

Evaluation of the Potential Role of Class II Histocompatibility Antigen HLA-DR in Platelet/Tumor Cell Interaction

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Abstract

It has been reported that HLA-DR is a potent inducer of thrombin generation. Human colorectal cells (GEO, WiDr, DLD-1, and MIP) that lack the constitutive expression of HLA-DR cause platelet aggregation through a thrombin-dependent mechanism. Treatment with recombinant human γ -interferon induced the expression of HLA-DR in the GEO, WiDr, and DLD-1 cells, whereas the MIP cell line remained HLA-DR negative. The concurrent analysis of tumor cell/platelet interaction after γ -interferon treatment showed a decrease in platelet proaggregating activity of either the responsive GEO (highly expressing HLA-DR) or the unresponsive MIP (HLA-DR negative) cells. Furthermore, the DLD-1 (moderately expressing HLA-DR) cells showed an increase of proaggregating activity after γ -interferon treatment, whereas WiDr (highly expressing HLA-DR) cells did not modify their activity. These results suggest a lack of a role of HLA-DR in the *in vitro* platelet proaggregating activity of human colorectal tumor cells.

Introduction

An association between cancer and thromboembolic disease has been reported by several investigators, and elements of the hemostatic system are proposed to be involved in tumor cell metastasis (1, 2). Several reports have suggested a role of platelets in the hematogenous metastasis of tumor cells from the primary tumor site (3). To explain the nature of such interaction(s), different mechanisms are now acknowledged, mainly generation of thrombin by tumor cells (4, 5). At least four separate factors are implicated in tumor cell-induced thrombin generation; these include tissue factors (6), cancer procoagulant (7), platelet proaggregating activity/procoagulant activity (8), and a factor Xa receptor (9). It has been recently suggested that adhesion molecules, such as glycoprotein Ib and glycoprotein IIb/IIIa complex, present on the platelet surface, are involved in the thrombin-dependent and -independent platelet aggregation induced by tumor cells (10-12). Activation of platelets will be ultimately responsible for trapping of tumor cells within a platelet mass, thus leading the cells to escape immunological surveillance within the circulation and providing them the possibility of attachment.

We have previously shown that human colorectal tumor cell lines were capable of inducing platelet aggregation through a thrombin-dependent mechanism (13), as demonstrated by the consistent lack of proaggregating activity when platelet suspensions were preincubated with a thrombin inhibitor (tosyl-arginine-methyl-ester).

In a recent study, it has been reported that the HLA-DR molecule is a potent inducer of thrombin generation (14) and that this procoagulant activity is contained in the areas of homology shared between the HLA-DR α and β subunits. The authors conclude that the expression of HLA-DR histocompatibility antigen may contribute to thrombotic disorders often observed in human cancer. Moreover, they propose the

opportunity to develop specific inhibitors with potential therapeutic use. It was, therefore, conceivable to perform a study evaluating whether the expression of class II histocompatibility antigen HLA-DR may also play a role in tumor cell-induced platelet aggregation.

Materials and Methods

Recombinant Human IFN- γ . The IFN- γ (Immuneron; Biogen, Inc., Cambridge, MA) was kindly supplied by Dr. J. W. Greiner (National Cancer Institute, NIH, Bethesda, MD). The IFN- γ was reconstituted in sterile water; the specific activity was approximately 2.4×10^7 units/mg protein based on an antiviral bioassay using encephalomyocarditis and human amnion WISH cells (standardized with NIH IFN- γ standard Gg23901350).

Cell Lines, Culture Conditions, and IFN- γ Treatment. Four human colorectal tumor cell lines were studied: the well differentiated GEO cell line, the moderately differentiated WiDr cell line, and the poorly differentiated MIP and DLD-1 cell lines (kindly provided by Dr. J. Schlom, National Cancer Institute). Cell lines were routinely grown in RPMI 1640 supplemented with glutamine (1X), 50 μ g/ml gentamycin, and 10% heat-inactivated fetal bovine serum. All established cell lines were routinely subpassaged every 5 days. Tumor cells were harvested by decanting the culture medium, washing the monolayer twice with RPMI 1640, and then treating them for 5 min with 1% trypsin EDTA at 37°C. The cell suspension was centrifuged at $300 \times g$ for 10 min, the supernatant was removed, and the cell pellets were washed twice with PBS without Ca^{++} and Mg^{++} (pH 7.2). Tumor cells were finally resuspended in PBS without Ca^{++} and Mg^{++} at a final concentration of 1×10^7 cells/ml. Viability was determined by trypan blue exclusion. The range of viable cells was always greater than 95%. The optimal dose and temporal kinetics for the IFN- γ mediated induction of class II HLA antigens on the surface of human colorectal tumor cells have been reported (15). Generally, a 48-72 h incubation in the presence of 1000 units IFN- γ /ml results in maximal *de novo* induction of class II HLA antigens.

For cell growth experiments, 3×10^5 cells/60-mm dish were seeded in complete medium. Twenty-four h later, the medium was changed, and IFN- γ (10 to 1000 units/ml) was added. At the indicated times, after harvesting cells with gentle trypsinization, cell count in triplicate was performed using a Coulter counter. Each experiment was performed at least three times.

Live Cell Enzyme-Immunoassay. The analysis of MAb binding on the cell surface was routinely performed using approximately 50,000 cells/well in 96-well plates. The primary MAbs used were anti-HLA-DR and anti-HLA-DP (Becton Dickinson, Inc., Mountain View, CA) and anti-HLA-ABC (W6/32; obtained from Cooper Biomedical, Inc., West Chester, PA). Anti-cytokeratin (Becton Dickinson, Inc.) was used as positive control. All assays included samples to which no primary MAbs or irrelevant primary MAbs (MOPC-21 and UPC-10; Sigma Chemical Co, St. Louis, MO) were added. The primary MAbs were incubated with the cells for 1 h at 37°C. The unbound MAb was removed by aspiration and extensive washing, followed by the addition of immunoperoxidase-conjugated goat anti-mouse IgG. The second antibody was incubated for 30 min at 37°C, and excess antibody removed by aspiration and washing. A substrate solution containing 0.014% H_2O_2 and 0.5 mg/ml *o*-phenylenediamine (Sigma Chemical Co.) was then added to each well, and reaction was stopped by the addition of 100 μ l of 2 N H_2SO_4 . Titration curves were constructed by plotting absorbance values (490 nm) versus MAb dilutions. All experiments were performed in triplicate at least three times.

² The abbreviations used are: IFN- γ , γ -interferon; PBS, phosphate-buffered saline; MAb, monoclonal antibody; PRP, platelet-rich plasma; rHu, recombinant human.

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Platelet Aggregation Studies. Blood samples were obtained from healthy volunteers by venipuncture of the antecubital vein, collected in sodium citrate 3.8% (1:9 v:v), and immediately centrifuged at $120 \times g$ for 15 min to obtain PRP. Platelet-poor plasma was obtained after removal of PRP and further centrifugation at $2000 \times g$ for 10 min. In all cases PRP was adjusted to a final count (counter PL100 Sysmex TOA) of 3.5×10^5 cells/ μl by dilution with autologous, platelet-poor plasma. Platelet proaggregating activity was evaluated by use of a four-sample Menarini 3210 Aggrecorder using siliconized glass cuvetts at 37°C and under continuous stirring at 1000 rpm. One hundred-fifty μl of tumor cell suspensions (1.5×10^6 cells) were added to 300 μl of PRP. As a control, the same PRP (300 μl plus 150 μl PBS w/o Ca^{++} and Mg^{++}) was stimulated by the addition of ADP at a final concentration of 2 μM .

Results

Fig. 1A shows the growth kinetics of the four colorectal tumor cell lines after treatment with 10, 100, and 1000 units/ml rHu IFN- γ for 48 h. Moreover, an example of time- and dose-dependent effect for the DLD-1 cell line is given in Fig. 1B. The results obtained are in perfect agreement with previous findings and suggest that a 48–72-h incubation in the presence of 1000 units IFN- γ /ml results in maximal effect.

Cell surface binding of MAb anti-HLA-DR was analyzed by live cell enzyme-immunoassay after a 48-h IFN- γ treatment (1000 units/ml). All four cell lines were constitutively negative for the HLA-DR expression. As shown in Fig. 2, rHu IFN- γ treatment induced the expression of HLA-DR in the GEO, WiDr, and DLD-1 cell lines. In agreement with previously published results (15), the MIP cell line was HLA-DR negative either before or after IFN- γ treatment. It is worthwhile to notice that the IFN- γ -induced expression of HLA-DR was similar for the GEO and WiDr cell lines (Fig. 2, A and B) and was significantly lower for the DLD-1 cells (Fig. 2C).

An example of platelet aggregation curves obtained by interaction with the four cell lines before and after treatment with rHu IFN- γ is reported in Fig. 3. As shown, a decrease of platelet proaggregating activity after IFN- γ treatment was observed with either the responsive GEO (highly expressing HLA-DR) or the unresponsive MIP (HLA-DR negative) cell lines, whereas the DLD-1 cell line (moderately expressing HLA-DR) exhibited an increase of platelet proaggregating activity following IFN- γ treatment. No significant differences were observed using the WiDr cell line (highly expressing HLA-DR). Moreover, despite the similarity in the amount of HLA-DR expressed after IFN- γ treatment (Fig. 2, A and B), the GEO and WiDr platelet proaggregating activity was highly dissimilar (Fig. 3, A and D).

Discussion

The analysis of the data reported shows a modulatory effect on platelet proaggregating activity following rHu IFN- γ treatment. Nevertheless, differences in tumor cell-induced platelet aggregation were observed among the four cell lines. In fact, after IFN- γ treatment, an inhibitory or stimulatory effect was achieved using the GEO and the

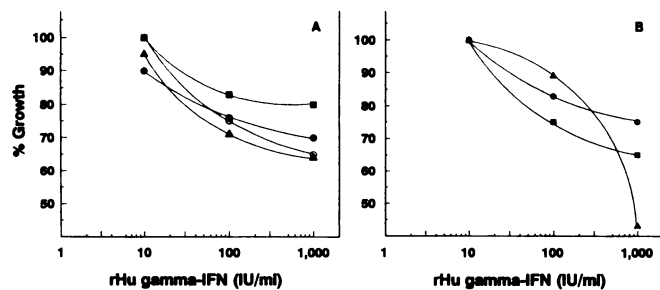


Fig. 1. Growth inhibition of colorectal tumor cells following rHu IFN- γ treatment. Points, mean of triplicate cell counts. A, dose-dependent effect on GEO (●), MIP (■), WiDr (▲), and DLD-1 (○) cells. B, time- and dose-dependent IFN- γ effect on DLD-1 cells after 24 h (●), 48 h (■), and 72 h (▲).

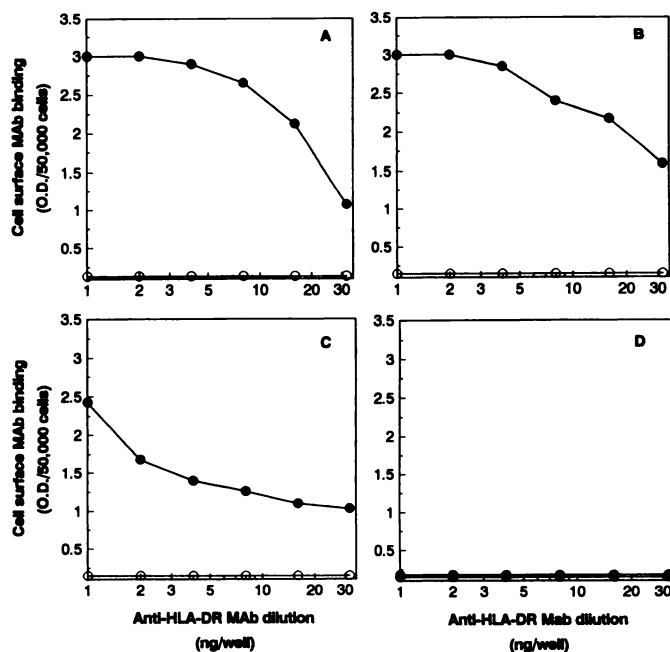


Fig. 2. Live cell enzyme-immunoassay of HLA-DR expression on GEO (A), WiDr (B), DLD-1 (C), and MIP (D) tumor cells after 48-h IFN- γ treatment (1000 units/ml). Points on the antibody dilution curves, mean of triplicate samples. Untreated cells (○); IFN- γ treated cells (●).

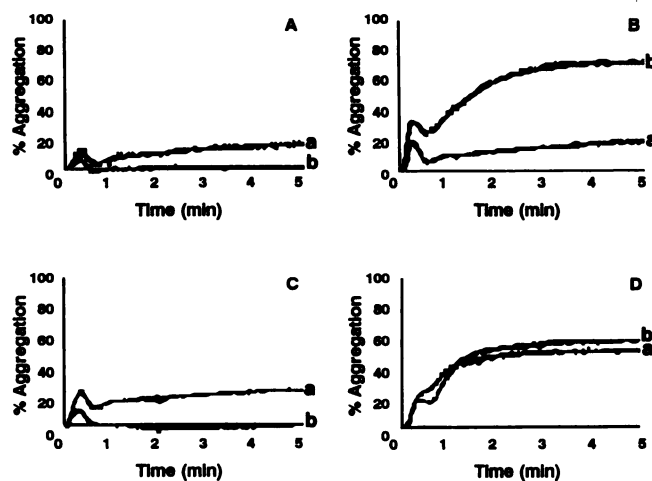


Fig. 3. Platelet proaggregating activity of human colorectal tumor cells (1.5×10^6) following a 48-h treatment with 1000 units/ml rHu IFN- γ . In this experiment, the aggregation waves were obtained on PRP samples obtained from a single blood donor. As control, stimulation of platelets was carried out with 2 μM ADP, yielding a platelet aggregation of 83%. A, GEO cell line; B, DLD-1 cell line; C, MIP cell line; D, WiDr cell line. a, untreated cells; b, IFN- γ treated cells.

MIP (Fig. 3, A and C) or the DLD-1 (Fig. 3B) cell lines, respectively. In contrast, no differences were observed with the WiDr cell line before and after IFN- γ treatment (Fig. 3D).

The evaluation of the HLA-DR expression after IFN- γ treatment showed that there was no correlation between its expression and tumor cell-induced platelet aggregation. This is supported by several findings: (a) the two cell lines highly expressing HLA-DR after IFN- γ treatment showed different patterns of platelet proaggregating activity in response to treatment, decreased in the case of the GEO cells and unmodified in the case of WiDr (Fig. 3, A and D); (b) the DLD-1 cells,

moderately expressing HLA-DR after IFN- γ , showed an increased platelet proaggregating activity when compared to control (HLA-DR negative) cells; and (c) a decreasing platelet proaggregating activity was observed in both GEO (expressing IFN- γ -induced HLA-DR) and MIP (HLA-DR negative either before or after IFN- γ treatment) cell lines.

The results presented here are against the hypothesis of an involvement of HLA-DR expression on the cell membrane in the *in vitro* platelet/tumor cell interactions. A recent study suggested the potential role of HLA-DR in the thrombotic disorders associated with human cancer, suggesting the opportunity for design of peptidic and nonpeptidic inhibitors with potential therapeutic use (14). However, in this study the HLA-DR procoagulant activity was evaluated using a purified preparation of this antigen; the possible interactions with other molecules and the steric conformation of HLA-DR expressed at the cell membrane level were not considered, which may be responsible for the unexpected results in different experimental models.

The modulatory effect of IFN- γ could be explained by differences in the responsiveness of the cell lines, probably due to differential expression of other molecules, such as adhesion molecules, which are known to be involved in platelet/tumor cell interactions. This hypothesis can be supported by the recent finding of an amplified ICAM-1 expression after IFN- γ treatment of DLD-1 cells (16), which is in agreement with our finding of an increased platelet proaggregating activity of this cell line after IFN- γ treatment.

It is generally believed that one of the most potent actions of IFN- γ is the *de novo* induction of class II HLA antigen expression on a variety of human cells. However, in some cases IFN- γ treatment does not result in induction of these antigens. The inability of IFN- γ to induce class II HLA expression on the MIP cell line does not seem to be reflective of a broad spectrum of cellular resistance because IFN- γ treatment can inhibit cell growth. As suggested by Zuckerman *et al.* (17), a *trans*-activating factor may be involved in the *de novo* induction of class II HLA antigens, and the inability of IFN- γ to alter surface antigen expression may be due to a lack of such a factor(s) and/or a disruption in recognition of secondary messenger(s) (15).

In conclusion, even if the modulatory effect observed on platelet tumor cell interaction following treatment with IFN- γ still remains unclear, the present data indicate a lack of a role of class II HLA-DR in the *in vitro* platelet proaggregating activity of human colorectal

tumor cells. Further investigations of the IFN- γ effect should be focused on different molecules, such as the family of adhesion molecules.

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