Glycoprotein 170 Induces Platelet-Activating Factor Receptor Membrane Expression and Confers Tumor Cell Hypersensitivity to NK-Dependent Cell Lysis

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Glycoprotein 170 Induces Platelet-Activating Factor Receptor Membrane Expression and Confers Tumor Cell Hypersensitivity to NK-Dependent Cell Lysis

Daniela Geromin,* Jean-François Bourge,* Annie Soulié,* Rob Pawliuk,† Christina Fleet,† Eugène Michel,‡ Yves Denizot,§ Christian Berthou,‖ Philippe Leboulch,† François Sigaux,* and Marilyne Sasportes2*

Multidrug resistance (MDR) confers resistance to anticancer drugs and reduces therapeutic efficiency. It is often characterized by the expression of the MDR1 gene product P-glycoprotein (or gp170) at the membrane of tumor cells. To further propose a potential complementary tool in cancer treatment, the sensitivity of gp170 tumor cells to NK-dependent lysis was investigated. Two kinds of cells were generated from wild-type K562 erythroleukemic cells: the first were derived from Taxol-selected cells and cloned, whereas the second were retrovirally transduced by the cDNA of the MDR1 gene. The last process was also applied to the human embryonal carcinoma cells called Tera-2 cells. First, both cloned and MDR-1 K562 cells appeared highly susceptible to naive NK cell killing. Interestingly, in addition, Tera-2 cells that were not sensitive to NK lysis could be killed when they expressed gp170 at their membranes. In previous data, we demonstrated that NK cell release of bimolecular complexes composed of perforin and platelet-activating factor (PAF) interacting with the PAF-R, which has to be expressed on the target cell membranes, were components of NK tumor cell killing. In the present study, we show that gp170 has the capacity to drive constitutive PAF-R expression on tumor cells, which could be responsible for hypersensitivity to NK lysis and accelerated cell death. The Journal of Immunology, 2004, 172: 3604–3611.

The resistance of tumor cells to anticancer agents remains a major cause of treatment failure in cancer patients (1).

Among the various multidrug resistance (MDR)3 human gene products that can be implicated in MDR, the MDR-associated proteins, lung resistance protein, MDR3, and MDR1 (1–5), the MDR1 P-glycoprotein (P-gp) appears to be the most frequently expressed. It belongs to the ubiquitous family of membrane transporters that contain an ATP-binding cassette (ABC) (ABC-transporters) and consume ATP during translocation of a wide variety of molecules from the cytosol to the extracellular space (6, 7). P-gp or gp170 occurs at the surface of many tumor cells (8). Several proposals have been put forth to explain the mechanism of action of P-gp in MDR. It is usually accepted that P-gp acts as a drug pump, which results in a decreased intracellular concentration of drug products (5, 9). This has been expanded to the flipase activity to try to understand how P-gp can remove a large range of diverse drugs and phospholipids without an apparent substrate specificity (10). However, there is not always a linear correlation between the extent of drug efflux and resistance to cell death. In addition, P-gp expression on tumor cells has been shown to be either constitutive (11–13) or, more frequently, acquired after chemotherapy treatment (14, 15). To explore therapeutic alternatives capable of being used in chemotherapy-resistant tumor cells, the question of tumor cell susceptibility to NK-mediated lysis in humans was addressed. This led us to investigate the impact of the NK-dependent lytic pathway on tumor cells treated by anticancer drugs and expressing gp170. In previous studies on that topic, some authors showed a decreased sensitivity to induced NK cell death (16), others described a resistance to NK cell death induced by the Fas/Fas ligand pathway together with a normal sensitivity to the perforin/granzyme B lytic one (17). In a recent work (18), we identified the platelet-activating factor (PAF) and its membrane receptor (PAF-R) together with perforin as a component in tumor cell killing activity by human naive NK cells. In this paper, we demonstrated that acquired gp170 associated to PAF-R membrane expression led to hypersensitivity to NK-mediated tumor cell killing.

Materials and Methods
Preparation of human NK effector cells

Human NK cells were purified as shown previously (18). Adult PBMC were prepared by standard Ficoll-Hypaque procedures (Lymphoprep, Axis, Oslo, Norway). After 1 h of adherence to plastic at 37°C in 5% CO2, nonadherent cells were loaded on a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged for 30 min at 500 × g. Cells were recovered from the low-density fraction and purified for NK cell lysis in the presence of anti-CD3 and anti-CD19 hybridomas at 1/100 dilution of an ascitic fluid and rabbit complement (Fillorga Laboratories, Paris, France). On purification, the cell population was analyzed by flow cytometry using anti-CD16 (FITC labeled; Leu11c), anti-CD56 (FITC

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labeled; Leu19) and anti-CD3 (FITC labeled; Leu4) mAbs from BD Immunocytometry Systems (San Jose, CA). NK cells were isolated to >93% purity.

**Assay for NK cell lysis**

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**Immunohistochemical study**

Immunohistochemical studies were performed on wt K562, neo-K562, MDR1-K562, and Tera-2 cells, as well as on MDR1-Tera-2 cells. The slides were incubated with an anti-P-gp mAb (UIC-2; Beckman Coulter, Fullerton, CA) or with an anti-PAF-R polyclonal Ab (Cayman Chemicals, Ann Arbor, MI) for 30 min. Slides were then washed three times, and detection was performed with the alkaline phosphatase immunohistochemical technique (kit LSAB; DAKO, Trappes, France).

**Flow cytometry analysis**

P-gp or PAF-R cell membranes were determined by flow cytometry. Cells were incubated for 30 min at 4°C with an anti-P-gp mAb (UIC-2; IgG2a; PE conjugate; Immunotech) or with an anti-PAF-R mAb (Coger, Paris, France). After washing with cold PBS, cells were analyzed. Data acquisition and analysis were performed on a BD Biosciences (Mountain View, CA) FACScan flow cytometer equipped with a 15-mW air-cooled 488-nm argon ion laser.

**Confocal laser microscopy**

Cytospin preparations were performed for double immunofluorescence. Cells were incubated 30 min with 3% paraformaldehyde. Slides were washed in PBS/0.02% Tween and incubated 30 min with an anti-P-gp mAb (UIC-2). After washing, cells were immunostained with a fluorescein goat anti-mouse IgG (Molecular Probes; Coger) for 30 min. Then, a Texas Red goat anti-mouse IgG (Molecular Probes; Coger) was added at the appropriate dilution for 30 min, and cells were washed in cold PBS. Cells were examined with a Bio-Rad (Hercules, CA) MRC 1000 laser confocal system without any windows filtering.

**Isolation and characterization of K562 Taxol-resistant clones**

A K562 Taxol-resistant cell line (gift from Dr. J. Soudon (Institut de Génétique Moleculaire, Hôpital Saint Louis)), was cloned by limiting dilution in a microtiter plate, at an average of 0.3 cells/well. Individual colonies were first split up, expanded, and analyzed by flow cytometry (FACScan analyzer, BD Biosciences) for gp170 expression at the cell membrane using UIC2 mAb.

**Cell transfection**

K562 and Tera-2 cells were transfected using a standard calcium phosphate transfection protocol. A total of 7 x 10^5 cells was plated in a six-well tissue culture dish. On the next day, the medium was changed. The cells were then transfected with SSR # 102 (a murine stem cell virus-based retroviral vector containing the neomycin resistance gene under the control of an internal phosphoglycerate kinase promoter) alone. This could also be done in combination with a retroviral vector (KA # 378) containing the MDR1 cDNA under the control of the promoter and enhancer elements from the Harvey murine sarcoma virus long terminal repeat. The expression of gp170 was measured by using a FACScan flow cytometer.

**Flow cytometry pH measurement**

Flow cytometry was performed with a BD Biosciences LSR flow cytometer equipped with a 15-mW air-cooled 488-nm argon ion laser and 575–595 bandpass filter for acidic carboxyseminalaphorodafluor-1 (SNARF-1).
orange fluorescence and 620-nm longpass for basic SNARF-1 red fluorescence. A calibration curve was generated for each experiment by staining the cells in high K⁺/H11001 buffers of varying pH values, and adding 10 μg/ml nigericin (Molecular Probes; Coger). To measure pH, 1 × 10⁶ cells are resuspended in 1 ml of HBSS and supplemented with 10 μl of SNARF-1 working solution in a polypropylene test tube (final SNARF-1 concentration, 200 nM), and then incubated for 5 min at 37°C. To calculate the pH test sample, the mean fluorescence ratio (F13/F12) is plotted against a

FIGURE 2. P-gp overexpression increases NK-dependent lysis. Resting NK cells were used in a standard cytotoxic assay against an MHC class I-negative, FasR-negative K562 cell line. Lysis was measured by target cell release of ⁵¹Cr-labeled cytoplasmic proteins. The percentage of lysis was greater in clones 7 and 42 compared with neo K562 (A), in the cell transfect MDR1-K562 compared with neo-K562 (B), and in MDR1-Tera-2 cells compared with neo-Tera-2 cells (C). The percentage of specific lysis was abolished in the various CML in the presence of EGTA.
A known pH curve. The pH test sample is then read from the calibration curve.

**Results**

**Taxol-selected gp170 K562-derived clones, retrovirally transduced MDR1-K562 and MDR1-Tera-2 cells are hypersensitive to NK cell lysis**

Eighteen clones were derived from Taxol-selected K562 cells; in addition, wt K562 cells were transduced by a retroviral construct containing the cDNA of the MDR1 gene and named MDR1-K562 cells. wt K562 cells transfected by an empty vector (neo-K562 cells) were used as controls. Tera-2 cells were submitted to the same process. Different profiles of clones were obtained regarding gp170 expression and using flow cytometry analysis. Two representative clones, clones 7 and 42, are illustrated in Fig. 1. The same analysis was performed with MDR1-K562 and MDR1-Tera-2 cells (Fig. 1). In addition, cloned and gp170 K562 tumor cells were studied for MDR and were found to disclose resistance to Taxol and other drugs like vincristine, doxorubicin, and daunorubicin (data not shown). The capacity of purified naive human NK cells to lyse gp170 targets cells was then explored. Upon interaction with their targets, NK effector cells release their granule protein content, essentially perforin and granzymes, into the intercellular space. The results showed that the gp170 target cells displayed a significantly higher susceptibility to NK cell damage. This appeared, when those cells were compared with wt K562 cells, as demonstrated by increases of 35 and 15% of $^{51}$Cr release for Taxol-selected clones 7 and 42, respectively (Fig. 2A), and a dramatic increase of 50% cytotoxicity for retrovirally transduced MDR1-K562 cells (Fig. 2B). Similarly, MDR1-Tera-2 cells could be significantly killed with an increase of 30% compared with Tera-2 cells at least at a 50:1 E:T ratio (Fig. 2C). These results indicated an agonistic effect of membrane gp170 protein on NK cell lytic activity directed against sensitive target cells. Other lytic pathways are susceptible to interfere with NK lysis of tumor cells expressing or not gp170 at the membranes. The Fas/Fas ligand system is one of them, through target cell apoptosis, providing Fas is present on the target membranes. This is also the case for the TNF and TRAIL pathways, which are known to act similarly. Therefore, it was investigated whether such death receptors were present at the membranes of the different target cells using specific mAbs and flow cytometry analysis. Using an anti-TNFR Ab to block the appropriate cell-mediated lympholysis (CML) performed, the TNFR was detected on wt and MDR1-K562 tumor cells as well as on clones 42 and 7, but did not influence the CML data (data not shown). wt and MDR-1 Tera-2 cells were lacking all the death receptors tested (data not shown). To confirm the major role of the perforin/granzyme B lytic pathway on induced target-cell lysis, various CML were performed using all targets with and without EGTA. The results clearly indicated that the percentage of specific lysis was abolished in the CML in which EGTA was added (Fig. 2). In addition, specific $^{51}$Cr release was also measured at different times (0, 10, 30, 60, and 120 min). In such conditions, a significant lysis was observed with MDR1-K562 targets as early as 60 min. In contrast, there was a low level of lysis with neo-K562 cells taken as a control at the same time (Fig. 3). These results further demonstrated that gp170 gave rise to an NK-accelerated target cell death.

**Drug-resistant tumor cells coexpress PAF-R and gp170 at their membranes**

We previously demonstrated the crucial role of PAF-R target-cell expression on the sensitivity to NK cell lysis (18). Detailed immunocytochemistry experiments were performed to visualize PAF-R proteins on tumor cells. A representative experiment is shown in Fig. 4. The most striking fact was that a different PAF-R...
distribution was observed whether K562 cells expressed gp170 or not. In the former case, PAF-R was localized at the membrane of gp170 or MDR1-K562 cells, contrasting with the diffuse cytoplasmic staining observed in wt K562 cells. Moreover, gp170 and PAF-R proteins were coexpressed by gp170 tumor cells using confocal microscopy analysis (Fig. 5). Similar results, which concerned PAF-R distribution and membrane coexpression of gp170 and PAF-R, were obtained with MDR1-Tera-2 cells (data not shown). Membrane PAF-R expression was further measured on gp170 clones, wt, neo, and MDR1-K562 cells, as well as on wt and MDR1-Tera-2 cells using flow cytometry analysis. PAF-R was constitutively expressed on 40.5 and 10.5% of clones 7 and 42, respectively, on 22.3% of MDR1-K562 cells and on 11.2% of MDR1-Tera-2 cells, in contrast to what occurred with wt and neo K562 and Tera-2 cells, which practically lacked PAF-R expression (Fig. 6). Similar data were obtained with MDR1-Tera-2 cells.

IFN-γ, which was responsible for induced PAF-R expression on wt K562 cells, turned out to be ineffective in further inducing that protein expression on gp170 K562 cells (data not shown).

Constitutive membrane PAF-R expression is responsible for gp170-positive tumor NK cell increased susceptibility

To provide insights into the role of PAF-R on NK cell-enhanced lytic activity, the effect of PAF-R antagonists on the lysis of gp170 targets by resting NK cells was investigated. The addition of
SR27417 PAF-R antagonist in the CML assay was followed by a potent inhibitory activity of NK cell lysis when applied to cloned K562 cells and MDR1-K562 targets (Fig. 7, A and B). This effect also occurred with MDR1-Tera-2 cells (Fig. 7C). Therefore, constitutive membrane PAF-R expression appeared to be implicated in the powerful perforin lytic activity induced by naive NK cells on gp170 targets.

gp170 induces membrane PAF-R expression on MDR1-transduced K562 cells through a pH-dependent process

The mechanism leading to gp170 and PAF-R expression was then analyzed. Using a quantitative RT-PCR assay, no significant variation was observed in the quantity of PAF-R transcripts found in gp170 tumor cells compared with those in wt K562 cells (data not shown). These results favored a translational or posttranslational mechanism for PAF-R membrane induction on gp170 tumor cells. Using the SNARF technique, a pH titration curve was performed (Fig. 8, A and C), and intracellular pH measurement was analyzed in wt and MDR1-K562 cells. An acidic pH of 6.2 was found in wt K562 cells (Fig. 8B, dotted line). Using the same technical conditions, the pH appeared more basic in MDR1-K562 and clone 7 cells, and reached 7.1 and 7.5, respectively (Fig. 8B, bold lines). The same results were found with Tera-2 cells. In addition, K562 and Tera-2 cells were respectively incubated at pH 6.5 and 7.5 during 1 h, and membrane PAF-R expression was evaluated by flow cytometry analysis. The results clearly indicated a heightened PAF-R membrane expression in both cells as soon as the pH increased from 6.5 to 7.5 (Fig. 9, A and B). This phenomenon coincided with a change in PAF-R localization. PAF-R was first diffuse within the cytoplasm of K562 cells at acidic pH 6.5 and then became concentrated under the cell membrane at more basic pH 7.5 as shown by immunostaining (Fig. 10).

Discussion

MDR occurs when cancer cells become resistant to chemotherapy by removing a broad range of lipophilic drugs from the cell interior (8, 19). These cells express high levels of the multidrug transporter MDR1 P-gp (or gp170), which pumps the lipidic drug from the cytosol to the plasma membrane (5, 9). These proteins belong to the ubiquitous family of membrane transporters that contain an ABC (ABC-transporters) and consume ATP during the drug translocation process (5, 9). Moreover, the induction of cell death in gp170 tumor cells resistant to chemotherapy constitutes a major challenge for treating cancer patients. This implies distinguishing among the major lytic pathways, those preferentially used by NK cells and triggered for that purpose. Such research has been the matter of several studies. Recently, Smyth and coworkers (17, 20, 21) have clearly shown that gp170 tumor cells were resistant to caspase-dependent cell death, while remaining sensitive to the caspase-independent perforin/granzyme B lytic pathway.

We analyzed the impact of gp170 expression on membrane cell death receptors. We especially looked at whether death receptors, such as TNF, Fas, and TRAIL receptors might potentially be induced through MDR1 gene expression. It has to be noted that wt as well as MDR1-K562 cells expressed the TNFR, while lacking Fas and TRAIL receptors at the membranes. In contrast, Tera-2 and MDR1-Tera-2 cells were negative for all the death receptors previously mentioned. From the results obtained, it could be concluded that MDR1 gene expression did not interfere with the presence or absence of death receptors and when present did not
influence their function. However, we have previously reported that the sensitivity of tumor target cells to NK-dependent lysis was dependent on the presence of membrane PAF-R. Conversely, lack of membrane PAF-R allowed tumor cells to escape NK immune surveillance. These results consisted of the demonstration of a ternary complex, present in vitro and formed by perforin, PAF, and PAF-R. This was supported in the NK cell assay by the rapid release from NK effectors of perforin-PAF complexes, in addition to IFN-γ, which was alone responsible for membrane PAF-R induction on tumor cell expression (18) and granzyme B, which could not be excluded.

A functional interaction of perforin with the 32-kDa granzyme B has been demonstrated. The effect of granzyme B was dependent on protein entry into target cells by endocytosis and through an endosome-disrupting agent such as perforin (22). Another proposal concerned the mannose 6-phosphate receptor, which has been recognized as a cell surface receptor for granzyme B (23). However, other authors (24) have shown that granzyme B-induced cell death could occur in the absence of MDR-associated protein receptor. Recently, a perforin-independent granzyme B-induced cell apoptosis has been described, in which granzyme B was directly interacting with the heat shock protein (Hsp70) expressed at the tumor cell surface (25). This resulted from the direct interaction of granzyme B with the C-terminal 14-aa Hsp70 sequence. Alkyllyso-phospholipids have been shown to increase Hsp70 membrane expression on tumor cells (26). In our study, we cannot exclude that the phospholipid PAF released by NK cells in front of tumor cells could up-regulate Hsp70 expression on these tumor cells (leukemic K562 cells constitutively express membrane Hsp70) and thus facilitate granzyme B-induced cell death.

We generated gp170 Taxol-selected clones and MDR1 retrovirally transduced K562 tumor cells. Unexpectedly, the latter cells displayed a very high sensitivity to NK-dependent lysis, compared with wt K562 target cells. In parallel, wt Tera-2 tumor cells, which are insensitive to NK lysis, when transduced by the MDR1 gene could also be killed through that lytic pathway. A hypothesis that concerned the flip-flop activity of gp170 protein was formed. This effect involves the transport of cytoplasmic phosphatidylcholines (27) such as PAF (28) to the cell surface. van Helvoort et al. (27) and Ragers et al. (28) have demonstrated the lipid translocase activity of MDR1 P-gp. These data led us to investigate its potential effect on the transport of PAF to the cell surface of the tumor cells, which could facilitate the pore-forming effect of perforin. Such an activity could not be confirmed in our hands, because PAF secretion was not detected in MDR1-transduced K562 cells. Furthermore, the level of endogenous PAF was reduced in gp170 cells as compared with wt tumor cells. Our observation that gp170 tumor cell lysis is inhibited by blocking PAF-R target cell function demonstrates the role of the previously mentioned complex in NK-induced cell death of tumor cells regardless of whether they express gp170 at the cell surface or not. The difference between the two types of cells was consistent with the higher sensitivity of gp170 tumor target cells to NK cell immune response. Moreover, it has to be noted that gp170 gave rise to an accelerated target cell death as early as 60 min.

To elucidate the functional relationship between gp170 and PAF-R proteins, PAF-R localization was compared in MDR1-positive and -negative cells. Regarding MDR1-positive cells, it appeared that selective membrane PAF-R targeting occurred in those cells. This phenomenon might result from an intracellular pH modification induced by gp170 as reported by Larsen et al. (29) and Bellhoussine et al. (30). It has indeed been mentioned that some tumor cells had an abnormal acidic cytoplasmic pH, responsible for the disruption of trans-Golgi network organization, which becomes normalized in tumor cells expressing gp170. This process was reproduced in our own experiments performed on pH modifications using wt, neo, and MDR1-K562 cells, and changes in PAF-R localization were visualized, as previously mentioned, using flow cytometry and confocal approaches. Concerning clones 42 and 7, it came out that they behaved like MDR1-K562 cells (data not shown). Modifications of pH were also undertaken with Tera-2 and MDR1-Tera-2 cells, and the results were comparable with those obtained with K562 and MDR1-K562. In contrast, PAF-R N-glycosylation has also been shown to be determinant in protein membrane targeting (31). We are now currently investigating whether purified PAF-R proteins extracted from transduced...
MDR1-K562 cells are modified in carbohydrate structure compared with wt K562 cells.

Our present concern was to analyze the impact of the NK-dependent lytic pathway on patient tumor cells that had acquired gp170 following chemotherapy. In the in vitro system studied, NK cells belong to healthy individuals and exert their usual cytotoxic function. In patients after chemotherapy, it has to be kept in mind that NK cell lytic activity is severely impaired, as reported by several authors (32–34). Therefore, in trials based on cellular immunotherapy, only naive NK cells from patients could be used before any treatment and expanded in vitro according to recent technical processes. In contrast, antitumoral drug pH modulation could be considered to favor PAF-R membrane expression and consequently NK cell lysis.

In conclusion, we showed that a tumor cell that acquired gp170 expression after chemotherapy treatment could remain highly sensitive to NK cell attack. These data would favor the insertion of an expression after chemotherapy, which could be considered to favor PAF-R membrane expression and consequently NK cell lysis.

Acknowledgments

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