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Induction of IFN-Regulated Factors and Antitumoral Surveillance by Transfected Placebo Plasmid DNA

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Delivery of DNA encoding therapeutic genes *in vivo* has great potential for treating malignancy as well as genetic diseases. Delivery of placebo DNA without a transgene is used as a control in gene therapy studies. It is tacitly assumed by most investigators that the protein expressed from the transfected DNA has phenotypic consequences, but that the consequences are not from the DNA itself. Here, we demonstrate that transfection of control plasmid DNA (that does not express a gene product) into tumor cell lines induces a dramatic (>10-fold) increase in the expression of the interferon (IFN)-regulated genes IRF7, STAT1, MIG (approved gene symbol CXCL9), MHCI (MICA), and CD11a (ITGAL) in tumor cell lines. Induction of these genes inhibits tumor development and tumor growth in immunocompetent mice that are immunized with apoptotic tumor cells. The antibody depletion study indicates that the underlying mechanism by which transfection of control DNA induces IFN-regulated genes is the induction of a secreting factor(s) such as IFN-β. Three lines of evidence indicate that DNA transfection-mediated induction of IFN-regulatory genes is independent of TLR9. The three lines of evidence are: (1) TLR9 is not expressed in either SCCVII or 4T1 cell line, (2) activation of TLR9 downstream signaling molecules is not associated with the induction of gene expression, and (3) the secretion factor(s) obtained from the conditioned medium of DNA-transfected SCCVII tumor cells induces the same type of gene expression in the 4T1 tumor cell line, which is refractory to the gene induction by DNA transfection. Our finding indicates that the 4T1 tumor cell line, which is resistant to the DNA transfection-mediated induction of IFN-regulated genes, can be used to determine the real therapeutic gene function.

Key Words: gene therapy, IRF7, STAT1, 4T1, gene regulation, TLR9

INTRODUCTION

Delivery of DNA into tumor cell lines *in vitro* and into tumors *in vivo*, using either chemical formulation or viral vectors, has become a standard protocol for laboratories, preclinical studies, and clinical trials for the development of gene therapy. In these protocols, a negative control or placebo, which does not contain the transgene or does contain only a reporter gene, a so-called control plasmid DNA or control viral vector, is also transfected or injected. Although it is generally assumed that the control plasmid DNA or control viral vector has no significant effect on tumor cells, this has not been examined directly.

The DNA-mediated induction of gene expression in nontumor cell lines uses two mechanisms. One mechanism is through DNA and TLR9 receptor interaction, which is the case for all the lymphoid cells [1–8]. The other mechanism is through transfer of DNA into the cells as demonstrated in nonlymphoid normal cell lines [9] and macrophages [10]. It is unknown whether incubation or transfer of control DNA into malignant tumor cell lines will induce the expression of immunoregulatory genes, trigger the immune system, and inhibit tumor growth. It is also unknown whether transfection of DNA will exclusively induce gene expression as shown in nontumor cell lines.

We hypothesize that transfection of plasmid DNA by itself will alter the expression of regulatory genes in tumor cell lines. The altered expression of regulatory genes in tumor cells will, in turn, change the phenotype of the cells, which will affect the sensitivity of

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tumor cells to attack by host antitumoral immune cells. Due to the broad genetic variation in the tumor cell lines, it is possible to select cell lines in which the gene expression is not affected by control plasmid DNA transfection. Such cell lines can be used for studying basic biological questions.

This report provides evidence that transfection of control DNA induces interferon (IFN)-regulated gene expression in some tumor cell lines. In particular, it shows that transfection of plasmid DNA induces the expression of IRF7, STAT1, MHCI (approved gene symbol MICA), CD11a (approved gene symbol ITGAL), and MIG (approved gene symbol CXCL9) in the SCCVII tumor cell line. The induction of these genes leads to immune protection against these transfected tumor cells. The DNA transfection-mediated induction of genes is through induction of a soluble secretion factor(s) such as IFN-β. The soluble secretion factor(s) obtained from DNA-transfected SCCVII cells can induce the expression of IFN-regulated genes directly in the 4T1 tumor cell line, which is resistant to plasmid DNA transfection-mediated gene induction. Because the DNA transfection-mediated gene induction is not observed in the 4T1 cell line, this cell line can be used as a valuable source for studying the molecular mechanism of gene induction between DNA-transfection-sensitive and -resistant cell lines. Importantly, the upregulation of IFN-regulated genes is through a TLR9-independent signal pathway.

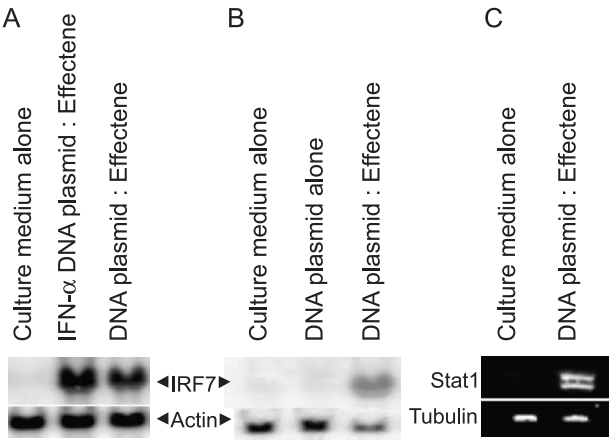

FIG. 1. Expression of IRF7 is upregulated by transfection with DNA plasmid–Effectene complex containing no transgene. Two micrograms of plasmid DNA was used to perform transfection of SCCVII cells. RNA or protein was isolated 1 day after transfection to perform gene expression analysis. (A and B) Northern blot analysis results; (C) Western blot analysis result. (A) Transfection of IFN-α gene or empty DNA plasmid induced upregulation of IRF7 expression in SCCVII tumor cells. (B) Incubation of SCCVII cells with DNA plasmid alone without transfection reagent could not induce expression of IRF7 in SCCVII cells. (C) DNA transfection induced STAT1 expression.

RESULTS

Induction of IRF7 Expression in Response to Transfected Plasmid DNA by Lipid

We previously reported that intratumoral injection of plasmid DNA containing the IFN-α gene (IFNA), followed by electric pulses, upregulated several genes of the IFN-α-induced signal transduction pathway in a subcutaneous SCCVII tumor model [11]. With the aim of determining the molecular mechanism of IFN action, we transfected tumor cells in tissue culture with two DNA plasmids: one that contains and one that lacks the IFN-α transgene. Effectene, a standard lipid cell transfection reagent, developed by Qiagen, Inc., was used for transfection. We refer to the transfection reagent-formulated plasmid DNA as DNA plasmid–Effectene complex. For brevity, we use the term "plasmid DNA" for empty or control plasmid DNA that lacks the transgene. Surprisingly, incubation of SCCVII cells with the DNA plasmid–Effectene complex upregulated the level of IRF7 mRNA expression *in vitro*, regardless of whether the plasmid contained the IFN-α transgene (Fig. 1A). Next, we assessed whether IRF7 induction was elicited by merely incubating the SCCVII cells with the plasmid DNA or if this response required that the plasmid DNA be transfected into the SCCVII cells. Neither DNA plasmid alone nor Effectene alone induced IRF7 expression, whereas the combination upregulated IRF7 mRNA levels by 13-fold, based on three independent experiments (Fig. 1B). Induction of STAT1 protein at a similar magnitude was also observed (Fig. 1C).

Upregulation of Multiple IFN-Regulated Genes by Transfection of Plasmid DNA, Regardless of DNA Substrates or Transfection Techniques

IRF7 is an IFN-mediated transcriptional factor. The induction of IRF7 by transfection of plasmid DNA suggests that other IFN-regulated signal transduction pathway genes are also possibly induced. To determine whether this is the case, we determined expression of MHCI and CD11a after transfection of plasmid DNA into the SCCVII tumor cells. Indeed, the expression of all these genes is upregulated by transfection of plasmid DNA (Fig. 2), but not by plasmid DNA or by Effectene alone (Figs. 2A and 2B). Transfection of a higher dose of plasmid DNA resulted in a higher level of gene expression than with a lower dose (Fig. 2C). To determine whether this is Effectene transfection-specific, we used electroporation, $CaPO_4$, and Lipofectamine to transfect plasmid DNA into the SCCVII tumor cells. All of these transfection methods resulted in the induction of gene expression (Figs. 2E, 2G, and 2H), although the magnitude of induction by electroporation-mediated DNA transfection was less than that by Effectene at the same DNA dosage (Fig. 2E vs 2C). Transfection of PCR DNA fragment also induced the level of CD11a expression.

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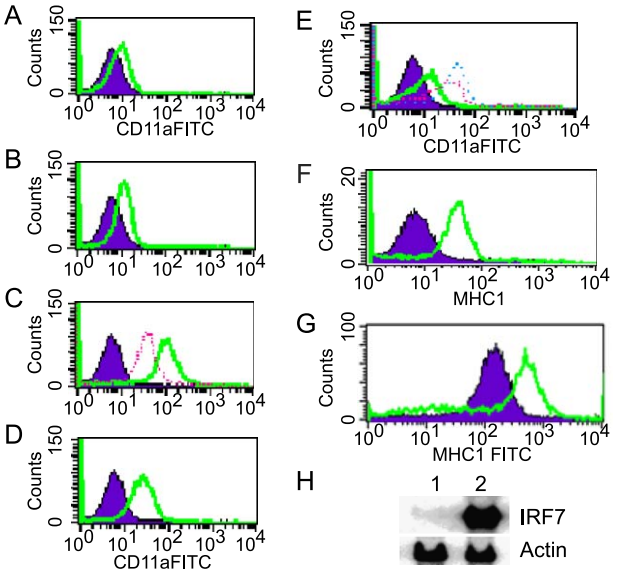

FIG. 2. Transfection of plasmid DNA into SCCVII tumor cells induces multiple IFN-regulated gene expression, regardless of transfection methods. (A–G) Analysis of gene expression by flow cytometry as determined by the shifted cell distribution; shaded area represents the gene expression level prior to DNA transfection. (A and B) Incubation of cells with plasmid DNA alone and Effectene alone, respectively, induced little change in CD11a expression. (C) Incubation of cells with DNA–Effectene complex induced a dose-dependent CD11a expression. The dotted and solid lines represent 2 and 10 μg plasmid DNA doses, respectively. (D) Transfection of DNA amplified by PCR induced CD11a expression. (E) Transfection of DNA with electroporation induced CD11a expression. Solid line and dotted lines indicate 2 and 10 μg plasmid DNA, respectively. (F and G) Transfection of DNA with Effectene and Lipofectamine, respectively, induced MHCI expression. (H) Transfection of DNA with $CaPO_4$ induced IRF7 expression. Gene expression was revealed by Northern blot analysis. Lane 1, cells were incubated with DNA alone; lane 2, cells were incubated with DNA–Effectene. Two micrograms of plasmid DNA was used to perform transfection of SCCVII cells. RNA or protein was isolated 1 day after transfection to perform gene expression analysis.

Tumor Cell Line-Dependent Gene Upregulation by Transfection of Control Plasmid DNA

Induction of gene expression by transfection of plasmid DNA occurs in every normal cell line examined so far [9]. The above results also demonstrate that transfection of control plasmid DNA will induce IFN-regulated gene expression in an SCCVII tumor cell line. This affects the accurate evaluation of a therapeutic gene effect on these tumor cells in a gene therapy study. This also compromises the understanding of the molecular mechanisms by which these IFN-regulated genes are induced by transfection of control plasmid DNA, as well as the biological function of the induction of these genes. To avoid this problem, it is necessary to find a tumor cell line in which transfection of plasmid DNA will not affect the expression of these genes. We hypothesized that the great genetic variation among tumor cell lines will allow for the discovery of a control DNA transfection-nonresponsive cell line. To test this hypothesis, we examined two other tumor cell lines, TRAMP and 4T1, derived from prostate and breast tumors, respectively. Similarly, as observed in SCCVVII tumor cells, transfection of plasmid DNA into TRAMP induced IRF7 expression (Fig. 3A). However, transfection of plasmid DNA into 4T1 cells did not induce any IRF7 expression (Fig. 3B). To exclude the possibility that this tumor cell line has defective IRF7 expression, the IFN-α gene was transfected into 4T1 cells, resulting in a significant upregulation of IRF7 expression (Fig. 3B). This result indicated that the 4T1 cell was resistant to the DNA transfection-mediated gene induction.

Inhibition of Tumor Development and Tumor Growth from DNA-Transfected SCCVII Tumor Cells in Immunocompetent Mice Immunized with Apoptotic Tumor Cells

Induction of the antigen-presenting molecule MHCI in tumor cells, through transfection of plasmid DNA, indicates that these transfected tumor cells may be sensitive to immune surveillance *in vivo*. To determine whether this is the case, we inoculated both DNA-transfected and nontransfected SCCVII tumor cells subcutaneously into immunocompetent mice. We determined the tumor incidence and tumor volume on days 5 and 12 after inoculation. Both transfected and nontransfected SCCVII tumor cells developed similar magnitudes of tumors in these mice (Fig. 4A). We reasoned that tumor development in the wild-type mice could be due to the lack of tumor-specific cytotoxic T cells, which inhibit tumor growth. To determine whether this is the case, we immunized

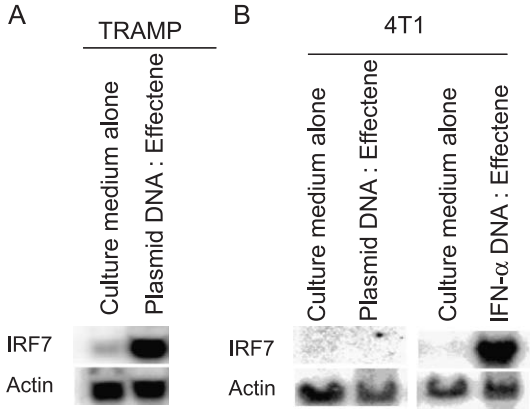

FIG. 3. Induction of IRF7 expression was observed in the prostate tumor cell line TRAMP but not in breast tumor cell line 4T1 after plasmid DNA transfection. Two micrograms of plasmid DNA was used to perform transfection, and RNA was isolated 1 day after transfection to perform gene expression analysis by using Northern blot. (A) Induction of gene expression by transfection of DNA. (B) Transfection of IFN-α-containing DNA induced IRF7 expression and transfection of control DNA failed to induce gene expression in 4T1 tumor cells.

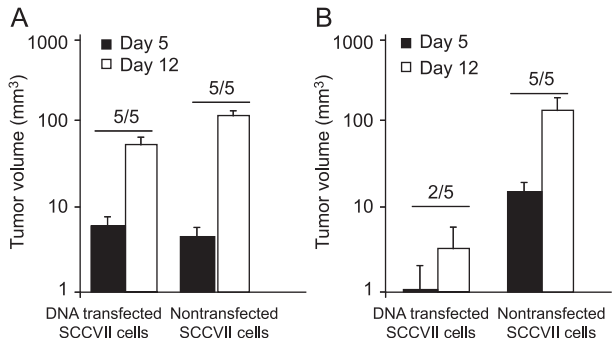

FIG. 4. Immunized mice showed inhibited tumor development from DNA-transfected tumor cells but not from the nontransfected cells. Immunocompetent C3H mice (n = 5) immunized or not with mitomycin C-treated SCCVII tumor cells were inoculated with either DNA-transfected or DNA-nontransfected SCCVII tumor cells 1 week after immunization. Numbers on top of the bar represent the tumor incidence. Tumor volume was determined on days 5 and 12 after inoculation of tumor cells. (A) Tumor incidence and tumor volume in the unimmunized C3H mice. (B) Tumor incidence and tumor volume in the immunized mice.

mice with mitomycin C-treated SCCVII cells. Inoculation of transfected tumor cells into these mice inhibited tumor development in 60% of mice (3/5) and reduced tumor growth by 96% (Fig. 4B). Inoculation of nontransfected tumor cells into these mice resulted in normal tumor development and growth, as found in the untreated mice (Fig. 4B). The lack of inhibition of the wild-type tumor cells may be due to the weak immunization strategy, in which only one administration was performed. To determine whether upregulation of IFN-pathway genes contributed to this antitumoral effect *in vivo*, we also challenged immunized mice with transfected and nontransfected 4T1 cells. There was no antitumoral protection or inhibition of tumor volume in either DNA-transfected or nontransfected 4T1 cells (data not shown). This indicated that the lack of upregulation of these genes in 4T1 cells after transfection inhibited the antitumoral immune surveillance.

DNA Transfection-Mediated IRF7 Induction is Transcriptionally Regulated

To determine the mechanism of DNA transfection-mediated gene induction in tumor cells, we determined whether the induction of a gene such as IRF7 by DNA transfection required *de novo* RNA and protein synthesis. To assess the requirement for protein synthesis, we incubated the transfected cells with translation inhibitors. Incubation with cycloheximide (CHX), puromycin, or emetine (EME) did not negate the response but in fact modestly increased the level of IRF7 mRNA (Fig. 5A). This suggested that rather than new proteins being required for the induction, one or more unstable proteins may modestly inhibit it. To determine the role of RNA synthesis, we incubated the cells with the transcription inhibitor actinomycin D (ActD), 18 h after these cells were treated with plasmid DNA–Effectene. We found that ActD strongly inhibited IRF7 induction, such that 6 h after treatment, IRF7 mRNA levels returned to basal levels (Fig. 5B). This finding indicated that the IRF7 induction response to plasmid DNA depended on *de novo* transcription.

Induction of Secretion Factor(s) by Transfection of DNA Triggers the Upregulation of IFN-Signal Transduction Pathway Genes

To determine the mechanism by which the DNA transfection-mediated gene expression occurred in SCCVII tumor cells, but did not occur in the 4T1 tumor cell line, we performed a Transwell study, in which Transwell inserts containing 4T1 or SCCVII cells were inserted into the DNA-transfected SCCVII cells. As expected, SCCVII conditioned medium induced the

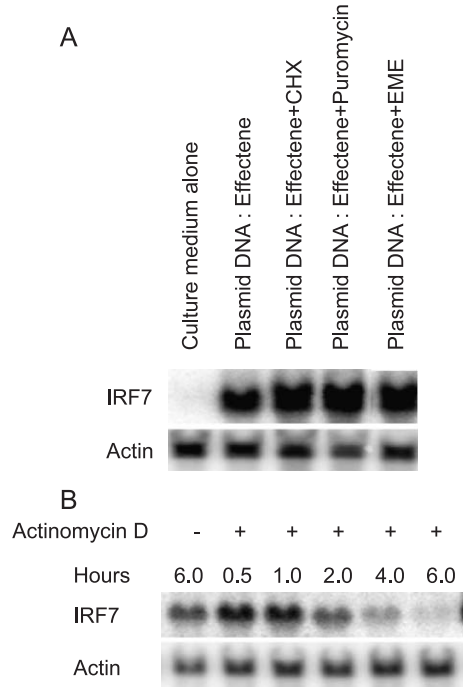

FIG. 5. RNA synthesis-dependent, but protein synthesis-independent, induction of IRF7 mRNA by plasmid DNA–Effectene. Two micrograms of plasmid DNA was used to perform transfection of SCCVII cells, and RNA was isolated by the end of treatment. (A) Induction of IRF7 mRNA by plasmid DNA–Effectene was only slightly enhanced by inhibitors of protein synthesis. DNA-transfected cells were treated with cycloheximide (CHX; 1 mg/ml), puromycin (3 mg/ml), and emetine (EME; 3 mg/ml) immediately after transfection of plasmid DNA with Effectene. (B) Inhibition of transcription inhibits the IRF7 induction by DNA transfection. Application of actinomycin D to the SCCVII cells 18 h after DNA transfection inhibited transcription. The treated cells were harvested for gene expression analysis at 0.5, 1.0, 2.0, 4.0 and 6.0 h after transfection.

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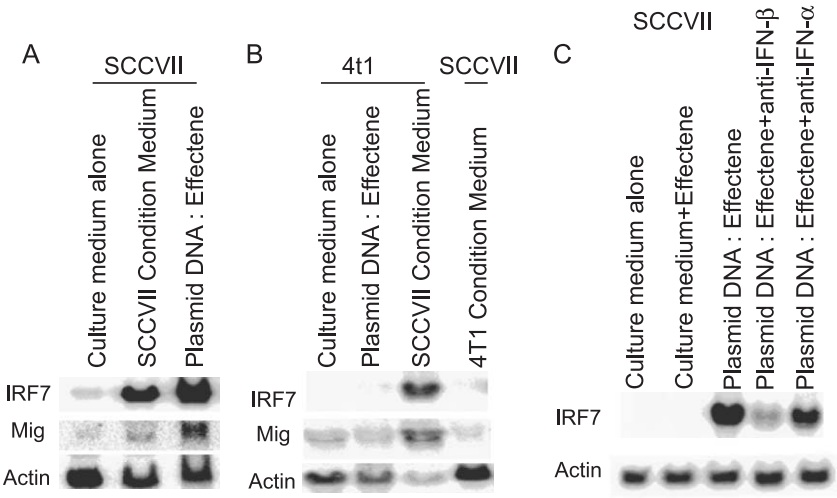

FIG. 6. DNA transfection into SCCVII tumor cells induced a soluble secretion factor(s) to induce the expression of IFN-regulated genes. Two micrograms of plasmid DNA was transfected with Effectene into tumor cells. The medium from these DNA-transfected cells is referred as conditioned medium. Nontransfected cells seeded in the inserts of Transwells were placed into the conditioned medium. Gene expression using Northern blot analysis was revealed 1 day after transfection or treatment with conditioned medium. (A and B) SCCVII conditioned medium induced IRF7 and MIG expression in both SCCVII and 4T1 cells, but 4T1 conditioned medium did not induce gene expression. (C) Neutralization antibody against IFN inhibits the induction of IRF7 expression by transfection of DNA.

expression of IRF7 in SCCVII cells (Fig. 6A). Surprisingly, SCCVII conditioned medium also induced IRF7 expression in 4T1 cells (Fig. 6B). This result suggests that a soluble factor that was secreted from the transfected SCCVII cells induced gene expression, regardless of DNA transfection-sensitive or -resistant cell lines. To prove that this was the case, we performed a similar study, in which both SCCVII and 4T1 cells containing inserts were placed into wells containing DNA-transfected 4T1 cells. IRF7 expression was not induced by 4T1 conditioned medium in SCCVII cells (Fig. 6B). This result confirmed the assumption that induction of IFN-regulated factors by transfection of plasmid DNA was preceded by the induction of a soluble secretion factor(s). To determine whether the soluble secretion factor is an IFN protein, immediately following DNA transfection, we applied IFN-neutralization antibody to the culture medium. Overdosed IFN-β antibody greatly inhibited DNA transfection-mediated induction of IRF7 by 70% (Fig. 6C), suggesting that IFN-β is the primary soluble factor that is induced by transfection of control plasmid DNA to mediate the gene expression. Partial reversal of this type of gene induction by using overdosed IFN-α antibody suggests that more than one soluble factor contributes to this effect.

Induction of IFN-Regulated Genes by Transfection of DNA is Independent of the TLR9 Signal Transduction Pathway

TLR9 is essential for double-stranded DNA-induced innate immune cell activation and molecular signaling [9,12–15]. To determine whether the induction of IFN-regulated genes in SCCVII cells but not in the 4T1 cells by DNA transfection was associated with TLR9 expression, we determined the expression of this gene. Northern blot analysis did not detect any expression of this gene (data not shown). To increase the sensitivity, we performed RT-PCR analysis. We did not detect TLR9 expression in either SCCVII or 4T1 cells (Fig. 7A). To confirm this observation further, we also analyzed the activation of both NF-κB and MAPK molecules, which are activated through TLR9 and DNA interaction. We detected two NF-κB-binding DNA complexes. The lower complex was detected in both cell lines, indicating a constitutive activation of this complex (Fig. 7B). However, the upper NF-κB-binding complex is constitutively activated only in 4T1 cells, and DNA transfection into 4T1 cells did not further increase the activation level of NF-κB (Fig. 7B). Although incubation of SCCVII cells with either DNA or DNA–Effectene complex induced the activation level of the upper NF-κB complex (Fig. 7B), only incubation with DNA–Effectene, not DNA alone, induced IFN-regulated gene expression (Fig. 1B). These data indicated that there was no association between NF-κB activation and DNA transfection-mediated IFN-regulated gene expression. We drew similar conclusions on the activation of the MAPK signaling pathway and DNA transfection-mediated IFN-regulated gene induction because transfection of plasmid DNA did not further induce any activation of p42/44 ERK kinase in the SCCVII cell line (Fig. 7D), and the activation of this protein in 4T1 cells was detected at a level that was hardly visible (Fig. 7C). However, the data did not exclude the possibility that the MAPK pathway was involved in the cell-specific induction of IFN-regulated genes because the MAPK activation was not detected in the 4T1 cells (Fig. 7C).

DISCUSSION

The central finding of this study was that the DNA plasmid–Effectene complex (DNA plasmid formulated with the transfection reagent Effectene) potently induced the IFN-regulated molecules in some tumor cell lines, regardless of transfection method or DNA substrate (Figs. 1 and 2). This finding was based on at least three independent experiments. This effect required entry of

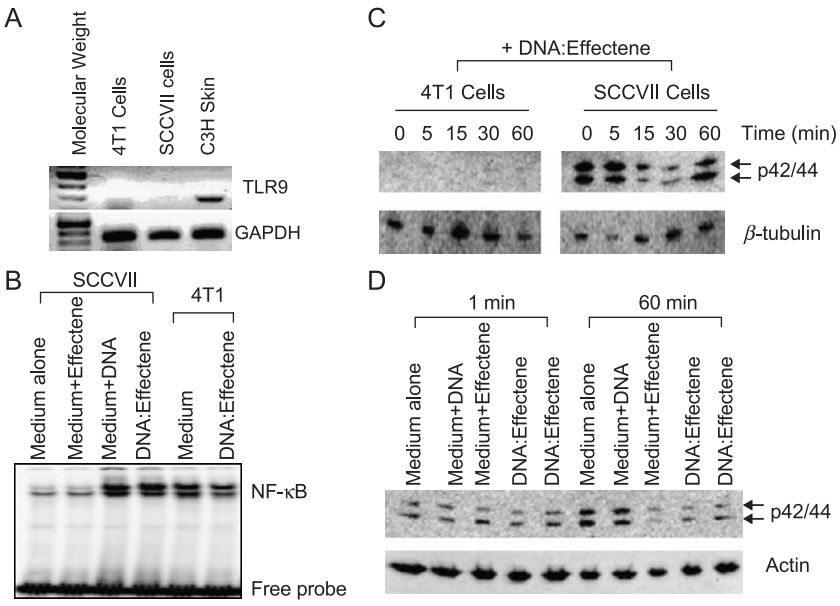

FIG. 7. DNA transfection-mediated induction of IFN-regulated genes was independent of TLR9 signaling cascade. (A) Detection of TLR9 expression from SCCVII tumor cells, 4T1 tumor cells, and C3H skin tissue using RT-PCR. GAPDH was used to serve as an internal control for RT-PCR assay. (B) Determination of NF-κB activation using EMSA. Cells either were not treated or were incubated with transfection reagent alone (Medium + Effectene), DNA alone (Medium + DNA), or the complex of transfection reagent and DNA (DNA:Effectene). Two hours after incubation, cells were subjected to isolation of nuclear extract for EMSA as described under Materials and Methods. (C) Time-course analysis of MAP kinase activation using Western blot analysis. Both SCCVII and 4T1 cells were subjected to transfection with DNA:Effectene. Two micrograms of DNA was used for each transfection. The samples were harvested at different time points. The antibody dilution used was 1:200. (D) Analysis of whether the activation of MAP kinases in SCCVII cells associates with DNA transfection. Cells were either not treated or incubated with transfection reagent alone (Medium + Effectene), DNA alone (Medium + DNA), or the complex of transfection reagent and DNA (DNA:Effectene). One minute and 1 h after transfection, cells were harvested and the protein extracts from these samples were subjected to Western blot analysis. The primary antibody dilution was 1:200. Actin was used as control for the assay.

the plasmid DNA into the cells by transfection, since neither plasmid DNA nor Effectene alone induced these transcripts (Figs. 2A and 2B). In contrast, it was shown that lymphoid cells became activated in response to plasmid DNA without the need for transfection, presumably because they possessed bacterial DNA-specific receptors [20]. However, in other cases, lymphoid cells required that the plasmid DNA be taken up, as demonstrated by the finding that induction of stress kinase pathway genes required endosomal uptake of CpG-ODN [6]. Induction of gene expression by transfection of plasmid DNA into tumor cells reported here and into normal cells reported by others [9,21] shared one similarity, in that IFN-regulatory genes were induced at the transcriptional level in both types of cells. However, there was also a distinction between these findings in tumor cells and the previous ones in normal cells. The previous report indicated that induction of genes by DNA transfection occurred in all examined normal cell lines. We found that this was not the case in tumor cell lines, because one of three tested tumor cell lines, 4T1, did not demonstrate an induction of gene expression by DNA transfection (Fig. 3B). The failed induction of IFN-regulated genes by DNA transfection in 4T1 cells was not due to any defect in this signal transduction pathway because both IFN and the conditioned medium induced the expression of genes in the IFN-regulated cascade in this cell line (Fig. 6B). This was significant because this cell line could be used by gene therapy investigators to assess accurately gene function on tumor cells *in vitro* and *in vivo*, rather than the function mediated by a mixture of control DNA and gene product. This is the first time that a cell line that is nonresponsive to DNA transfection is clearly defined. The finding of this cell line is also significant because 4T1 tumor cells are highly metastatic *in vivo*, compared to SCCVII and TRAMP, suggesting that the gene induction in response to DNA transfection might predict the sensitivity to immune therapy, although this is purely speculation.

Another important finding of this work was that induction of IFN-regulated genes in tumor cells inhibited the tumor development and tumor growth of immunized mice, but not of the unimmunized mice. This result suggested that an effective approach to the prevention of tumor growth may not rely on vaccination alone, but may also require sensitization of tumor cells to attack by immune cells. This result will certainly help to reshape the preclinical design for developing a successful, clinically effective vaccination approach against tumors.

These data clearly indicate that the induction of IRF7 and other genes was a secondary event to the induction of soluble factors, because lack of these soluble secretion factors in the conditioned medium derived from DNA-transfected 4T1 cells failed to induce the expression of these genes (Fig. 6B). The most logical candidates for these soluble factors are IFNs. This is supported by at least two lines of evidence. First, IFN was induced in lymphoid cells by incubation with bacterial DNA [22]; second, neutralization of IFN partially reversed the induction of

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IRF7 expression in tumor cells (Fig. 6C). However, it was possible that other soluble factors might also contribute the induction of these genes. Others found the induction of the proinflammatory cytokines TNF-α; interleukin (IL)-1, IL-6, and IL-12; and IFNs in lymphoid cells [1–4,6–8,13,23–26], but it was difficult to translate these findings to tumor cells derived from epithelial cells, because of their completely different nature. More work is needed to define the soluble factors that are induced by DNA transfection. Discovery of these soluble factors will be beneficial for designing a safe formulation for gene delivery and for designing a more immunogenic formulation for the treatment of tumors.

It is known that DNA binds TLR9 on the cell membrane, recruiting MyD88 and activating downstream signaling molecules such as NF-κB and MAP kinases in immune cells [9,15,27]. Our result indicated that it is possible that there is another DNA-mediated signaling pathway that does not require TLR9 binding for inducing gene expression. The evidence that supports such a claim is that TLR9 was not expressed in SCCVII cells but transfection of DNA induced the expression of multiple genes. However, the genes regulated by DNA transfection in tumor cells were limited to IFN-regulated genes; further study is needed to determine whether other signaling pathways are affected by DNA transfection. Although the current data indicate that there is no association between the activation of MAPK, such as ERK1/2, and transfection DNA-mediated gene induction (Fig. 7D), current data do not exclude the possibility that the activation of this signal pathway controlled the cell-specific gene induction (Fig. 7C). Further analysis of the MAPK pathway in knockout cell lines is necessary to make a definitive conclusion.

MATERIALS AND METHODS

Chemical reagents. Actinomycin D and inhibitors of protein synthesis, including CHX, puromycin, and EME, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Polyclonal anti-IFN-α and -β antibodies were purchased from PBL Biomedical Labs (Piscataway, NJ, USA). Antibodies used for flow cytometry were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies used for determination of MAP kinase activation were purchased from Cell Signaling Technologies (Beverly, MA, USA). Effectene transfection reagent was purchased from Qiagen (Valencia, CA, USA). Lipofectamine was purchased from Invitrogen. The $CaPO_4$i transfection kit was purchased from Promega (Madison, WI, USA).

Cell growth and transfection. SCCVII cells, known as SCCVII/SF, were derived from a spontaneously arising murine squamous cell carcinoma [16]. TRAMP is a spontaneously arising murine prostate carcinoma and was developed at the Baylor College of Medicine (Houston, TX, USA). 4T1 is a murine mammary cancer cell line that was developed at the Karmanos Cancer Institute (Detroit, MI, USA). The cells were maintained in DMEM with 10% fetal bovine serum (GIBCO, Invitrogen). DNA plasmid–Effectene, DNA–Lipofectamine, and DNA–CaP_i complexes were prepared according to the manufacturers' instructions for a six-well plate, with 2 μg DNA/well. Cells at 70% confluence were incubated with DNA plasmid–Effectene complex for 24 h, unless specified otherwise. For transcriptional-inhibition studies, cells were treated with DNA plasmid–Effectene complex for 18 h, then actinomycin D (100 μg/ml) was applied to the cells for 0.5, 1, 2, 4, or 6 h before RNA was isolated from the cells. For inhibition of protein synthesis studies, inhibitors of protein synthesis at concentrations of 100 μg/ml for CHX and 300 μg/ml of EME and puromycin were applied to cells that had been incubated for 18 h with DNA plasmid–Effectene. For the *trans*-inducing study using conditioned medium (medium from DNA-transfected cells), cells in the bottom well were transfected with plasmid DNA–Effectene complex, and the untransfected cells containing a Transwell insert were inserted into the bottom well of a six-well plate. Cells were harvested 24 h after transfection to prepare RNA for Northern blot analysis. For the secretion protein neutralization study, cells were incubated with 10 μg anti-IFN-β or anti-IFN-α antibody during and after DNA transfection before they were collected for RNA isolation. For electroporation transfection of plasmid DNA into SCCVII cells, a BTX electroporator (San Diego, CA, USA) was used to pulse 3×10^5 SCCVII cells in a volume of 300 μl DMEM, using parameters of 1 pulse, 150 V/cm, and 75-ms duration.

Gene constructs and plasmid manufacture. The empty DNA plasmid used in these experiments was the control DNA plasmid pcDNA3.1, purchased from Invitrogen. All plasmids were manufactured with the Qiagen EndoFree plasmid preparation kit. Residual salts were removed from plasmids by dialysis against sterile water (USP) with a Millipore dialysis tube (Millipore Corp., Bedford, MA, USA).

RNA isolation, Northern blot, and RT-PCR analysis of gene expression. RNA isolation was performed with TRIzol reagent as described previously [17]. Probes for actin, STAT1, MIG, and IRF7 were derived from previous experiments [11]. Northern blot analysis of gene expression was detailed in an earlier publication [18]. In brief, 10 μg of total cellular RNA was subjected to 1% agarose–formaldehyde gel electrophoresis at 60 V for 2 h. The RNA was then transferred to a positively charged Nylon membrane (Roche Applied Science, Indianapolis, IN, USA), prehybridized, and hybridized at 42°C in hybridization buffer (Ambion, Austin, TX, USA). The membrane was stripped with the use of a StripEZ PCR labeling kit (Ambion) and rehybridized with other randomly prime-labeled probes (DECA Primer II DNA Labeling Kit; Ambion) with [32P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech). The Northern blot results were quantified by scanning the expression signal intensity with a PhosphorImager analyzer (Model 445 SI; Molecular Dynamics, Sunnyvale, CA, USA).

For RT-PCR analysis of TLR9 expression, 5 μg total RNA from each sample was used to generate cDNA using the RT-for-PCR Kit purchased from Clontech (Palo Alto, CA, USA). The sequences of forward and reverse primers used for amplification of TLR9 from cDNA are 5′-AGAATCCTCCATCTCCCAAC-3′ and 5′-TGGACAGTTCCACTTGAGGT-3′, respectively. The primer sequences for amplifying GAPDH are 5′-AGGCTGAGAACGGGAAG-3′ and 5′-TCATGAGTCCTTCCACG-3′. The TLR9 fragment was denatured for 2 min at 95°C and amplified for 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C for a total of 35 cycles, followed by a final extension for 10 min at 72°C. GAPDH was similarly amplified except for the annealing temperature, which was 57°C. The PCR products were analyzed in a 1% agarose gel. The agarose gel was documented using a Bio-Rad (Hercules, CA, USA) gel documentation imager after the gel was stained with ethidium bromide.

Western blot analysis. The procedure is the same as described previously [18]. In brief, SCCVII cells were lysed with RIPA buffer by freezing and thawing. The sticky genomic DNA was homogenized by syringe, using a 27½-gauge needle. One hundred micrograms of protein per sample was loaded onto an 8% SDS–PAGE gel and separated with 200 V using a Bio-Rad protein psiII electrophoretic apparatus. The separated proteins were transferred onto a nitrocellulose membrane using 100 V for 30 min. The membrane was subjected to incubation with 5% dry milk in TBS buffer, followed by the primary antibodies and anti-rabbit secondary antibody conjugated to horseradish peroxidase. The chemiluminescence substrate was incubated with the membrane for 30 s and the chemiluminescent signal was captured using a Kodak 410 imager (Perkin–Elmer, Shelton, CT, USA).

Electrophoretic mobility shift assay (EMSA). The EMSA method was used to characterize the activities of NF-κB in nuclear extracts as described by others [19]. To prepare nuclear extracts, 1×10^6 cells were washed with cold PBS and suspended in 0.4 ml hypotonic lysis buffer containing protease inhibitors for 30 min. The cells were then lysed with 12.5 μl of 10% Nonidet P-40. The homogenate was centrifuged and the supernatant was discarded. The nuclear pellet was resuspended in 25 μl ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the extract was centrifuged, and supernatants containing nuclear extracts were secured. The protein was measured by the Bradford method. The nuclear extracts containing 10 μg protein were incubated with 16 fmol of ^{32}P-end-labeled double-stranded NF-κB oligonucleotide at 37°C for 30 min. The DNA–protein complex formed was separated from free oligonucleotides on 6.6% native polyacrylamide gels. The dried gels were visualized, and radioactive bands were quantitated by a PhosphorImager using ImageQuant software. The NF-κB binding sequence for EMSA was 5′ -TTGTTACAAGGGACTTTCCGCTGGGGACTTCCAGGGAGGCGTGG-3′ .

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