Histologically, AP2 B16 tumors demonstrated diffuse growth and a
paucity of host inflammatory cells, which was not associated with
areas of coagulation necrosis (Fig. 3E). In contrast, IRF-3 B16 tumors
possessed a larger capsule, displayed dense infiltration of both neu-
traphils and lymphocytes, and contained fewer blood vessels. Also
apparent were areas of necrosis in which tumor cells close to the
inflammatory cells had degenerated. Tumor infiltration of both mono-
nuclear cells and neutrophils may participate in the killing of residual
tumor cells, which suggests that one strategy to improve the efficacy
of IRF-3-based therapy is to combine it with immunotherapy.
Expression of Cytokine mRNA in IRF-3-transduced B16 Mel-
anoma Cells in Vitro. To test whether IRF-3 induced cytokine ex-
pression in B16 melanoma cells in vitro, RNase protection analysis
was performed at various times after infection with Sendai virus, a
well-known inducer of cytokine gene expression and activator of
IRF-3 function (9, 24). Expression of TNF-α, IL-6, IP-10, and IFN-β
mRNA was markedly enhanced in B16 cells expressing IRF-3. At
their peak, 4–12 h after induction, IFN-β mRNA levels were en-
hanced more than a 1000-fold, compared with the AP-2 B16 popula-
tion (Fig. 4A, Lanes 10-12, and B), whereas in the AP-2 B16 cells,
only a weak response to virus induction was observed (Fig. 4A, Lanes
2-7, and B), which suggests that IRF-3 expression reconstituted a
defective IFN response. Although the magnitude of the response was
decreased for TNFα and IL-6, the same enhanced production of
mRNA was observed in the IRF-3-transduced B16 cells, and, again,
IRF-3 expression appeared to restore an otherwise deficient TNF and IL-6 response (Fig. 4, A, C, and E). The expression of Sendai-induced IP-10 was also enhanced in IRF-3-expressing B16 melanoma cells; IP-10 was induced at 18–24 h after virus induction in control B16 cells, (Fig. 4A, Lanes 6 and 7), whereas in IRF-3-expressing B16 cells, the kinetics and magnitude of IP-10 mRNA expression were dramatically enhanced, with mRNA induction beginning as early as 4-h postinfection and reaching a peak at 12 h postinfection (Fig. 4A, Lanes 10–14, and D). Together, these data indicate that IRF-3 expression reconstitutes a defective cytokine response pathway in B16 melanoma cells. The potential of IRF-3-transduced B16 cells to secrete these cytokines on in vivo stimulation could modulate the microenvironment of the tumor by attracting and activating inflammatory cell infiltration. Recent studies have demonstrated that in addition to viruses, multiple activators including lipopolysaccharide, cellular stress, and DNA damage can activate IRF-3 function (25). Therefore, it is conceivable that IRF-3 activation, albeit at a low level, may occur in vivo as a consequence of stress within the tumor microenvironment. Alternatively, simply increasing the intranuclear concentration of IRF-3 may be sufficient to increase the effect of IRF-3 on IFN and cytokine gene expression.

In summary, we used a syngeneic murine B16 tumor model to evaluate the capacity of transcription factor reprogramming of cytokine gene expression to modulate the tumorigenicity of a poorly immunogenic tumor model. Recruitment of inflammatory effector cells to the tumor microenvironment as a consequence of cytokine release may inhibit tumor cell growth and metastasis by directed cell-mediated killing (26–28). The reconstitution of cytokine profiles in IRF-3-expressing B16 melanoma contributes to the enhanced recruitment of lymphocytes to the local tumor site compared with AP2-B16 in which cytokine induction remains defective. Our results demonstrate that transcription factor reprogramming of gene expression may be a potential therapeutic strategy to augment the host antitumor response.

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References


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