Class I Major Histocompatibility Complex Enhancement by Recombinant Leukocyte Interferon in the Peripheral Blood Mononuclear Cells and Plasma of Melanoma Patients

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ABSTRACT

In vivo administration of escalation doses of recombinant α-interferon (IFN-α) during a phase I trial in malignant melanoma patients caused dose-dependent increases in the mRNA accumulation, synthesis, steady state cellular content, and plasma membrane expression of class I major histocompatibility complex molecules in peripheral blood mononuclear cells. In addition, circulating levels of class I molecules were also enhanced. These findings show that (a) antigenic enhancement by biomodifiers may occur in vivo, in humans and (b) the mechanism of class I major histocompatibility complex enhancement by IFN-α is similar in vitro and in vivo. Furthermore, because peripheral blood mononuclear cells of different melanoma patients display different susceptibility to IFN-α, the entity of their antigenic modulation may represent a useful parameter to evaluate the efficacy of different therapeutic regimens and/or assess the individual susceptibility to the molecular changes induced by IFN-α.

INTRODUCTION

The interferons have been the first biomodifiers to become available in highly purified recombinant form (1). Of the three types of interferons, from leukocytes (IFN-α, β), and immune T-lymphocytes (IFN-γ), IFN-α has been the first and most widely used in clinical studies.

Even though the nonconventional cytostatic and immunomodulatory properties of IFN-α have widened its applicative spectrum well beyond the conventional field of antiviral therapy (reviewed in Ref. 2), its antineoplastic effects do not favorably compare with those of conventional cytostatic drugs, except in certain hematological malignancies (3). Some or all of a number of variables in clinical protocols may be involved in determining a poor clinical response in solid tumors. Among these variables, of major relevance appear to be the inappropriate dosage, schedule, and route of administration; the particular subtype of IFN-α utilized; the histiotype and stage of differentiation of the neoplastic disease; as well as the individual susceptibility of tumor patients (2, 4, 5).

At variance from conventional antiblastics, there are no preset, generally accepted rules to optimize treatment protocols with biomodifiers. One significant advantage in this direction would be a consensus method to objectively evaluate the in vivo efficacy of IFN-α. Unfortunately, the levels of IFN-α in blood, although routinely assessed, do not appear to be suitable to this aim, since they have a short half-life (2, 4, 6) and do not provide any indication as to the biological activity of IFN-α on its cellular targets. A direct estimate of a molecular response, on the contrary, might represent a method of choice to measure interferon efficacy.

Among the gene products the expression of which is enhanced by IFN-α, class I MHC antigens have been long known for their elective susceptibility to in vitro up-regulation in a variety of cell types (7, 8) including PBMCs (8). Should this occur also in vivo, in humans, class I antigens might be useful to monitor the efficient delivery of pharmacological doses of IFN-α to different cell types and anatomical districts. Because PBMCs can be easily collected prior to and following interferon treatment, we have tested mRNA accumulation, synthesis, plasma membrane expression, total cellular steady state of class I MHC products in these cells, and their circulating levels in the plasma of nine malignant melanoma patients enrolled in a phase I trial involving treatment with escalation doses of recombinant IFN-α.

MATERIALS AND METHODS

Interferon, Melanoma Patients, and Experimental Plan. Recombinant IFN-α was from Hoffmann La Roche, Nutley, NJ. Patients with metastatic melanoma (stages III and IV of the M. D. Anderson Hospital classification) had received no previous chemotherapy except patient LNG, who had terminated a 4-(dimethylamino)azobenzene-4-carboxamide cycle 3 months before IFN-α administration. Nine melanoma patients were given i.m. injections of 3 × 10⁶ units of IFN-α three consecutive times at 24-h intervals. The treatment was repeated weekly for two more cycles at dosages of 9 × 10⁶ and 18 × 10⁶ units, respectively. Heparinized blood was collected prior to the first cycle and 24 h after the last injection of each of the three cycles and immediately tested, as described below, for class I MHC expression at plasma membrane, translational, or mRNA levels.

Because the IFN-α-mediated plasma membrane enhancement of class I molecules lasts longer than mRNA and translational increases (9–12), the present study was aimed to measure changes in the steady state cellular accumulation of class I protein products. The time interval between IFN-α administration and testing (24 h) was not preliminarily tested but was chosen in order to measure changes in the expression of class I antigens, keeping into account several parameters affecting antigenic modulation, such as (a) the limited period (3–6 h) of pharmacologically active IFN-α levels in blood (2, 4, 5); (b) the time (up to 12 h) required by IFN-α to trigger the cellular processes resulting into enhancement of class I expression at protein level (11); and (c) the finite (about 24 h) half-life of antigenic modulation at cell surface after IFN-α withdrawal (9, 11). However, because many of the above referenced studies have been performed in continuous cell lines of different lineages, the conditions selected by us for PBMC testing may be suboptimal.

Reagents. MoAb W6/32 recognizes a class I determinant highly dependent on the association of the heavy (M, 44,000) chain with β₂-m (M, 12,000) (13). MoAb Namb-1 is to a distinct determinant carried by β₂-m (14). MoAb Ep2 is to a proteoglycan not expressed on PBMCs (15). MoAbs were purified from ascitic fluids by DEAE chromatography (Pharmacia, Uppsala, Sweden), following the instructions of the
obtained by routine Ficoll-Isopaque density gradient centrifugation of heparinized blood. PBMCs were immediately lysed at a concentration of $1 \times 10^9$/ml by incubation for 30 min in phosphate (0.01 M)-buffered (pH 7.0) saline (0.9%) containing 1% Renex 30 (Atlas, Houston, TX) and 100 μM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation at 7000 × g for 5 min. Equalization of protein concentration in different extracts was confirmed by the BCA assay (Fierce, Rockford, IL). Both PBMCs and plasma were stored at −20°C in aliquots. The double determinant immunassay to quantitate soluble class I antigens was performed on polyclonal chlorite microtitrator plates as described (18). Briefly, 10 μg of MoAb W6/32 to class I molecules (catcher) per well were absorbed on polyvinyl chloride plates; then the antigen source in duplicate (50 μl of undiluted extract or plasma) was added. After unbound antigen was washed, $^{125}$I-labeled MoAb Namb-1 (20 ng) was incubated (tracer). Following extensive washing, specifically bound MoAb Namb-1 was measured in a gamma-counter. Specificity controls included the use of irrelevant antibodies as catchers as well as tracers, and of soluble extracts from the class I-negative K562 cell line. Titration experiments were preliminarily performed to exclude testing in antigen excess. Indirect immunofluorescence was performed as described (19), with MoAbs at a concentration of 50 μg/ml on aceton-fixed cytospin centrifugates using $1 \times 10^5$ cells as target. Freezing of fixed specimens at −20°C for up to 6 months did not alter MoAb W6/32 reactivity with PBMCs.

Protein Synthesis and mRNA Analysis. Metabolic labeling of PBMCs with $[^{35}]$S)methionine and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of indirect immunoprecipitates were performed as described previously (10, 11). Total cellular RNA was obtained from guanidinium isothiocyanate lysis of PBMCs, run on a 2.2 M formaldehyde/1.4% agarose gel, transferred to nylon filters, and hybridized as described (11).

RESULTS

Effect of in Vivo IFN-α Treatment on the Levels of Class I MHC Molecules in the PBMCs and Plasma of Melanoma Patients. Nine melanoma patients underwent the three cycles of IFN-α administration as outlined in “Materials and Methods.” PBMCs and plasma samples were collected prior to IFN-α treatment and 24 h after the last administration of each cycle (Fig. 1). As expected, the class I molecular complex was detected in all extracts as well as plasma samples. The levels in 5 $\times$ 10^4 PBMCs or 50 μl of plasma from untreated patients were found to be approximately equal to those present in soluble extracts from 2 to 4 $\times$ 10^5 WI-L2 B-lymphoid cells and showed minimal variations among all patients. Levels of class I MHC antigens were found significantly elevated after IFN-α treatment at the highest dosage in all samples. In all cases in which, for technical reasons, cell extracts were not available, a significant, although occasionally weak, enhancement in class I plasma levels was detected. The entity of class I increases varied in different patients and was greater in PBMCs than in plasma. The patients responsive at low doses (MST and DNG) also showed the greatest absolute increases in class I levels in both extracts (up to 8-fold) and plasma (approximately 3-fold).

In addition, increases in IFN-α dosages caused, in these two patients, only marginal or no increases in class I expression. The dose response of class I enhancement in extracts and plasma, in the 5 cases for which both types of samples were available, were often, although not invariably, correlated. In all patients but BRR, elevations in plasma levels were either concomitant or subsequent to class I enhancement in extracts. Very similar results were obtained when PBMCs from five melanoma patients (MST, BRR, DNG, CCN, RFF) were cytospin centrifuged, fixed in cold absolute acetone, and tested in indirect immunofluorescence with MoAb W6/32. In all cases expression of class I antigens was demonstrated as an incomplete ring of plasma membrane associated fluorescence in >95% of pretreatment PBMCs. Cells collected after IFN-α administration displayed, on the other hand, a strong, anular, ribbon-like stain. This increase in staining intensity was detected in the entire mononuclear cell population. Representative results of this kind of analysis are shown in Fig. 2. In two additional cases, PBMCs were fractionated according to a procedure described in detail elsewhere (20). After recovery of adherent cells (1 h incubation in plastic flasks), positive selection of surface immunoglobulin cells was carried out by panning on anti-human immunoglobulin antibody-coated polystyrene dishes. Finally, adherence-negative/immunoglobulin-positive cells (T-enriched fraction) were recovered from the supernatant. These experiments (not shown) confirm that the increases in class I MHC expression are of similar magnitude in all mononuclear cell types. In addition, the negligible alterations in differential blood counts before and after IFN-α treatment (not shown) argue against the possibility that changes in the cellular composition of the mononuclear cell pool might account for inter- or intrapersonal differences in the levels of class I MHC expression (not shown).

Parenthetically, it should be noted that only patient DNG (good class I responder) showed a complete clinical response to IFN-α treatment. This patient is still alive with apparently no sign of the disease 3 years after treatment. All the remaining patients, including patient MST, showing good class I enhancement, had only minimal or no clinical response. Whether a
correlation exists between class I responsiveness and clinical outcome remains to be evaluated in a larger number of patients.

**Effect of in Vivo IFN-α Treatment on the Synthesis and mRNA Accumulation of Class I MHC Products by the PBMCs of Melanoma Patients.** In order to obtain information about the mechanisms involved in IFN-α up-regulation, the synthesis of class I protein products and the accumulation of specific mRNAs were assessed, in a distinct cycle of treatment, in two selected cases (BRR and MST, respectively). In patient BRR, PBMCs were isolated prior to (day 0) and following (day 4; 24 h after the last IFN-α administration) a cycle of three consecutive daily administrations of 18 × 10^6 units of IFN-α; immediately processed in identical conditions by a metabolic pulse (2 h) in the presence of [35S]-methionine (50 μCi/ml), with no added IFN-α; and solubilized by the non-ionic detergent Renex 30. Immunoprecipitation was done in parallel, with MoAb W6/32, from equal amounts of soluble extracts. As shown in Fig. 3, a residual IFN-α enhancement of class I heavy chain synthesis (slightly less than 2-fold) is still detectable at day 4 as compared to day 0 PBMCs, during the 2-h metabolic pulse. A similar quantitative increase of the associated β2-m component is also visible after longer exposure times (not shown). No increases in class I MHC synthesis could be demonstrated in the PBMCs from three additional, different melanoma patients by prolonging (8–24 h) metabolic labeling, most likely due to a rapid in vitro reversal of the IFN-α-mediated stimulation of class I synthesis (not shown). These results are consistent with the hypothesis that PBMCs exposed in vivo to IFN-α still display enhanced levels of translatable class I mRNAs 24 h after the latest interferon administration, and for at least the initial 24 h of in vitro permanence. To test this hypothesis directly, PBMCs were isolated as above at days 0 and 4 from patient MST and lysed immediately with guanidinium isothiocyanate solution. The isolated total cellular RNA was then tested by Northern blotting to quantitate the presence of specific class I transcripts. The relative hybridization intensities of the 1.6-kilobase (Kb) pS8-generated and 1.7-kilobase β-actin bands in the absence (A) and presence (B), respectively, of IFN-α were estimated by densitometric scanning of preflashed, underexposed Kodak XAR-5 films. Densitometric values (arbitrary units) generated by the two probes in the same lane were plotted in graphic form with respect to each other. The lesser the amount of mRNA loaded, the closer the points are displayed to the origin of the axes. Under the assumption that hybridizable β-actin transcripts are not affected by IFN-α treatment, the slope of the lines is directly proportional to class I mRNA expression from three additional, different melanoma patients by prolonging (8–24 h) metabolic labeling, most likely due to a rapid in vitro reversal of the IFN-α-mediated stimulation of class I synthesis (not shown). These results are consistent with the hypothesis that PBMCs exposed in vivo to IFN-α still display enhanced levels of translatable class I mRNAs 24 h after the latest interferon administration, and for at least the initial 24 h of in vitro permanence. To test this hypothesis directly, PBMCs were isolated as above at days 0 and 4 from patient MST and lysed immediately with guanidinium isothiocyanate solution. The isolated total cellular RNA was then tested by Northern blotting to quantitate the presence of specific class I transcripts. As expected, an at least 2-fold increase in the hybridization intensities of the pS8 32P-labeled probe was detected in PBMCs collected at day 4 (Fig. 3).

**DISCUSSION**

In this report we show that in vivo administration of IFN-α up-regulates the steady state protein levels and plasma membrane expression of class I MHC molecules in the PBMCs of metastatic melanoma patients.

![Image of Fig. 2: Effect of in vivo administration of IFN-α on plasma membrane expression of class I MHC antigens in the PBMCs of melanoma patients. PBMCs from patient BRR were obtained before interferon administration (A) and immediately cytospun, fixed in cold absolute acetone, and frozen at −20°C. The patient underwent the complete schedule (three cycles) of interferon treatment (see text), and 18 days later, at 24 h distance from the last IFN-α administration of the third cycle (18 × 10^6 units of IFN-α), PBMCs were again isolated and processed as above (B). For IIF testing with MoAb W6/32 to the class I MHC molecular complex, samples were defrosted and stained in parallel, under identical conditions. × 640.](image-url)
melanoma-bearing patients. We also provide evidence, in two selected cases, that this enhancement is related to an increase, although of lesser magnitude and limited life span, in the accumulation and translation of class I-specific transcripts. This quantitative discrepancy between mRNA and protein accumulation, however, was not unexpected (see also “Materials and Methods”), since class I enhancement occurs, \textit{in vitro}, through the induction of labile transcriptional activators (21). In view of this, a reversible transcriptional up-regulation and/or class I mRNA turnover represents the most likely interpretation also for the presently detected \textit{in vivo} enhancement of class I molecules.

At the cellular level, the up-regulation of class I molecules was detected in all Ficoll-Isopaque PBMCs, as well as in fractionated cells, suggesting that IFN-\(\alpha\) is active in both lymphocytes and monocytes. This is at variance, for instance, with the more specialized effect of IFN-\(\gamma\) on class II MHC molecules, shown to occur only in the latter cell lineage (22).

From a structural standpoint, it is of interest that MoAb W6/32 detects, both alone and in combination with antibodies to \(\beta_2\)-m, the heavy/light chain association form of class I molecules (13). Because HLA heavy chain and \(\beta_2\)-m may associate only in presence of endogenous (viral) antigenic peptides (23), the enhancement of this type of fully assembled molecules is more easily related to the primary antiviral activity of IFN-\(\alpha\) than to its antitumor effects. Nevertheless, a significant class I up-regulation in PBMCs may still be of practical interest in oncology, since it may help to rule out that an inefficient modality of administration and/or a defective individual susceptibility to IFN-\(\alpha\) might be involved in a poor clinical response.

Because they recirculate from the periphery into the bloodstream (24), mononuclear cells may be exposed to IFN-\(\alpha\) in different anatomical districts, thus providing a useful index of appropriate delivery of pharmacologically active doses of IFN-\(\alpha\) not only to circulating cells but also outside the vascular compartment.

Finally, it should be noted that higher IFN-\(\alpha\) dosages did not elicit greater effects on class I expression in at least two patients. Thus, our results are consistent with the possibility of an IFN-\(\alpha\) overdosage in susceptible individuals. Overdosage has been suggested previously to depend upon an ambiguity intrinsic to many clinical trials with these biomodifiers (2). In these trials, IFN-\(\alpha\) dosages are often scaled up, in analogy to clinical protocols with conventional antibotics, until a cytostatic effect is obtained. This may be counterproductive, however, since a modulatory effect on the antigenic phenotype is largely dose independent and may play a major role in the overall antitumor effects of many biomodifiers (2, 5).

One possible alternative approach, previously described, to evaluate the biological efficacy of IFN-\(\alpha\) \textit{in vivo} is the assessment of natural killer activity (24). Unfortunately, increase in natural killer activity measures interferon susceptibility only in a subpopulation of lymphocytes and is labor intensive, time consuming, and difficult to perform outside specialized centers. On the contrary, the determination of class I HLA levels in PBMCs seems a simple and quantitative test. Being adaptable to be run in a standard cytofluorimeter, this assay is within the scope of most hematology laboratories.

The increase of class I antigens mediated by IFN-\(\alpha\) is not limited to the cellular compartment but also involves the soluble form of these molecules, the plasma levels of which were found invariably elevated following IFN-\(\alpha\) treatment. This finding was expected, since the presence of class I antigens in blood has been known for at least 15 years (25), and several agents, including IFN-\(\alpha\), enhance their release in the tissue culture medium by lymphoid and tumor cells (9, 10, 26). As to the cellular source of class I molecules in plasma, their origin, at least in part, from PBMCs, is suggested by the related time sequence and magnitude of class I enhancement in most PBMCs and plasma samples.

Although the biochemistry and function of soluble class I antigens are, at present, poorly understood (26–28), one study performed with reagents and methods similar to ours (26) has recently shown that circulating class I molecules are comprised of both the classical HLA-A,B,C antigens and the nonconventional, alternatively spliced class I MHC products. Our data are equally compatible with IFN-\(\alpha\) up-regulating either or both subsets. Whatever the nature of soluble class I molecules detected by us, their enhancement by IFN-\(\alpha\) and their presence in the circulation in the form of a class I/\(\beta_2\)-m complex confirm, \textit{in vivo}, their release from nucleated cells in response to specific biological stimuli. These findings support the notion, still controversial (28, 29), that soluble class I antigens have a role in antigen-specific responses (27).

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