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*J Immunol* 2006; 176:3426-3437; doi: 10.4049/jimmunol.176.6.3426

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Immunization with Murine Breast Cancer Cells Treated with Antisense Oligodeoxynucleotides to Type I Insulin-Like Growth Factor Receptor Induced an Antitumoral Effect Mediated by a CD8<sup>+</sup> Response Involving Fas/Fas Ligand Cytotoxic Pathway<sup>1</sup>

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We have demonstrated that in vivo administration of phosphorothioate antisense oligodeoxynucleotides (AS[S]ODNs) to type I insulin-like growth factor receptor (IGF-IR) mRNA resulted in inhibition of C4HD breast cancer growth in BALB/c mice. The present study focused on whether in vivo administration of C4HD tumor cells pretreated with IGF-IR AS[S]ODN and irradiated could provide protection against C4HD wild-type tumor challenge and also on elucidating the mechanism mediating this effect. Our results showed that mice immunized with IGF-IR AS[S]ODN-treated C4HD cells experienced a growth inhibition of 53.4%, 61.6%, and 60.2% when compared with PBS-treated mice, wild-type C4HD cell-injected mice, or phosphorothioate sense oligodeoxynucleotide-treated C4HD cell-injected mice, respectively. The protective effect was C4HD-specific, because no cross-protection was observed against other syngeneic mammary tumor lines. The lack of protection against tumor formation in nude mice indicated that T cells were involved in the antitumoral response. Furthermore, cytotoxicity and splenocyte proliferation assays demonstrated that a cellular CD8<sup>+</sup>-dependent immune response, acting through the Fas/Fas ligand death pathway, could be mediating the antitumor effect induced by immunization with AS[S]ODN-treated cells. Immunization also induced splenocytes to produce Ag-dependent IFN-γ, indicating the presence of a type 1 response. We demonstrated for the first time that IGF-IR AS[S]ODN treatment of breast cancer cells induced expression of CD86 and heat shock protein 70 molecules, both involved in the induction of the immunogenic phenotype. Immunization with these tumor immunogens imparted protection against parental tumor growth through activation of a specific immune response. The Journal of Immunology, 2006, 176: 3426–3437.

Immunotherapy constitutes a strategy of promising efficiency in cancer patients (1, 2). Therefore, extensive efforts are currently being made to increase antitumor immunity through genetically modified cell-based vaccines (3).

Several tumor Ags recognized by specific CTLs have been identified, and some are now being tested in cancer clinical trials (4). Most of these Ags, however, are only expressed by certain types of tumors. To design a cancer vaccine with potential application in a wide range of tumors, a method should be developed that is capable of increasing immune reactivity against tumor-self Ags without the need for prior identification. For this reason, one of the approaches to developing a cancer vaccine is the use of tumor cells proper as a source of Ag. On one hand, vaccinations with whole cell extracts have been tested widely without convincing results (5), apparently because tumors Ags arising during cancer progression are totally ignored by the immune system (6). On the other hand, immunization with tumor cells that have been modified by either cytokines or costimulators can elicit a CTL response against Ags that were not immunogenic if parental tumor cells were used for immunization (7, 8). These observations indicate the existence of tumor Ags that are normally “silent,” meaning that they are not able to induce a CTL response in the context of tumor cells (7, 9).

T cell response was demonstrated to be dependent mainly on the costimulatory signals present at the time of Ag recognition. The Ag-specific signal is delivered by interaction between the MHC/peptide on the surface of any nucleate cell and the TCR on T cells. Costimulatory signals are mediated by cytokines and adhesion molecules. One well-characterized costimulatory signal is provided by the interaction between CD28 on T cells and its primary ligands CD80 (B7.1) or CD86 (B7.2) on APCs (10, 11) or on a non-APC-derived tumor (12). In the absence of appropriate costimulatory signals, engagement of the TCR itself typically leads to ignorance, anergy, or apoptosis of the Ag-specific T cells. Lack of sufficient Ag signals is usually found in tumor cells where expression of MHC class
I, CD80, or CD86 costimulatory molecules is reduced or lost (13, 14). Increase of their expression in tumor cells has made these molecules effective for augmenting tumor immunogenicity (15, 16). However, the need to transfect tumor cells with one or several expression vectors makes the process quite complicated.

In contrast, targeting tyrosine kinase receptors as type I insulin-like growth factor (IGF-I)\(^4\) receptor (IGF-IRs) in anticancer therapy has proved to be effective, and the use of blocking Abs (17), selective tyrosine kinase inhibitors (18), and antisense strategies (19–21) has yielded promising results. Nevertheless, much more could be achieved if, in addition to the antitumor effect, a particular drug could also switch a tumor cell from a nonimmunogenic into an immunogenic phenotype, i.e., by uncovering Ags that were “silent” during tumor growth.

We have recently demonstrated that breast cancer growth can be inhibited by direct in vivo administration of phosphorothioate antisense oligodeoxynucleotides (AS[S]ODNs) to IGF-IR mRNA using C4HD tumors from an experimental model of hormonal carcinogenesis in female BALB/c mice (21). C4HD tumor belongs to a progesterin-dependent mammary tumor model and requires medroxyprogesterone acetate (MPA) administration to proliferate (22).

IGF-IR is a tyrosine kinase cell surface receptor that plays a crucial role in the establishment and maintenance of transformed phenotype (23). Conversely, down-regulation of IGF-IR function provides a selective target for therapies aimed at extermination of cancer cells. IGF-IR targeting unexpectedly has also seemed to produce a host response with several of the characteristics of an immune response. Injection into syngeneic rats of C6 glioblastoma cells stably expressing antisense IGF-IR transcripts elicited a protective host response that led to rejection of tumor formation by subsequent injection of C6 wild-type cells (24, 25). Furthermore, injection of C6 IGF-IR antisense-transfected cells causes regression of established tumors (24, 25). Similar results were obtained with established mouse N2A neuroblastomas growing in syngeneic C57BL/6 mice (26). There is general consensus that a fully activated IGF system interferes with immune recognition of tumor cells (24, 26–29). However, the mechanism by which tumor growth inhibition is achieved by immunization with IGF-IR antisense-treated cells has not yet been elucidated.

In the present work we explore whether inhibition of breast cancer growth could be achieved by activation of a host’s immune response through administration of breast tumor immunogens obtained by in vitro treatment of tumor cells with IGF-IR AS[S]ODN. We demonstrated that serial injection of IGF-IR AS[S]ODN-treated C4HD cells provides protection against C4HD wild-type tumor challenge in vivo in BALB/c mice. In addition, we found that cytotoxic effect against tumor cells was CD8\(^+\) dependent and that it involved activation of the Fas/Fas ligand (FasL) death pathway. Immunization also induced splenocytes to produce Ag-dependent IFN-\(\gamma\), indicating the presence of a type 1 response. Also, we demonstrated that IGF-IR blockade with AS[S]ODN induced up-regulation of the costimulatory molecule CD86 and of peptide chaperone heat shock protein 70 (Hsp70) in C4HD cells, which we have found to be the mechanisms of cell-enhanced immunogenicity. Hence, blockade of the expression of a tyrosine kinase receptor in breast cancer modulated not only the mitogenic behavior, but also the immunogenic phenotype, by increasing the expression of essential molecules for Ag recognition. Our major goal was to demonstrate for the first time that in vivo injection

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\(^4\) Abbreviations used in this paper: IGF-I, type I insulin-like growth factor; IGF-IR, IGF-I receptor; AS[S]ODN, phosphorothioate antisense oligodeoxynucleotide; MPA, medroxyprogesterone acetate; FasL, Fas ligand; Hsp70, heat shock protein 70; S[S]ODN, phosphorothioate sense oligodeoxynucleotide; DTH, delayed-type hypersensitivity; PI, propidium iodide; BFA, brefeldin A; CCA, concanamycin A.
Delayed-type hypersensitivity (DTH) assay

Mice were immunized as described above, and 1 wk after last immunization they were challenged s.c. with 2 × 10^5 irradiated C4HD wild-type cells in the left footpad and with PBS in the right footpad. DTH response was determined 48 h later, measuring footpad swelling with a dial caliper. Results were expressed as footpad swelling index obtained by subtracting right footpad thickness (baseline) from that of the left (experimental). Seven mice were included in each experimental group.

Splenocyte proliferation assay

Mice were immunized as described above, and 2 wk after last injection splenocytes were isolated and cultured in flat-bottom 96-well plates for 5 days at 2 × 10^4 cell/ml in presence or absence of 4 × 10^8 C4HD cells that had previously been incubated with 50 μg/ml mitomycin C (Sigma-Aldrich) at 37°C for 1 h. The medium used was RPMI 1640 (Life Technologies) supplemented with 10% FCS, 50 μM 2-ME, 10 nM HEPES, 2 mM glutamine, and 50 μg/ml gentamicine (all from Sigma-Aldrich). In the last 16 h, cells were pulsed with 0.5 μCi of [3H]thymidine. Cells were then harvested and incorporation of [3H]thymidine was used as a measure of DNA synthesis. Assays were performed in quadruplicate.

Determination of cytokine production

Mice were immunized as described above, and 2 wk after last injection splenocytes were isolated and cultured (4 × 10^7/ml) in flat-bottom 48-well plates in presence or absence of mitomycin-C-inactivated C4HD cells (8 × 10^6/ml) for 48 h. At the end of the culture, cells were centrifuged and supernatants were aliquoted and stored at -70°C until they were used. The content of IFN-γ and IL-5 released was quantified by sandwich ELISA using paired cytokine-specific mAbs according to the manufacturer’s instructions (BD Pharmingen).

Flow cytometric analysis

Proliferating splenocytes, which had been cocultured in the presence of C4HD cells, were washed with PBS containing 3% BSA and 0.02% sodium azide. Cells were incubated on ice with PE-conjugated anti-mouse CD8 or anti-mouse CD4 and with FITC-conjugated anti-mouse CD3 at 10 μg/ml (BD Pharmingen) for 30 min at 4°C. Cells were then washed and analyzed using a FACSCalibur cytometer (BD Biosciences). A total of 10^4 cells/sample was analyzed. Background staining was evaluated in cells incubated with the FITC- or PE-conjugated isotype control. Data analysis was performed using CellQuest software (BD Biosciences). After blocking IGF-IR function, expression of MHC class I and costimulatory molecules CD80 and CD86 was performed. C4HD cells growing in 10 nM MPA were incubated with 2 μM IGF-IR AS[5ODN or IGF-IR S[5ODN or with anti-IR3 Ab (5 μg/ml; GR11L, sodium azide-free; Oncogene) (36) or with an irrelevant Ab as control (IgG1; MOPC-21; Sigma-Aldrich). After 2 days, cells were harvested with 1 nM PBS plus EDTA and were then stained with FITC-conjugated anti-mouse CD80 (B7.1; 16-10A1) or anti-mouse CD86 (B7.2; GL-1) or with PE-conjugated anti-mouse MHC class I (H-2^d; 34-5-8S) or with the corresponding isotype controls (BD Pharmingen). The results are expressed as mean fluorescence intensity determined as mean fluorescence intensity of the isotype control cells from the mean fluorescence of each specific Ab-treated cells.

Detection of apoptosis and necrosis

C4HD cells were treated with 10 nM MPA or MPA plus 2 μM IGF-IR AS[5ODN or IGF-IR S[5ODN for 2 days and then were harvested with 1 mM PBS plus EDTA. UV-irradiated C4HD cells were used as control of apoptotic cells. Apoptosis and necrosis were investigated through cell surface binding of fluorescent annexin V by using the Annexin V-FITC binding assay (Immunotech) (37) and propidium iodide (PI) staining (38).

Cytotoxicity assay

Lysis of C4HD or LM3 target cells (mouse splenocytes, effector cells) was determined in vitro using a standard 51Cr release assay. Splenocytes obtained from mice subjected to the original immunization protocol were cocultured for 5 days with wild-type, mitomycin-cytotoxic C4HD cells. C4HD or LM3 cells were labeled with 100 μCi of 51Cr (Na^2; 1,020 Ci/mCi specific activity, 0.5 Ci/mg; NEN Du Pont) for 1 h at 37°C and were washed four times. Cells were then plated at 1 × 10^5 cells/well of a round-bottom 96-well plate in E:T ratios of 100, 50, 25, 12, and 6 and were incubated for 4 h at 37°C. In some cases, effector cells (1 × 10^6 cells/well) were preincubated with 10 μg/ml brefeldin A (BFA; Sigma-Aldrich), 200 nM concanamycin A (CCA; Sigma-Aldrich), neutralizing mAb reactive with FasL (10 μg/ml; clone M7L3), or isotype-matched control Ab (both from eBio-science) for 2 h before addition of C4HD target cells labeled with 51Cr. In the case of in vitro effector lymphocyte subset depletion, effector cells were depleted of L3T4-positive cells or of L3T8-positive cells by Dynabeads Mouse CD4 (L3T4) or Mouse CD8 (L3T8), respectively (Dynab Int.) according to the manufacturer’s instructions, and they were resuspended in the original volume before addition of C4HD target cells labeled with 51Cr. After incubation, radioactivity released was counted in a gamma counter. Wells containing only target cells in the absence or presence of 1% Triton X-100 determined spontaneous and maximal 51Cr release, respectively. Percentage of specific target cell lysis was calculated by the formula (([E-S]/(T-S)) × 100, where E is the average experimental release, S is the average spontaneous release, and T is the average total release.

Hsp70 expression

Primary cultures of C4HD cells were obtained as previously described (19, 21, 30). Protein lysates were prepared as previously described (21) from C4HD cells treated for 48 h with 10 nM MPA, with 10 nM MPA plus 2 μM AS[S5ODN or S[S5ODN, with 10 nM MPA + 5 μg/ml of an anti-IGF-IR Ab, or with an irrelevant Ab. Proteins were solubilized in sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.7 M 2-ME, and 0.01% bromophenol blue; all from Sigma-Aldrich), subjected to SDS-PAGE, and electroblotted onto nitrocellulose. Membranes were immunoblotted with the following Abs: Hsp70 (clone 7) acquired from BD Transduction Laboratories, and actin pan Ab-5 (clone ACTN50) was obtained from NeoMarkers. After washing, membranes were incubated with HRP-conjugated secondary Ab (Vector Laboratories). ECL was performed according to the manufacturer’s instructions (Amer sham Biosciences).

Statistical analysis

The differences between control and experimental groups were analyzed by ANOVA followed by Tukey t test between groups. Linear regression analysis was performed on tumor growth curves, and the slopes were compared using ANOVA followed by a parallelism test to evaluate the statistical significance of the differences. A value of p < 0.05 was accepted as statistically significant.

Results

Immunization with IGF-IR AS[S5ODN-treated C4HD cells inhibits in vivo C4HD wild-type tumor growth

We have in previous works shown that targeting IGF-IR with antisense strategies in vitro and in vivo inhibits growth of breast cancer cells (19, 21). We have also recently demonstrated that i.v. or intratumoral administration of AS[S5ODN to IGF-IR mRNA resulted in significant growth inhibition of the progestin-dependent murine mammary tumor C4HD in vivo (21). Currently, one of the most exciting aspects regarding the potential therapeutic use of antisense strategies targeting IGF-IR is the growing body of evidence indicating that a host immune response is also involved in their antitumor effect (24). Therefore, we explored whether the serial injection of IGF-IR AS[S5ODN-treated C4HD cells could provide protection against C4HD wild-type tumor challenge in vivo. C4HD cells growing in 10 nM MPA were treated in vitro with 2 μM IGF-IR AS[S5ODN for 48 h and then inactivated by irradiation. Mice were then injected s.c. with 2 × 10^6 AS[S5ODN-treated C4HD cells at 6, 4, and 2 wk before challenge with a fragment of C4HD tumor (1 mm^3). Simultaneously to tumor challenge, mice were implanted with the MPA depot. Control groups included mice injected with PBS or with 2 × 10^6 C4HD cells treated either with 10 nM MPA (named wild-type C4HD cells) or with 2 μM IGF-IR S[S5ODN plus MPA and thereafter irradiated. The effect of IGF-IR AS[S5ODN on protein expression was assessed by Western blot of C4HD cell lysates, which demonstrated that IGF-IR levels were reduced by 65–90% after 2 μM AS[S5ODN treatment as compared with wild-type C4HD cells, whereas S[S5ODN had no effect on IGF-IR levels (21). As shown in Fig. 1A, no protection against parental tumor formation was found in mice injected with PBS, with wild-type C4HD cells, or with S[S5ODN-treated C4HD cells. However, a significant delay in tumor growth was observed in mice injected with AS[S5ODN-treated C4HD cells. At the end of the
tumor volume was accordingly calculated. Each point represents the mean inactivated by irradiation with 50 Gy of 60Co. BALB/c mice were then injected s.c. in the left flank with 2 × 10^5 C4HD cells, with 2 × 10^5 wild-type C4HD cells growing in 10 nM MPA were treated in vitro with 2 μM IGF-IR AS[S]ODN or S[S]ODN for 48 h and then were inactivated by irradiation with 50 Gy of 60Co. BALB/c mice were then injected s.c. in the left flank with 2 × 10^5 AS[S]ODN-tREATED C4HD cells. C4HD cells growing in 10 nM MPA were treated in vitro with 2 μM IGF-IR AS[S]ODN or S[S]ODN for 48 h and then were inactivated by irradiation with 50 Gy of 60Co. BALB/c mice were then injected s.c. in the left flank with 2 × 10^5 AS[S]ODN- or S[S]ODN-treated C4HD cells, with 2 × 10^5 wild-type irradiated C4HD cells, or with PBS at 6, 4, and 2 wk before challenge with C4HD tumor (A), 60 tumor (B), or 3 × 10^5 LM3 cells (C). Simultaneously with C4HD tumor challenge, mice were implanted with the MPA depot. Monitoring of tumor growth began 1 wk after tumor challenge (day 1 in the curve shown in this figure). Tumor length and width were measured as described in Material and Methods, and tumor volume was accordingly calculated. Each point represents the mean volume ± SE of eight independent tumors.

experiment (day 21), growth inhibition in mice injected with AS[S]ODN-treated C4HD cells was 53.4% compared with PBS-treated mice, 61.6% compared with mice injected with wild-type C4HD, and 60.2% when compared with mice injected with S[S]ODN-treated C4HD cells. Growth rates in mice injected with AS[S]ODN were significantly lower (p < 0.001; 41.7 ± 4.9 mm^3/day) than those found in mice injected with PBS (90.7 ± 10.8 mm^3/day), with wild-type C4HD cells (112.7 ± 13.6 mm^3/day), or with S[S]ODN-treated C4HD cells (113.7 ± 14.2 mm^3/day). At day 21, tumor growth delay in mice injected with AS[S]ODN-treated C4HD cells was 5 days compared with mice injected with PBS and 6 days compared with either wild-type C4HD cells or S[S]ODN-treated C4HD cells.

To evaluate whether the protective antitumor activity after AS[S]ODN-treated C4HD cell immunization was specific to this cell type, we challenged immunized mice with other syngeneic murine mammary tumors 60 (31) and LM3 (32). We observed that all animals developed tumors despite the immunization protocol they had received (Fig. 1, B and C). Indeed, there were no differences in tumor volume, latency, or growth rates among different groups for each type of tumor. These results suggested that the immune memory developed by mice immunized with AS[S]ODN-treated C4HD cells did not induce cross-protection against other syngeneic breast tumor cells. The fact that immunization was effective only against the parental tumor line indicated that AS[S]ODN-treated C4HD cells presented Ags that were absent in the other breast cancer cell lines.

**Evidence of specific host cellular immune response to wild-type C4HD cells both in vitro and in vivo**

Up to this point, our results strongly suggested that injection with AS[S]ODN-treated C4HD cells could elicit a specific host immune response against parental C4HD cells in vivo. Thus, to accurately account for the mechanism involved in tumor rejection, we then assessed several parameters of immune function. We first evaluated whether injection of IGF-IR AS[S]ODN-treated cells induced a humoral immune response by flow cytometry analysis of Ab binding to C4HD cells in culture. Neither PBS-injected control mice nor mice immunized with wild-type C4HD cells, with S, or with AS[S]ODN-treated C4HD cells produced significant levels of IgM or IgG Abs against C4HD cells (data not shown).

To then establish the requirement of T cells in the protective antitumor response in normal BALB/c mice, we repeated the exact experimental conditions of immunization, only this time using nude mice. Immunization of nude mice with AS[S]ODN-treated cells did not prevent C4HD tumor formation, and animals from all groups developed tumors equally (Fig. 2A). The results clearly show that AS[S]ODN-treated C4HD cells did not protect nude mice from tumor development as they had in euthymic mice (Fig. 2A compared with Fig. 1A). Hence, T cells were essential to provide the antitumor effect observed in BALB/c mice. Additionally, this suggested that increased NK activity found in nude mice (39) did not contribute to antitumor activity of AS[S]ODN-treated C4HD cell immunization.

We next tested the DTH response as a measure of the overt potential of in vivo cellular immunity. BALB/c mice were subjected to the original immunization protocol. One week after last immunization, mice were challenged s.c. with 2 × 10^5 irradiated C4HD wild-type cells in the left footpad and with PBS in the right footpad. As shown in Fig. 2B, DTH reactivity increased clearly 48 h later in mice injected with AS[S]ODN-treated C4HD cells compared with control groups, suggesting a cellular immune response in the antitumoral effect.

To confirm these in vivo findings, we went on to perform several in vitro assays. We first assessed the capacity of splenocytes obtained from immunized mice to proliferate in response to the Ag C4HD wild-type cells. Only splenocytes from mice injected with AS[S]ODN-treated C4HD cells proliferated strongly in response...
FIGURE 2. Cell-mediated immunity triggered by immunization with IGF-IR AS[S]ODN-treated C4HD cells: in vivo (A and B) and in vitro (C) assays. A. Immunization in nude mice: nude mice were immunized as described in Fig. 1 with AS[S]ODN- or S[S]ODN-treated C4HD cells, with wild-type irradiated C4HD cells and with PBS, at 6, 4, and 2 wk before challenge with a fragment of C4HD tumor (1 mm³). Tumor length and width were measured as described in Material and Methods, and tumor volume was accordingly calculated. Each point represents the mean volume ± SE of eight independent tumors. B. DTH response: BALB/c mice were immunized as in Fig. 1, and 1 wk after last immunization mice were challenged s.c. with 2 × 10⁷ irradiated C4HD wild-type cells in the left footpad and with PBS in the right footpad. DTH response was determined 48 h later, measuring footpad swelling with a dial caliper. Results were expressed as footpad swelling index obtained by subtracting right footpad thickness (baseline) from that of the left (experimental). Data are presented as mean ± SE. Seven mice were included in each experimental group. *, p < 0.001. C. Proliferation of splenocytes: BALB/c mice were immunized as described in Fig. 1, and 2 wk after last immunization mice were challenged s.c. with 2 × 10⁷/ml of mitomycin-treated C4HD cells at 8 × 10⁷/ml for 48 h. Production of IFN-γ in supernatants was measured by ELISA. Each value represents the mean of six mice ± SE; *, p < 0.001. This is an experiment representative of a total of three.

FIGURE 3. Production of IFN-γ by splenocytes from immunized mice. Splenocytes, obtained from immunized mice, were cocultured at 4 × 10⁸/ml in presence (+) or absence (−) of mitomycin-treated C4HD cells at 8 × 10⁷/ml for 48 h. Production of IFN-γ in supernatants was measured by ELISA. Each value represents the mean of six mice ± SE; *, p < 0.001. This is an experiment representative of a total of three.
isolated from mice injected with AS[S]ODN-treated C4HD cells were effective in lysing C4HD cells at different E:T ratios, exhibiting the highest (~40%) cytotoxic activity at 100:1 E:T ratio (Fig. 4A). In contrast, splenocytes isolated from all three control groups showed basal cytotoxic activity (3–7%) at 100:1 E:T ratio and displayed no activity at any other E:T ratios (Fig. 4A). Cytotoxicity was specific to C4HD cells, because syngeneic breast cancer LM3 cells were not significantly lysed (<5%) by any of the splenocyte groups (Fig. 4A). These experiments were repeated three times.

To identify the T cell subset(s) responsible for the cytotoxic effect, we depleted CD4⁺ or CD8⁺ T cells by immunomagnetic separation. The cytotoxic profiles of unseparated CD4⁺ or CD8⁺ depleted splenocytes at a 100:1 E:T ratio are shown in Fig. 4C. Depletion of CD8⁺ cells from splenocytes of mice immunized with AS[S]ODN-treated C4HD cells resulted in total suppression of cytotoxic activity. In contrast, depletion of CD4⁺ cells from splenocytes of mice immunized with AS[S]ODN-treated C4HD cells did not affect cytotoxicity significantly (Fig. 4C). CD4⁺ or CD8⁺ depleted splenocytes isolated from all three control groups showed cytotoxic activity (5–9%) as low as that of the whole population. These results suggested that CD8⁺ T cells were the primary effectors mediating cytotoxic activity.

To investigate the effector mechanism that caused cytotoxicity of C4HD cells, splenocytes obtained from immunized animals were incubated with either BFA or CCA before cytotoxicity assay. BFA is an inhibitor of protein transport to the cell surface blocking FasL expression, whereas CCA is an inhibitor of exocytosis, which proved to be a selective inhibitor of perforin-based cytotoxicity (40). As shown in Fig. 4D, BFA inhibited lysis of C4HD cells by whole splenocytes from mice immunized with AS[S]ODN-treated C4HD cells as well as by splenocytes depleted of CD4⁺ cells. In
contrast, CCA did not significantly reduce the cytotoxic effect of either whole or CD4\(^+\)-depleted splenocytes from mice immunized with AS[S]ODN-treated C4HD cells. Both inhibitors had no toxic effect against either the effector or the target cell, detected by trypan blue dye exclusion or by \(^{51}\)Cr release from C4HD cells, respectively (data not shown). Cytotoxicity from both CD4\(^+\)-depleted and undepleted splenocytes was also effectively inhibited by anti-FasL Ab (Fig. 4D). These observations clearly showed that the specific cytolytic activity of CD8\(^+\) T cells induced by immunization with IGF-IR AS[S]ODN-treated cells proceeded through a Fas/FasL-dependent mechanism.

Blockage of IGF-IR expression induced phenotype changes by increasing levels of CD86 and Hsp70

To evaluate whether suppression of IGF-IR expression could have changed the cellular phenotype by inducing the molecules necessary to provoke immune recognition, we examined CD86, CD80, and MHC class I expression in C4HD cells. For this purpose, C4HD cells growing in 10 nM MPA were treated in vitro with 2 \(\mu\)M IGF-IR AS[S]ODN or with S[S]ODN for 48 h, and the presence of CD86, CD80, and MHC class I was assessed by immunofluorescence and flow cytometry analysis (Fig. 5, A–C). When

![Image](https://example.com/image1.png)

**FIGURE 5.** Induction of the costimulatory molecule CD86 and Hsp70 expression in IGF-IR AS[S]ODN or anti-IGF-IR Ab-treated C4HD cells. C4HD cells growing in 10 nM MPA were treated in vitro with 2 \(\mu\)M IGF-IR AS[S]ODN or S[S]ODN for 48 h. Cells were stained with anti-CD86 (A), anti-CD80 (B), and anti-MHC class I, H-\(^{2}\) (C) Abs and analyzed by flow cytometry. D, C4HD cells growing in 10 nM MPA were treated in vitro with 5 \(\mu\)g/ml anti-IGF-IR Ab or with 5 \(\mu\)g/ml isotype control Ab for 48 h, and cells were stained with anti-CD86. Expression levels were determined by comparing fluorescence intensity (x-axis) of CD86, CD80, and MHC class I-stained cells (solid line) to isotype control-stained cells (gray area). The results are expressed as mean fluorescence intensity determined by subtracting the mean fluorescence of the isotype control cells from the mean fluorescence of each specific Ab-treated cell. The experiment shown is representative of a total of three. E and F, C4HD cells were lysed and a total of 50 \(\mu\)g of protein was electrophoresed and immunoblotted with an Hsp70 Ab. Western blot using an anti-actin Ab was conducted using identical protein lysates as a loading control. Densitometric analysis of Hsp70 was normalized to actin levels and expressed as percentage of control value (i.e., C4HD cells growing in 10 nM MPA). These are representative experiments of a total of four; *, \(p \leq 0.05\).
compared with S[S]ODN-treated cells, AS[S]ODN-treated C4HD cells expressed significant increase of CD86 levels (Fig. 5A). In contrast, AS[S]ODN treatment did not affect CD80 and MHC class I expression (Fig. 5, B and C). There was no difference in MHC class I, CD80, or CD86 expression between C4HD cells growing in MPA alone and S[S]ODN treated-cells (data not shown). Then, an alternative strategy was applied to block IGF-IR signaling. C4HD cells growing in 10 nM MPA were treated in vitro with 5 μg/ml anti-IGF-IR Ab (36) or with isotype control Ab for 48 h. The expression level of CD86 was then evaluated, resulting in a significant increment in CD86 expression in anti-IGF-IR Ab-treated C4HD cells with respect to control Ab-treated cells (Fig. 5D). These data showed that blockage of IGF-IR activation or expression in C4HD tumor cell induced a key costimulatory molecule, CD86, necessary for T cell activation.

Another mechanism that could be promoting cell immunogenicity is the induction of Hsp70 expression. Hsp70 has been previously reported to potently activate protective CD8+ CTLs that mediate tumor immunity in animal models (41). Therefore, to further explore the mechanism involved in the cellular immune response induced by IGF-IR AS[S]ODN, we investigated Hsp70 expression levels in C4HD cells growing in vitro. As shown in Fig. 5E, IGF-IR AS[S]ODN treatment resulted in significant increase (54 ± 9.4%) in Hsp70 expression compared with C4HD wild-type or C4HD S[S]ODN-treated cells. We then used an alternative strategy to block IGF-IR signaling. Treatment of C4HD cells with an anti-IGF-IR Ab also resulted in significant induction (51 ± 7%) of Hsp70 expression in C4HD cells compared with control Ab-treated cells (Fig. 5F). These results proved that in vitro blockage of IGF-IR induced Hsp70 protein expression regardless of the strategies used. As a whole, these findings confirm that IGF-IR signaling abrogation, either through AS[S]ODN or Ab strategy, leads to an increase in CD86 and Hsp70 expression.

Because a decrease in the number of IGF-IRs was reported to cause apoptosis in several tumors as well as induction of immunogenicity in host (26, 42), we explored whether IGF-IR AS[S]ODN treatment of C4HD cells had any influence on cell survival in vitro. For this purpose, C4HD cells growing in 10 nM MPA were treated in vitro with 2 μM IGF-IR AS[S]ODN or with S[S]ODN for 48 h and were then analyzed by flow cytometry for evidence of apoptotic cell death using annexin V-FITC/PI staining. Cell staining with these two distinct fluorescent markers enabled us to distinguish live (annexin V-negative and PI-negative), apoptotic (annexin-V-positive and PI-negative), and necrotic (annexin V-positive and PI-positive) cell populations within the culture. Fig. 6 shows that C4HD cells treated with either S[S]ODN or AS[S]ODN displayed low annexin staining (7.9 and 9.4%, respectively), showing absence of apoptosis in both cases. To analyze a positive control of apoptosis, C4HD cells were irradiated with UV (50 J/m2) and cultured for 24 h. This treatment resulted in a marked increase in apoptotic (29.2%) and necrotic cells (35.1%) present in the culture (Fig. 6, lower panel). During culture, apoptotic cells eventually lost membrane integrity and switched from negative to positive for PI staining.

To demonstrate that increased expression of CD86 and Hsp70 induced by IGF-IR AS[S]ODN leads to immunogenicity of these tumor cells, we immunized mice with C4HD cells treated with IGF-IR AS[S]ODN, only this time simultaneously blocking CD86 and Hsp 70 induction. For that purpose, we treated C4HD cells with different concentrations of AS[S]ODN to CD86 and Hsp70 in presence of IGF-IR AS[S]ODN. A concentration of 2 μM CD86 AS[S]ODN effectively prevented CD86 increase induced by IGF-IR AS[S]ODN treatment in C4HD cells (Fig. 7A). Accordingly, induction of Hsp70 expression, caused by IGF-IR AS[S]ODN treatment, was prevented by using a 4 μM concentration of Hsp70 AS[S]ODN (Fig. 7B). Like before, mice were s.c. injected with 2 × 10^6 C4HD cells at 6, 4, and 2 wk before challenge with a fragment of C4HD tumor (1 mm³) for immunization. Experimental groups comprised C4HD cells growing in 10 nM MPA in presence of IGF-IR AS[S]ODN, IGF-IR AS[S]ODN + CD86 AS[S]ODN, IGF-IR AS[S]ODN + Hsp70 AS[S]ODN, or IGF-IR AS[S]ODN + CD86 AS[S]ODN + Hsp70 AS[S]ODN, which were then irradiated. Control groups included mice injected with PBS or C4HD cells treated with either 10 nM MPA or MPA + 2 μM IGF-IR S[S]ODN + 2 μM CD86 S[S]ODN + 4 μM Hsp70 S[S]ODN and thereafter were irradiated or injected with apoptotic UV-irradiated C4HD cells. As shown in Fig. 7C, none of the control groups showed any tumor growth inhibition compared with PBS-injected animals. In contrast, as was expected, significant growth inhibition was observed when mice were immunized with IGF-IR AS[S]ODN-treated cells. However, when mice were injected with IGF-IR AS[S]ODN + CD86 AS[S]ODN + Hsp70...
AS[S]ODN-treated cells, no protection against tumor formation was observed. Interestingly, partial protection from tumor development was observed when mice were immunized with IGF-IR AS[S]ODN, IGF-IR AS[S]ODN + CD86 AS[S]ODN, IGF-IR AS[S]ODN + Hsp70 AS[S]ODN, IGF-IR AS[S]ODN + CD86 AS[S]ODN + Hsp70 AS[S]ODN, IGF-IR AS[S]ODN + CD86 AS[S]ODN + Hsp70 AS[S]ODN, IGF-IR AS[S]ODN + CD86 AS[S]ODN + Hsp70 AS[S]ODN + CD86 C4HD-treated cells, wild-type C4HD cells, UV-irradiated C4HD, or with PBS. The doses were administered at 6, 4, and 2 wk before challenge with a fragment of C4HD tumor (1 mm³). Simultaneously to tumor challenge, mice were implanted with the MPA depot. Monitoring of tumor growth began 1 wk after tumor challenge (day 1 in the curve shown in this figure). Tumor length and width were measured as described in Material and Methods, and tumor volume was accordingly calculated. Each point represents the mean volume ± SE of eight independent tumors.

Discussion

Currently one of the most exciting aspects of targeting IGF-IR in cancer treatment is that a host’s immune response appears to be involved in the antitumor effect of blocking IGF-IR expression by using antisense strategies (24, 26). There are clear precedents for the concept that inhibition of IGF-IR signaling can render certain tumor cells immunogenic. Although several pieces of evidence indicate that T cell activation is responsible for the antitumor effect of blockage IGF-IR expression, the mechanism mediating this effect has not yet been determined. In the present study, we have demonstrated that serial injection of AS[S]ODN to IGF-IR mRNA-treated C4HD cells provides protection against C4HD wild-type tumor challenge in vivo. Also, with that we have characterized the immune response elicited by in vivo administration of IGF-IR AS[S]ODN-treated breast cancer cells, as well as a mechanism involved in the induction of the immunogenic phenotype of treated tumor cells.

Our results are in line with a series of earlier reports by Baserga and coworkers (24, 25) that demonstrated that rat C6 glioblastoma cells, stably expressing antisense IGF-IR transcripts, elicited a protective host response after injection into syngeneic rats, which served to protect against tumor formation by subsequent injection of C6 wild-type cells. Furthermore, injection of C6 IGF-IR antisense cells causes regression of established tumors (24, 25). In another study, injection of antisense IGF-IR plasmids into established mouse N2A neuroblastomas growing in syngeneic A/J mice resulted in inhibition of tumor growth (26). Interestingly, when those mice in which complete tumor regression occurred after antisense injections were rechallenged with s.c. inoculation of unmodified N2A cells, no tumors formed in any of these animals until two months later (26). The mechanism by which IGF-IR antisense cells might induce immune recognition in a syngeneic host has not been clearly elucidated. Particularly in the C6 brain tumor model, Baserga and coworkers (42) have postulated that apoptosis induced by antisense against IGF-IR could be a specific means to achieve a host antitumor response. Based on this hypothesis, they proposed that a soluble factor is released into the systemic circulation by the cells undergoing apoptosis, which could have direct antitumor cytotoxicity and/or induce cellular immunity. By contrast, we were not able to find any apoptotic signal in C4HD AS[S]ODN-treated cells (Fig. 6). This prompts us to propose that in our model of breast cancer, what determines the induction of an immune response is not the way in which the cells die, but rather their transformation into a new cell phenotype as a consequence of IGF-IR suppression that allows the immune system to recognize the tumor cell and therefore activate the effector machinery to destroy the growing tumor.

In the present study, we clearly found that a cellular immune response is involved in the mechanism of C4HD tumor rejection. First, there was no evidence of Ab production against tumor cells in mice immunized with AS[S]ODN-treated cells, suggesting the
absence of a humoral type response. Second, protection from tumor growth after immunization with AS$\odot$ODN-treated C4HD cells was absent in nude mice, which lack T lymphocytes. Moreover, the marked increase in DTH reactivity seen in BALB/c mice injected with AS$\odot$ODN-treated C4HD cells compared with control groups indicated induction of in vivo cellular immunity. Third, splenocytes from mice injected with AS$\odot$ODN-treated C4HD cells proliferated strongly in response to C4HD cells, duplicating CD8$^+$ T lymphocytes (but not CD4$^+$), whereas lymphocytes from control groups remained unresponsive to C4HD cells. Furthermore, whereas splenocytes isolated from mice injected with AS$\odot$ODN-treated C4HD cells exhibited high cytotoxic activity, splenocytes isolated from control groups showed basal levels of cytotoxic activity. Cytotoxicity was specific to C4HD cells, because syngeneic breast cancer LM3 cells were not significantly lysed by splenocytes from mice injected with AS$\odot$ODN-treated C4HD cells, confirming in vivo findings using syngeneic breast tumors 60 and LM3. Additionally, the results demonstrating that depletion of CD8$^+$ T cells, but not of CD4$^+$ T cells, caused complete inhibition of cytotoxic activity clearly indicate that this activity is mediated by the CD8$^+$ T cell population. These findings are consistent with a previous study by Resnicoff et al. (24), which described the induction of a CD8$^+$ cellular response in the antitumor effect of antisense blocking of IGF-IR expression in C6 cells with no detected humoral response. Similarly, Trojan et al. (28) found that injection of C6 tumor cells that had been transfected in vitro with an antisense construct to IGF-I resulted in regression of established wild-type tumors. Marked infiltration of regressing tumors by CD8$^+$ CTLs was demonstrated by immunostaining of tumor sections.

Previous studies have established two major pathways for CTL cytotoxicity, which are dependent on perforin or FasL (43). The FasL-dependent pathway, which seems to be mainly operative with CD4$^+$ T cells (44) but is also operative with CD8$^+$ T cells, is mediated by FasL expressed on activated CTLs, which triggers Fas-mediated apoptosis in target cells (45). This pathway of cellular cytotoxicity is thought to be primarily involved in immune regulation and maintenance of tolerance. In this context, Fas expression level is thought to be a determining factor in induction of apoptosis (46). The data presented here clearly demonstrate that C4HD tumor cells can undergo cytolysis induced by CD4$^+$ T cell-depleted splenocytes, obtained from mice immunized with AS$\odot$ODN-treated C4HD cells, through the Fas/FasL, but not through the perforin-dependent lytic pathway. Evidence of Fas/FasL pathway involvement was provided by the fact that cytotoxicity against C4HD cells, which constitutively express MHC class I and Fas molecules (data not shown), was blocked by preincubation with BFA, an inhibitor of protein transport, and with a FasL blocking Ab, but not with CCA, an inhibitor of the secretory pathway (perforins) (40). The prevailing concept is that FasL-mediated cytotoxicity by CD4$^+$ T cells is restricted to MHC class II-expressing APCs and that it acts immunoregulatorily, whereas FasL-mediated cytotoxicity by CD8$^+$ T cells is directed to infected or transformed cells expressing MHC class I, and thus acts for immunosurveillance (47). In line with our results, Bergmann-Leitner and Abrams (48) clearly demonstrated that human CD8$^+$ T cells had the ability to lyse a colon adenocarcinoma cell in an Ag/MHC class I-restricted manner, involving a Fas/FasL-based cytotoxic mechanism in which IFN-$\gamma$ was crucial for the modulation of the lytic phenotype.

It is well known that type 1 cytokines (IFN-$\gamma$) mediate inflammatory response and tumor rejection, whereas type 2 cytokines (i.e., IL-5) induce humoral immune response. Moreover, it has been reported that as in the case of CD4 T cells, CD8 T lymphocytes can be classified into distinct effector cell types based on their cytokine-secreting profiles after tumor Ag encounter (49, 50). Type 2 CD8 T cells preferentially secrete IL-4, IL-5, and IL-10 and kill predominantly through the perforin pathway, whereas type 1 CD8 T cells predominantly secrete IFN-$\gamma$ and kill through either perforin or a Fas-mediated mechanism (49, 50). Our data strongly demonstrated that immunization of mice with AS$\odot$ODN-treated C4HD cells resulted in induction of a type 1 response, because its splenocytes produced augmented levels of IFN-$\gamma$ in response to C4HD wild-type cells, with no induction of IL-5.

One of the major goals of this work was to disclose the possible mechanisms by which AS$\odot$ODN to IGF-IR mRNA-treated breast cancer cells acquired immunogenic characteristics. On one hand, CTL activation is determined by a complex balance between the strength of the antigenic signals and the degree of costimulatory molecules. Ag silencing will occur when combination of signals from peptide-MHC class I molecules and costimulation is insufficient, a situation commonly observed in tumor cells (9). Several investigators have shown that a more effective antitumor response is elicited against tumor cells that are modified to express the costimulatory molecule B7 (CD80 and CD86) (15, 51). The present data showed that either AS$\odot$ODN or Ab to IGF-IR treatment induced the expression of the costimulatory molecule CD86 in breast cancer cells. Previously, Trojan et al. (52) found B7 up-regulation in rat C6 glioma, in human primary glioma, and in mouse teratocarcinoma cells transfected with a vector encoding antisense IGF-I. However, they did not characterize whether B7 increment corresponded with CD80 and/or CD86 expression. They also demonstrated an increment in MHC class I levels in C6 cells after transfection with antisense IGF-I plasmid. Nevertheless, we did not find any change in MHC class I expression after blocking IGF-IR. Lack of MHC class I regulation was described by Resnicoff et al. (24) in IGF-IR antisense-treated C6 cells in vitro. On the other hand, heat shock proteins (gp96, Hsp70, Hsp90) are associated with a broad range of peptides derived from cells, in such a manner that the Hsps chaperone the antigenic repertoire of the cell from which they are obtained (53). The effectiveness of mouse immunization with tumor-derived Hsp-peptide complex is exclusively dependent on the presence of functional APCs in the immunized host (54). Among APCs, bone marrow-derived dendritic cells can translocate Ags from the endocytic to the cytosolic compartment of the cells, thereby directing endocytosed proteins into the MHC class I presentation pathway, a process known as cross-presentation (53). It was demonstrated that gp96 and Hsp70-chaperoned peptides can be presented to CTLs by dendritic cells in the context of MHC class I molecules (55). Based on these previous reports, we investigated whether the immunogenic phenotype acquired by C4HD tumor cells after blockage of IGF-IR could be due in part to induction of Hsp70 expression. In the present study, we for the first time found that in vitro blockage of IGF-IR expression either by AS$\odot$ODN or by blocking Ab induces Hsp70 protein expression in breast tumor cells. Moreover, we observed that blockage of IGF-IR AS$\odot$ODN capacity to induce Hsp70 and CD86 in C4HD cells resulted in abolishment of the in vivo antitumor effect provided by inhibition of IGF-IR expression. We also assessed the in vitro durability of IGF-IR AS$\odot$ODN effect after its removal from the culture medium and observed that up to day 4 both CD86 and Hsp70 levels were still high, whereas at day 6 expression levels returned to basal (data not shown). Nevertheless, it is worth pointing out that when irradiated tumor cells are injected as immunogens, the immune machinery, which includes among others macrophages and dendritic cells, is quickly activated (within ~24 h) to process such tumor cells into Ags (56). Within this period of time, Hsp70 and CD86 levels are clearly high, as a
result of IGF-IR AS[+]ODN treatment, enhancing Ag presen-
tation. These data for the first time demonstrated that IGF-IR AS[+]ODN-treated breast cancer cells induced expression of CD86 and Hsp70 molecules, both involved in the induction of the immunogenic phenotype. Furthermore, we were able to demonstrate that in breast cancer cells, it was not induction of apoptosis in AS-
[+]ODN-treated cells that caused immune activation, as had been proposed in C6 glioblastoma cells (42). Clearly, apoptosis was not detected in AS[+]ODN-treated cells (Fig. 6) nor was there any protection when mice were immunized with apoptotic UV-irradiated C4HD cells (Fig. 7). Therefore, it is tempting to hypothesize that activation of the host’s immune system, which we achieved through injection of IGF-IR AS[+]ODN-treated C4HD cells, de-
rives partly from induction of CD86 and Hsp70 expression in C4HD cells. Reinforcement of the costimulatory and Ag-traffick-
king machinery might have provided a functional signal to the im-
mune system, thereby breaking the tolerance to tumor Ags, which otherwise remain immunologically hidden, and eliciting a protec-
tive CTL-mediated tumor immunity.

In summary, we have demonstrated that breast cancer growth can be inhibited by administration of tumor immunogens obtained by treating breast cancer cells with IGF-IR AS[+]ODN, acting through activation of the host’s cellular adaptive immune re-
sponse involving the production of IFN-γ and a CD8+ cell-mediated cytotoxicity via Fas/FasL pathway.

Acknowledgments

We are grateful to Dr. Alfredo Molinolo for his continuous assistance and support. We also thank N. Lope for her expert assistance with animal care, R. Ruggiero for helpful discussions of the immunization protocol, Laborato-
rios Gador for providing Medrosterona, and C. Lanari for providing the

Disclosures

The authors have no financial conflict of interest.

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