Immunotherapy of cancer by antisense inhibition of Ii protein, an immunoregulator of antigen selection by MHC class II molecules

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Ii protein suppression is a promising antisense drug-based therapy that dramatically enhances the immunogenicity of tumor cell major histocompatibility complex class II-presented antigenic epitopes. The strength of this approach is that the antisense only needs to be transiently effective in a fraction of the tumor cells. The systemic antitumor immune response generated subsequently eradicates both directly treated cells and distant tumor deposits. The drugs and mechanisms of this therapy are considered, in addition to practical developmental questions.

Keywords Cancer vaccine, Ii antisense, immunotherapy, major histocompatibility complex class II, T-helper cells

Introduction

Thwarting a tumor's escape from immune surveillance

Tumors escape the host immune surveillance, which normally prevents evolution of a malignancy by blocking presentation of immunogenic tumor-associated antigenic epitopes. Additionally, tumors suppress the antitumor immune response by developing active immunosuppressive mechanisms against dominant T-cell-recognized antigenic epitopes of the tumor.

Protein Ii can be inhibited in two ways against these tumor growth-favoring mechanisms. Presentation of tumor-associated antigenic epitopes is enhanced by rearranging the antigen-processing pathway to allow major histocompatibility complex (MHC) class II presentation of tumor self-proteins to T-helper (Th) cells. This method also favors presentation of cryptic and subdominant epitopes to which immunosuppression had never developed previously. The approach can even cure mice of an aggressive prostate tumor that is poorly immunogenic. The biological mechanism of this therapy and the steps taken to bring it to the clinic are reviewed.

Overview of mechanism and therapeutic potential

Normally, antigenic epitopes of cellular self-proteins are transported from the cytoplasm into the endoplasmic reticulum for binding to newly synthesized MHC class I molecules, but not to MHC class II molecules, which are blocked by the Ii protein. The Ii protein is effectively suppressed with antisense oligonucleotides or reverse gene constructs. This process can be 'rearranged' in the endoplasmic reticulum of such Ii-suppressed tumor cells, to permit MHC class II molecules to bind tumor-associated antigenic epitopes for subsequent presentation to self-surveying T-cells. New epitopes presented by MHC class II molecules stimulate Th cells to enhance the activity of tumor-specific cytotoxic T-lymphocytes (CTLs) and create long-lasting antitumor immunological memory. The repertoire of MHC class II epitopes is also expanded to include 'cryptic epitopes', to which a cancer patient's immune system has never been exposed. The presentation of such epitopes by MHC class II molecules can reverse immunological tolerance to the tumor and cure an established tumor, at least in mice. While this antisense drug is directly therapeutic when injected into tumors that are either naturally MHC class II-positive or made such by co-transfection of genes for MHC class II transactivator (CIITA) or interferon (IFN)γ, it also enables many additional therapies. Ii suppression can enhance various DNA vaccines for tumor or infectious disease antigens. In addition, novel peptide therapeutics can be mined from the repertoire of induced MHC class II epitopes found in MHC class II+/Ii- cultured cells of tumors or antigen-presenting cells (APCs) loaded with antigens relevant to autoimmune disease. In short, antisense-induced Ii suppression enables a wide range of antigenic epitope diagnostics and therapeutics.

Evidence for mechanisms

Antigen processing and presentation pathways potentially blocked during tumorigenesis

All nucleated cells express MHC class I molecules. At the time of their synthesis, these molecules bind antigenic peptides derived from cytoplasmic proteins, which are processed into peptides by proteosomes and transported by the transporter of antigenic peptides (TAP) into the endoplasmic reticulum [1,2]. β_2-Microglobulin binds to MHC class I molecules, locking them into a conformation that tightly holds the antigenic peptide for the duration of its presentation at the cell surface [3].

MHC class II molecules are normally expressed on professional APCs, such as dendritic cells (DCs), macrophages and B-cells, to induce a CD4+ Th cell response. Such Th cells induce DCs to a stage of activation defined as 'licensing', which stimulates and activates specific CTLs [4•,5•,6].

Normally, MHC class II molecules are blocked by the Ii protein at the time of their synthesis, and receive exogenous antigen selected by the APCs in a post-Golgi compartment. There, certain proteases that cleave the foreign antigen also

cleave and release Ii fragments from the MHC class II molecules in a concerted exchange process, during which antigenic peptide is inserted into the binding site of class II molecules [7,8]. This pathway prevents expression of endogenous peptides by MHC class II molecules in tumor cells. Interestingly, an increase in Ii in hairy leukemic cells and the inverse correlation of Ii expression and tumor-infiltrating lymphocytes in human colon carcinomas suggest an immunosuppressive role for Ii in such malignancies [9,10]. MHC class II molecules appear to influence the antitumor response [11-13,14•]. The absence of, or defects in these antigen processing and presentation pathways have been individually reported to promote non-recognition of tumors.

Tumor defense by immunosuppression

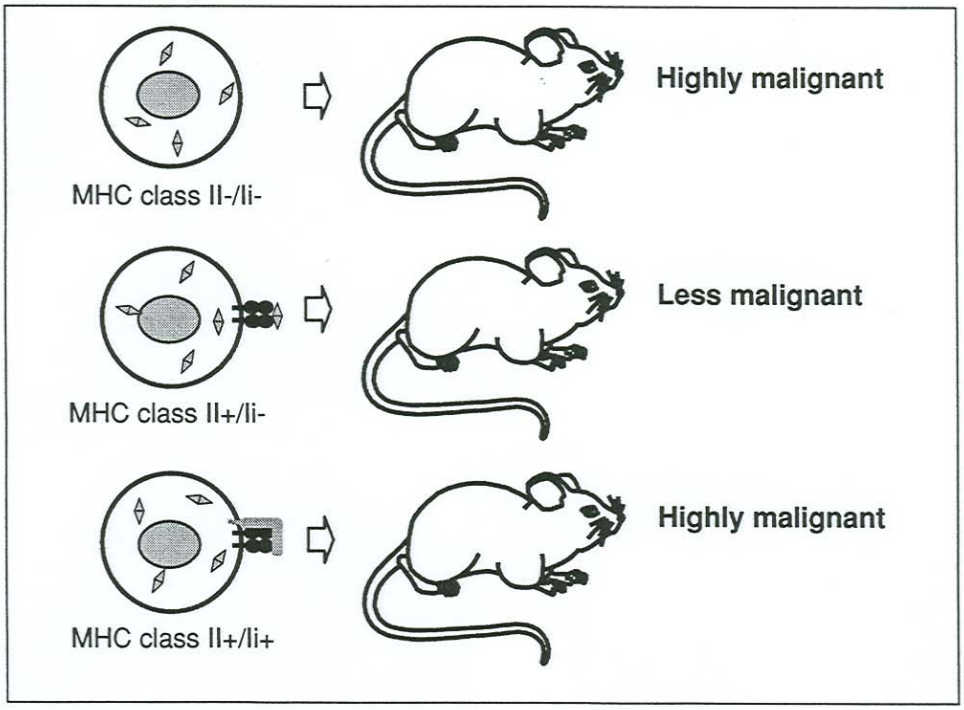
Dominant epitopes within a population of tumor-associated antigenic epitopes often induce suppression, which shuts off all antitumor immune responses. This immunosuppression is sometimes associated with the T-cell subset Th3 CD4+/CD25+ immunoregulatory cells [15••,16,17••,18], which secrete interleukin (IL)-10 and transforming growth factor (TGF)β. This is in contrast to the IFNγ-secreting Th1 cells, a Th subset that promotes CTL responses [19•,20-22].

One can hypothesize that by downregulating expression of the Ii protein, a much larger repertoire of MHC class II-restricted epitopes is presented, including cryptic epitopes, which have never been seen before by the immune system. The immune system also recognizes newly exposed, lower-affinity Th cell-recognized epitopes, to which tolerance has never been developed. The response to both types of such non-immunosuppressed epitopes leads to a robust Th1 response, stimulating tumor-specific CTLs and providing long-term immunological memory.

Pioneering experiments

Suzanne Ostrand-Rosenberg and colleagues discovered the principle that MHC class II+/Ii- tumor cells, made MHC class II-positive by transfection of genes for syngeneic MHC class II α and β chains, and not expressing Ii protein, are potent anticancer vaccines [23-25]. These researchers characterized the immunological mechanisms in detail (Figure 1). Mice vaccinated with MHC class II gene-transfected Sal 1 sarcoma cells rejected subsequent challenges with the parental MHC class I+/MHC class II- Sal I cells [23]. However, supratransfecting the engineered MHC class II+ tumor cells with the *Ii* gene abrogated the vaccine potential of the modified cells. The destruction of tumor cell immunogenicity following re-introduction of the *Ii* gene presumably resulted from its ability to block the binding of endogenous tumor-associated peptides in the endoplasmic reticulum and consequent development of a Th cell response to epitopes from those peptides. Both CD4+ Th cells and CD8+ CTLs were found to be essential, as deletion of either T-cell subpopulation in adoptive transfer of immunity experiments abrogated the protective effect [25]. This study is consistent with the established principle that the activation of CD4+ Th cells by MHC class II+/Ii- tumor cells is required for optimal activation and expansion of CD8+ CTLs. Finally, the importance of Th cells activated by MHC class II+/Ii- tumor cells in prolonging immunological memory was demonstrated by the protection of mice against tumor challenge for extended periods of time after vaccination [25].

Figure 1. Tumor malignancy and Ii expression.



When tumor cells are MHC class II+ and Ii+, the tumor is highly malignant, as Ii blocks presentation of tumor antigen through MHC class II. The same situation applies to tumor cells that are MHC class II- and Ii-. Only when they are MHC class II+ and Ii- do tumor cells present the tumor antigen by MHC class II to activate CD4+ T-cells, which in turn activate CD8+ CTLs.

Endogenous proteins from different intracellular compartments of a tumor cell can be presented by MHC class II+/Ii- tumor cells [26,27]. This is demonstrated by the finding that cells engineered to express the gene for hen egg lysozyme (HEL) with a leader sequence targeting the endoplasmic reticulum, presented MHC class II HEL epitopes to HEL-specific CD4+ T-cells. Similar to the studies described above, co-expression of the Ii protein in these cells inhibited presentation of the HEL epitopes [27].

Antisense suppression of Ii in tumors induced for MHC class II and Ii expression

Given the numerous different MHC class II alleles in humans, even only at the human leukocyte antigen (HLA)-DR locus, generating the MHC class II+/Ii- phenotype by transfecting a patient's tumor with genes matching MHC class II α and β chain types is not a practical clinical approach. We developed an alternative approach using a non-polymorphic gene construct active in all humans. Expression of endogenous MHC class II molecules can be induced using *CIITA* or *IFN*γ genes, while the co-induced Ii protein is suppressed with a reverse gene construct (*Ii*-RGC) targeting Ii mRNA. *Ii*-RGC acts at the mRNA level to prevent translation of the Ii protein. Initially, Ii antisense oligonucleotides were used to suppress Ii expression in MHC class II+/Ii+ tumor cells [28]. In these studies, using the Sal 1 sarcoma model, MHC class II+ cells treated with Ii antisense oligonucleotides demonstrated good vaccine potency against challenge by parental tumor.

Subsequently, we created expressible Ii antisense constructs (*Ii*-RGC) for inclusion into DNA vaccine vectors. These constructs were cloned into expressible plasmids and/or engineered into adenoviruses to evaluate different methods of tumor gene transfection [29,30]. The *Ii*-RGCs were evaluated by stable or transient DNA transfections using several murine tumor cell lines; the most active, *Ii*-RGC(-92,97) (A in the AUG start codon is position 1), was selected for *in vivo* studies.

While some tumor cell lines were MHC class II+/Ii+, many of the lines we tested were MHC class II-/Ii-. In these cell lines, the *CIITA* or *IFN*γ gene, or both, were co-transfected *in vitro* with *Ii*-RGC(-92,97) to create the MHC class II+/Ii- phenotype, as detected by immunostaining [29-31]. *In vivo* induction of this phenotype in established tumors was also generated by intratumoral injection of *Ii*-RGC and *CIITA* plasmids delivered in liposomes [29,31], or using recombinant adenoviral vectors containing *Ii*-RGC(-92,97), *CIITA* and *IFN*γ [30]. While the *CIITA* gene used in the mice studies is human, its gene product fortuitously functions well on the murine promoters for MHC class II and *Ii* genes [30].

Ii suppression therapy of animal tumors

We tested our therapeutic strategy for both tumor prevention (vaccination to protect against tumor challenge) and tumor cure (therapy of established tumors). In a prevention model using Sal 1, tumor cells treated with antisense oligonucleotide-suppressed Ii protein were much more potent than Sal 1 cells treated with sense and mismatch antisense oligonucleotides [28]. In cure models, the *in vivo* activities of these therapeutic constructs were tested by intratumoral injection of plasmids or adenoviral vectors in established subcutaneous tumors of both the Renca renal adenocarcinoma and RM-9 prostate carcinoma murine models [29,31].

In tumor cure models, complete regression of established tumors was achieved. Renca tumor regression was observed in approximately 50% of mice following four intratumoral injections of *CIITA* and *Ii*-RGC plasmid constructs over 4 days administered with a subtherapeutic dose of *IL-2* plasmid [29]. In these tumor nodules, *in situ* induction of the MHC class II+/Ii- phenotype was confirmed by immunohistochemical staining of tumor sections [29]. The injection of established Renca tumors with recombinant adenovirus, containing *CIITA*, *IFN*γ and *Ii*-RGC, combined with a low suboptimal dose of *IL-2* adenovector, induced complete tumor regression in approximately 60 to 70% of mice and complete protection against Renca tumor re-challenge [30]. These studies using the weakly immunogenic Renca MHC class I+/class II- model confirm that induction of the MHC class II+/Ii- phenotype triggers an antitumor immune response with long-lasting systemic immunity.

In the aggressive, poorly immunogenic MHC class I-/class II- RM-9 prostate tumor model, *in situ* induction of the MHC class I+/class II+/Ii- phenotype by intratumoral injection of the plasmids *pCIITA*, *pIFN*γ and *pIi*-RGC caused a significant but transient inhibition of tumor growth, even when suboptimal doses of pIL-2 were added to the tumor nodule treatment (Table 1) [31]. Complete responders were observed only when tumor nodules were first irradiated prior to gene therapy [31]. In a subsequent experiment, established RM-9 subcutaneous tumors were selectively irradiated and treated 1 day later with intratumoral plasmid gene therapy using a mixture of the plasmids *pCIITA*, *pIFN*γ and *pIi*-RGC combined with a subtherapeutic dose of *pIL-2* for 4 consecutive days. Table 1 demonstrates that intratumoral treatment with all four plasmids induced complete tumor regression in more than 50% of the mice only when tumor irradiation was administered 1 day prior to gene therapy. Mice rendered tumor free by radiation and intratumoral gene therapy and re-challenged on day 64 were protected against RM-9 challenge but not against syngeneic EL-4 tumor challenge (Table 1) [31]. These findings demonstrate that in the RM-9 model, radiation enhanced the therapeutic efficacy of intratumoral gene therapy for *in situ* induction of tumor-specific immunogenicity.

IL-2 at subtherapeutic doses is probably acting as an adjuvant to strengthen and sustain the activation of T-cells. Our recent studies demonstrate that both CD4+ Th cells and CD8+ CTLs are essential for the induction of a complete antitumor response in the RM-9 model, specifically, the *in vivo* depletion of either T-cell subset abrogates this response [GG Hillman *et al*, unpublished data]. These studies are consistent with induction of both a Th response and a CTL response by our gene therapy approach, resulting in long-lasting tumor immunity. Furthermore, in both the Renca and RM-9 model, omission of the *Ii*-RGC vector in the intratumoral gene therapy protocol led to a significantly lower incidence of complete tumor regressions, emphasizing the essential role of Ii suppression in the induction of a complete and systemic antitumor immune response [30,31].

Table 1. Antitumor response of RM-9 tumor-bearing mice treated with radiation and plasmid gene therapy, and response of cured animals to re-challenge.

Treatment group	Proportion of tumor-free mice		
	Post-treatment	Post-challenge	
		RM-9	EL-4
Control	0/10	-	-
pCIITA + pIFNγ+ pIL-2 + pIi-RGC	0/7	-	-
Radiation	0/11	-	-
Radiation + *pCIITA + pIFNγ+ pIL-2 + pIi*-RGC	7/13	7/7	-
Radiation + *pCIITA + pIFNγ+ pIL-2 + pIi*-RGC	3/6	-	0/3
Naive mice	N/A	0/5	0/5

Established RM-9 tumors of 0.3 to 0.4 cm were irradiated with 8 Gy photons on day 6 after cell injection. From day 7, tumors were injected with the plasmids *pCIITA + pIFNγ+ pIL-2 + pIi*-RGC for 4 consecutive days. The proportion of tumor-free mice is presented at the end of the observation period, on day 64, following radiation and plasmid therapy. Tumor-free mice and naive mice were re-challenged with RM-9 cells or genetically identical EL-4 cells on day 64, the proportion of tumor-free mice by day 30 after challenge is reported [31]. **N/A** not applicable.
(Reproduced with permission from Mary Ann Liebert and Hillman GG, Xu M, Wang Y, Wright JL, Lu X, Kallinteris NL, Tekyi-Mensah S, Thompson TC, Mitchell MS, Forman JD: ***Radiation improves intratumoral gene therapy for induction of cancer vaccine in murine prostate carcinoma.*** *Human Gene Therapy (2003)* ***14****(8):763-775. © 2003 Mary Ann Liebert).*

Role of radiation

The role of radiation in enhancing intratumoral gene therapy for the induction of cancer immunity is particularly intriguing. Possible mechanisms for radiation enhancement of gene therapy include the following: (i) the DNA damaging and debulking effect slows tumor growth to allow time for the immune response to be effective [31,32]; (ii) radiation-induced tissue damage mobilizes inflammatory cells in the tumor vicinity that can be readily activated by *in situ* gene therapy [32]; (iii) radiation is hypothesized to limit suppressive immunoregulatory T-cells; and (iv) radiation increases gene transduction efficiency and duration of expression of surviving tumor cells, thus improving the efficiency of *in situ* genetic modification, leading to an immune response that eradicated remaining tumor cells. Stevens and colleagues demonstrated that radiation improves the transfection efficiency of plasmid DNA in normal and malignant cells *in vitro* resulting from radiation-induced DNA breaks and DNA repair mechanisms [33,34]. They demonstrated that radiation followed by plasmid or adenoviral transfection caused enhanced integration of the transgene. Preliminary studies in the Renca model using intratumoral injections of the *IL-2* adenovector demonstrated that radiation potentiates the genetic modification of the tumor cells by increasing both the level and duration of expression of transfected genes [GG Hillman, unpublished data].

Unique features of Ii antisense therapy

Making antisense therapy effective clinically for other cancer molecular targets has two daunting obstacles. One is the requirement for delivering antisense reagents to all tumor cells, and the other is the requirement for suppressing the target gene continuously [35•,36]. Comparatively, Ii suppression for tumor immunotherapy has three advantages. Firstly, it stimulates tumor antigen-specific CD4+ Th cell activation without interrupting MHC class I presentation for CD8+ CTL activation. Simultaneous activation of both CD4+ and CD8+ T-cells creates a more robust tumor cell immunotherapy. Secondly, inhibition of Ii protein expression does not need to occur in all tumor cells, unlike antisense targeting other tumor genes. Thirdly, the Ii inhibition does not need to be continuous, as a transient inhibition of Ii protein (3 to 5 days) in a portion of tumor cells is sufficient to generate a strong antitumor immune response [30,31]. After a specific antitumor immune response has been generated, the immune system will eradicate the residual tumor cells until all tumor cells sharing the same tumor antigens have been killed.

Future directions

Issues to address in the design of clinical trials

The biological mechanism of this therapy leads to special considerations for the design and evaluation of clinical trials. For initial trials, patients with metastatic disease poorly responsive to other means of therapy will be targeted. Although chemotherapy in such patients suppresses the anticancer immune response, once leukocyte counts have rebounded, this mode of immunotherapy eliciting an immune response within injected tumor masses, is possible. Readily accessible, ie, subcutaneous axillary masses in breast carcinoma might be preferred because they can be approached with ease. However, many radiologists feel that any tumor they can visualize is a candidate for intratumor injections, for example, colon adenocarcinoma. While melanoma has been a classic target for tumor immunotherapy procedures, the frequency of accessible masses, frequency of patients with metastatic disease, and a variable and sometimes long clinical course, indicates against melanoma for initial trials.

Injection of a tumor with *Ii*-RGC is expected to lead to presentation of normal tissue antigens, as well as tumor determinates. While we have not observed histological signs of autoimmunity in mice with cancers treated by this method, such reactions against self-tissues probably occur. Those reactions on balance might promote tumor cure. Vitiligo is observed in some melanoma patients vaccinated against melanoma-associated tumor antigens. Signs of autoimmunity to normal tissue antigens of the tumor will be monitored in trials of this immunotherapy.

Additional therapeutic uses for Ii suppression

This technology can also be used to identify novel MHC class II epitopes in tumor and autoimmune disease-related antigens. Specifically, tumor- and autoimmune disease-related antigenic epitopes can be identified by high performance liquid chromatography (HPLC) tandem mass

spectrometry of acid-eluted peptides from immune purified MHC class II molecules. The eluted-peptide HPLC patterns can then be compared with those of MHC class II+/Ii+ cells to identify the putative Ii suppression of specific peaks [37•]. The molecular weight of a peptide in such a peak can be precisely determined by tandem mass spectrometry and a sequence assigned from the weight.

Ii suppression can also enhance the efficiencies of DNA vaccines and of gene-transfected DC vaccines. The biological effect of Ii suppression might enhance the immune response to a co-delivered DNA vaccine containing a gene for a malignant or infectious antigen [38-40]. When a cell (eg, a professional APC) is transfected with a gene encoding an antigen and an Ii suppression construct, this cell expresses the antigen endogenously, while Ii is suppressed to produce the MHC class II+/Ii- phenotype. Consequently, the transfected cell can now present antigenic epitopes through MHC class II and I to activate both CD4+ Th cells and CD8+ CTLs, respectively. The result is a stronger DC or DNA vaccine.

Conclusion

We have developed a novel antisense approach to convert tumor cells into MHC class II+ and Ii- APCs. Suppression of Ii gene expression in the endoplasmic reticulum leads to the simultaneous presentation of endogenous tumor antigens by both MHC class I and II molecules and generates a robust and long-lasting antitumor immune response. Injecting murine tumors with genes that induce the MHC class II+/Ii- phenotype in tumor cells causes complete tumor regression (ie, cure) in a significant number of animals with renal and prostate tumors.

Compared with other antisense applications, our Ii antisense method has two major advantages; Ii only needs to be suppressed temporarily and it does not need to be suppressed in all tumor cells. Analogous human Ii antisense gene constructs that are suitable for most patients and cancers have been developed.

Conversion of cancer cells into APCs via induction of the MHC class II+/Ii- phenotype *in vivo* by this method is simple to achieve. The induction of MHC class II molecules and Ii by *CIITA* and suppression of Ii by *Ii*-RGC antisense is a clinically practical strategy, as both *CIITA* and *Ii* genes are monomorphic. Transduction of a focal population of cells within a tumor mass stimulates an immune response that potentially leads to destruction of all tumor cells within that mass as well as in distant metastases. This is particularly relevant to the anticipated clinical use where local treatment to induce a potent systemic antitumor immune response is the goal.

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• of special interest

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