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High-Dose Chemotherapy-Induced Platelet Defect: Inhibition of Platelet Signal Transduction Pathways

LINDA KAROLAK, ANITA CHANDRA, WASIUDDIN KHAN, BEN MARKS, WILLIAM P. PETROS, WILLIAM P. PETERS, CHARLES S. GREENBERG,[1] and YUSUF A. HANNUN[1]

Division of Hematology/Oncology, Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710

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SUMMARY

Patients receiving high-dose chemotherapy and autologous bone marrow transplantation acquire a platelet secretion defect. The role of chemotherapeutic agents and their metabolites in mediating this platelet defect was investigated. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), but not cyclophosphamide or *cis*-platinum, was found to inhibit platelet aggregation *in vitro* in response to activation by either ADP, thrombin, or collagen. Inhibition by BCNU was dose dependent and required preincubation of platelets with BCNU. After a 60-min preincubation, 30 μM BCNU produced 50% inhibition of platelets in platelet-rich plasma. The cyclophosphamide metabolites acrolein and 4-hydroperoxycyclophosphamide also inhibited platelet aggregation in a dose-dependent manner, with a requirement for preincubation. Platelet inhibition occurred at clinically relevant concentrations of BCNU and metabolites of cyclophosphamide. The effects of acrolein were totally prevented by coincubation with the sulfhydryl-protecting agents *N*-acetylcysteine and 2-mercaptoethanesulfonic acid, whereas the effects of BCNU were incompletely prevented. The mechanism of platelet inhibition was investigated next by examining protein phosphorylation in response to platelet agonists. Acrolein inhibited thrombin- and phorbol ester-induced phosphorylation of a 40-kDa polypeptide and other substrates, indicating a cellular defect in protein kinase C signaling. BCNU did not interfere with protein phosphorylation, indicating preservation of initial signaling pathways. Thus, chemotherapeutic agents and their metabolites inhibit platelet function by inhibiting distinct components of the intracellular activation pathways.

HDC with autologous bone marrow transplantation has emerged as an important treatment modality for advanced solid tumors (1). HDC is associated with significant toxicities including endothelial cell damage, venoocclusive disease, and hemorrhagic myocarditis (1–3). We have recently identified an acquired platelet defect induced by HDC [composed of BCNU (carmustine), *cis*-platinum, and cyclophosphamide], whereby platelets acquire a defect in aggregation and secretion in response to a number of platelet activators such as ADP, arachidonic acid, collagen, and thrombin (4). This defect appears to play a causative role in the pathogenesis of hemorrhagic myocarditis such that transfusion of patients with platelets prevents the occurrence of this life-threatening complication (1). The pathogenesis of this acquired platelet defect could not be evaluated due to the absence of *in vitro* models for its study. Such studies are critical in understanding the mechanism of toxicity of HDC. Moreover, these mechanisms may also operate in the pathogenesis of endothelial cell dysfunction. Understanding these mechanisms may also suggest strategies for treatment and/or prevention of these complications.

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[1] Established Investigator, American Heart Association.

In this study, we demonstrate that the chemotherapeutic agent BCNU can reproduce *in vitro* the platelet defect acquired with HDC. Neither cyclophosphamide nor *cis*-platinum, the other two agents used in HDC, affected platelet function. However, two metabolites of cyclophosphamide, acrolein and 4-HC, were also able to induce a defect in platelet aggregation. Acrolein inhibited cellular protein kinase C activity, whereas BCNU inhibited more distal components of the signaling pathway.

Experimental Procedures

Materials

cis-Platinum, cyclophosphamide, acrolein, *N*-acetylcysteine, and MESNA were from Sigma. 4-HC was from Ben Venue Laboratories and luciferin/luciferase was from Chrono-log Corp.

Methods

Preparation of human platelets. Platelets were obtained from healthy adult volunteers who denied receiving any medications in the 2 weeks before blood collection. Approximately 50–100 ml of blood were added to acid/citrate/dextrose buffer (5) in a 9:1 ratio (v/v) and were centrifuged at 200 × *g* for 20 min to obtain PRP. Washed platelets

ABBREVIATIONS: HDC, high-dose chemotherapy; PRP, platelet-rich plasma; PMA, phorbol-12-myristate-13-acetate; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; C_{max}, maximum calculated plasma concentration; MESNA, 2-mercaptoethanesulfonic acid; 4-HC, 4-hydroperoxycyclophosphamide.

were obtained according to the method of Siess *et al.* (6) with slight modification as described (5).

Platelet aggregation and secretion studies. Platelet aggregation and secretion were measured in a Chronolog Lumi-Aggregometer as described (5). For studies with PRP, collagen or ADP was used as platelet activator. For studies in washed platelets, thrombin was the agonist of choice. Aggregation and secretion data were obtained from at least three independent experiments.

Platelet phosphorylation studies. Phosphorylation of the 40-kDa protein and other platelet proteins was carried out in intact platelets in response to thrombin or PMA as described (7).

Measurement of plasma levels of cyclophosphamide and BCNU. Twelve plasma samples were collected from 0 to 20 hr after the administration of each cyclophosphamide dose. Samples were analyzed by modification of the El-Yazigi and Martin method (8). Briefly, drug was extracted from plasma by using silica cartridges, after addition of the internal standard (diphenhydramine). Concentration in the ethyl acetate eluent was determined with a Perkin-Elmer model 8500 gas chromatograph with nitrogen-phosphorus detection. The linear standard curve for this assay extended from 1 to 40 μg/ml.

BCNU (carmustine) concentrations were determined from nine blood samples collected before and up to 90 min after infusion. Drug was stabilized at the bedside and analyzed by the method of Jones *et al.*[2] The range of the linear standard curve for this assay was 0.5 to 40 μg/ml.

Pharmacokinetic analysis of each drug was conducted by weighted nonlinear least squares regression (PCNONLIN; Statistical Consultants, Lexington, KY). A two-compartment model with zero-order input and first-order elimination best described the cyclophosphamide concentrations. Each dose of cyclophosphamide was modeled independently because of clearance induction during therapy. A one-compartment model (zero-order input, first-order elimination) best characterized BCNU disposition.

Results

***In vitro* model for platelet inhibition by HDC.** Previously, an acquired platelet defect was detected in the setting of HDC involving BCNU, cyclophosphamide, and *cis*-platinum. Incubation of PRP with these reagents for 5 min did not result in any significant inhibition of platelet aggregation (Fig. 1). Upon incubation of platelets with these reagents for 2 hr, BCNU (100 μM) was found to inhibit potently the platelet aggregation in response to ADP. Neither *cis*-platinum nor cyclophosphamide inhibited platelets under these conditions at concentrations up to 1 mM (Fig. 1). However, two major metabolites of cyclophosphamide (9), 4-HC (100 μM) and acrolein (100 μM), inhibited platelet aggregation by 50 and 60%, respectively, after the 2-hr incubation (Fig. 1). These results suggested that prolonged incubation of platelets with either BCNU or metabolites of cyclophosphamide results in inhibition of platelet aggregation.

To evaluate further the requirement for preincubation of platelets with these agents in the induction of inhibition, the effects of the duration of preincubation on the ability of these agents to induce platelet inhibition were investigated. Preincubating PRP with 30 μM BCNU resulted in a time-dependent inhibition of platelet aggregation such that BCNU produced up to 80–90% inhibition by 1–2 hr (Fig. 2). Inhibition of platelets by BCNU was concentration dependent, with an IC_{50} value of 32 μM (standard deviation, 11 μM) (Fig. 2C).

[2] R. B. Jones, S. Matthes, and E. J. Shpall. BCNU pharmacokinetics in 72 patients treated with high-dose combination chemotherapy and autologous bone marrow support: analytical methodology and patient data. Submitted for publication.

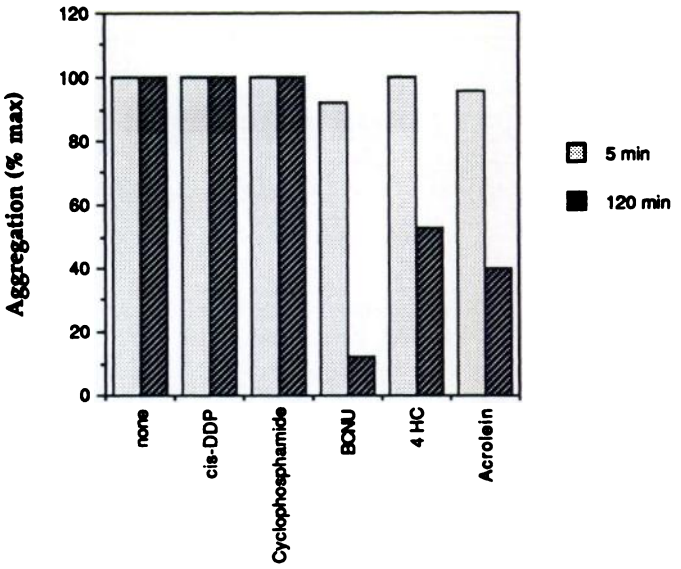

Fig. 1. Inhibition of platelets by BCNU, 4-HC, and acrolein and requirement for preincubation. PRP was stimulated with 5 μM ADP and aggregation was monitored with a Lumi-Aggregometer. Platelets were preincubated with either vehicle, *cis*-platinum (*cis-DDP*) (1 mM), BCNU (100 μM), 4-HC (100 μM), acrolein (100 μM), or cyclophosphamide (1 mM) for 5 or 120 min as indicated; PRP was then stimulated with 5 μM ADP. The extent of aggregation is plotted as percentage of maximal aggregation obtained with ADP alone.

Preincubating platelets with 100 μM 4-HC resulted in a time-dependent inhibition of platelet aggregation in response to collagen, with 50% inhibition occurring after 10 min and total inhibition occurring after 2 hr of preincubation (Fig. 3A). Inhibition of PRP by 4-HC after 30 min of preincubation was concentration dependent with near-total inhibition at 300 μM 4-HC and an IC_{50} value of 9 μM (standard deviation, 3 μM) (Fig. 3B).

Another metabolite of cyclophosphamide, acrolein, is implicated in the pathogenesis of hemorrhagic cystitis (10). Preincubation of PRP with acrolein (1 mM) resulted in a time-dependent inhibition of platelet aggregation (Fig. 4A). The acrolein effect appeared to require a shorter preincubation than that of BCNU or 4-HC, possibly indicating a different mechanism of action. Alternatively, the chemical instability of acrolein may result in a shorter half-life that limits the duration over which acrolein can interact with platelets.

The effects of acrolein were also concentration dependent. Aggregation of PRP preincubated with acrolein for 60 min was inhibited in a dose-dependent manner with an IC_{50} value of 393 μM (standard deviation, 123 μM) (Fig. 4B). Inhibition of platelet aggregation was accompanied by inhibition of ATP secretion from dense granules in a concentration-dependent manner, with 100 μM acrolein producing approximately 50% inhibition (Fig. 4C). Acrolein appeared to be somewhat more potent in inhibiting secretion than aggregation; the reasons for this finding and its significance are unknown.

cis-Platinum did not inhibit platelets at concentrations of up to 1 mM and after preincubation for up to 4 hr (Fig. 1 and data not shown).

Because the studies described above suggested that preincubation of platelets in plasma with BCNU, 4-HC, or acrolein results in platelet inhibition, it became important to determine

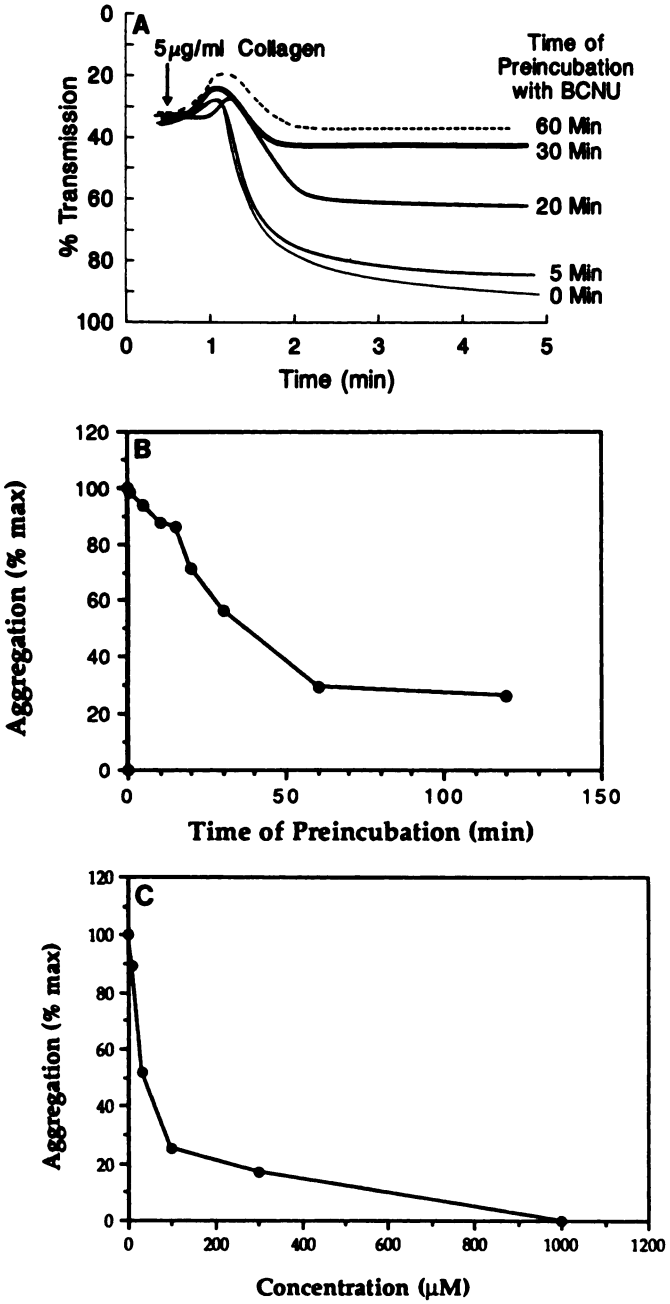

Fig. 2. Effects of BCNU on aggregation in PRP. A, Time dependence. Aggregation tracings of PRP preincubated with 30 μM BCNU for the indicated times and stimulated with 5.0 μg/ml collagen. B, An independent experiment in which aggregation was measured and plotted as percentage of maximal aggregation observed with collagen alone. C, Concentration dependence. PRP was preincubated with different concentrations of BCNU for 30 min and then stimulated with collagen (2.5 μg/ml).

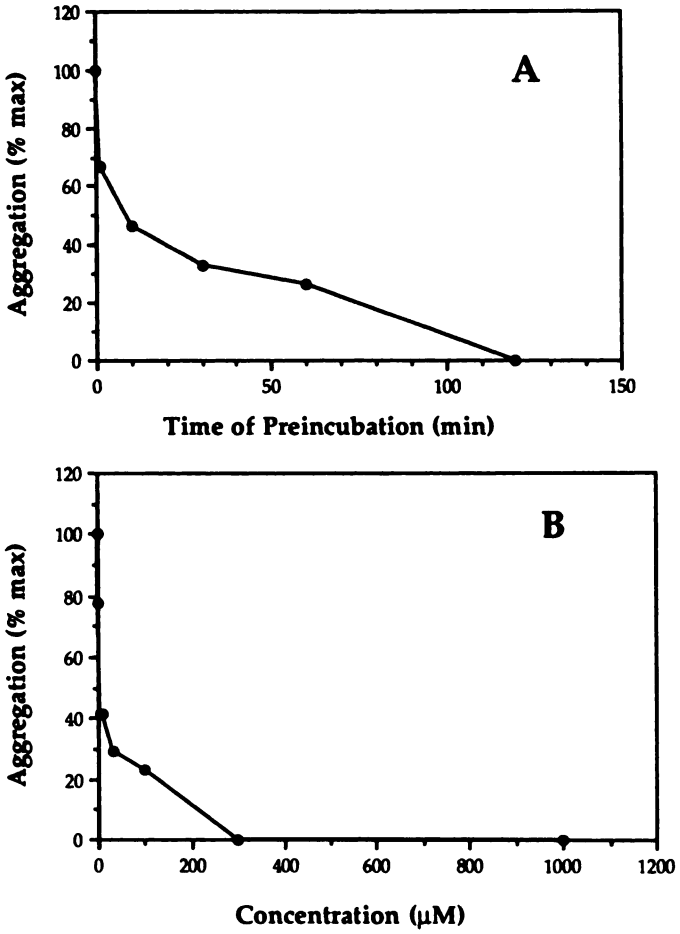

Fig. 3. Effects of 4-HC on aggregation in PRP. A, Time dependence. PRP was preincubated with 100 μM 4-HC for the indicated times and then stimulated with collagen (2.5 μg/ml). B, Concentration dependence. PRP was incubated with the indicated concentrations of 4-HC for 30 min and then stimulated with collagen.

whether these effects are dependent on the presence of plasma proteins or other factors. Moreover, determining the parameters of inhibition in washed platelets is critical for further biochemical studies. Therefore, additional studies were conducted in a washed platelet preparation. Preincubation of washed platelets with 30 μM BCNU resulted in a time-dependent inhibition of platelet aggregation in response to γ-thrombin (Fig. 5A). A 30-min preincubation resulted in 50% inhibition, whereas preincubation for 60 min resulted in total inhibition of aggregation. Inhibition of platelet aggregation was dependent on the concentration of BCNU, with an IC_{50} value of 10 μM (standard deviation, 3.3 μM) (Fig. 5B).

Similar results were obtained with acrolein. Preincubation of washed platelets with 30 μM acrolein resulted in progressive inhibition of platelet aggregation with increased duration of preincubation. Total inhibition was seen after 15 min of preincubation (Fig. 6A). Inhibition of platelet aggregation was dependent on the concentration of acrolein, with an IC_{50} value of 8 μM (standard deviation, 3 μM) (Fig. 6B). Similar studies were also obtained with 4-HC, showing a concentration dependence of inhibition as well as increased inhibition with prolonged preincubation (data not shown).

These data suggest that preincubation of platelets alone with these reagents is sufficient for the development of inhibition. Both BCNU and acrolein appeared more potent in inhibiting washed platelets, compared with PRP, which may be related to the presence of plasma proteins in PRP.

The effects of acrolein in inducing hemorrhagic cystitis appear to be due to interaction of acrolein with sulfhydryl groups. Thus, sulfhydryl-protecting agents such as MESNA and *N*-acetylcysteine provide protection from the development of hemorrhagic cystitis (11, 12). To evaluate whether a similar mech-

anism is operating in the inhibition of platelet function, washed platelets were incubated with acrolein in the presence or absence of MESNA or *N*-acetylcysteine. Incubation of washed platelets with 100 μM acrolein produced near-total inhibition of aggregation in response to thrombin (Fig. 7A). However, the

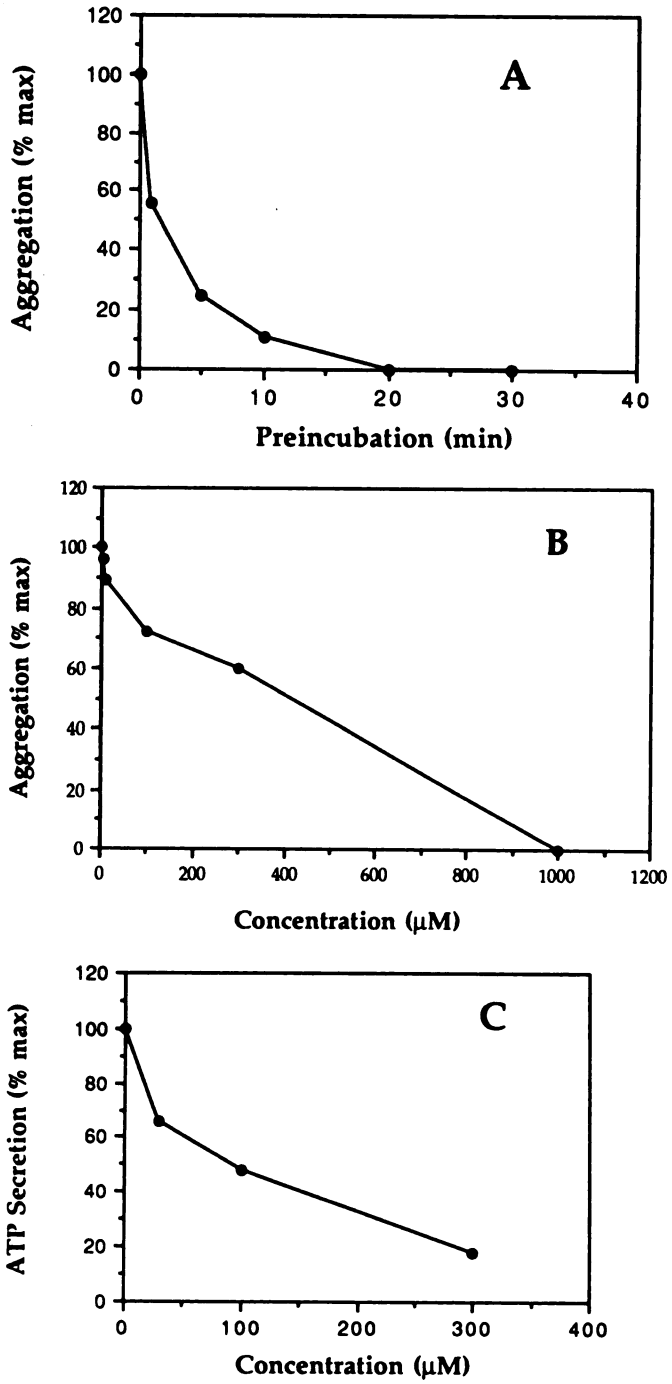

Fig. 4. Effects of acrolein on aggregation in PRP. A, Time dependence. PRP was preincubated with 1 mM acrolein for the indicated times and then stimulated with collagen (2.5 μg/ml). B, Concentration dependence. PRP was preincubated with the indicated concentrations of acrolein for 60 min and then stimulated with collagen (2.5 μg/ml). C, Effects on secretion. PRP was preincubated with the indicated concentrations of acrolein for 60 min and then stimulated with collagen. ATP secretion was monitored using Chronolume and results are expressed as percentage of maximal secretion obtained with collagen alone.

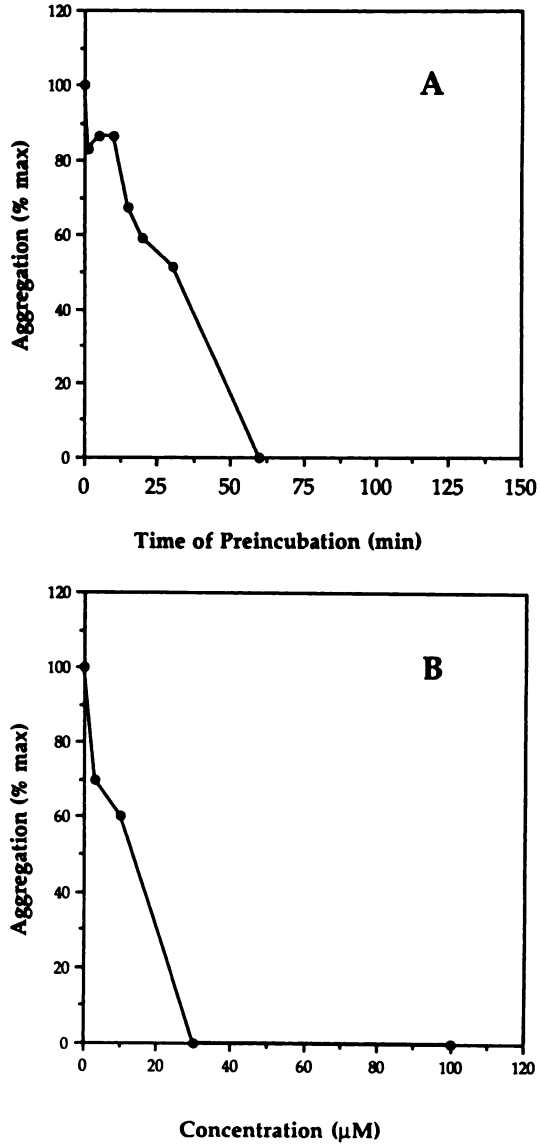

Fig. 5. Effects of BCNU on washed platelets. A, Washed platelets were preincubated with 30 μM BCNU for the indicated times and then stimulated with 8 nM γ-thrombin. B, Washed platelets were preincubated with the indicated concentrations of BCNU for 60 min and then stimulated with γ-thrombin (8 nM).

inclusion of 1 mM MESNA or 100 μM *N*-acetylcysteine resulted in total protection of platelet function from the action of acrolein (Fig. 7A).

A similar mechanism appears to partly account for the effects of BCNU. Incubation of washed platelets with BCNU for 30 min resulted in approximately 60% inhibition of aggregation (standard deviation, 7%). The inclusion of 1 mM MESNA or 100 μM *N*-acetylcysteine resulted in significant protection from the effects of BCNU (22 ± 4% and 27 ± 6% inhibition, respectively) (Fig. 7B). However these agents were not able to totally prevent the inhibition by BCNU, suggesting that perhaps the action of BCNU is mediated through sulfhydryl inactivation as well as other mechanisms.

Plasma levels of BCNU and cyclophosphamide. The actual *in vivo* mediators of the platelet defect are difficult to establish at this point. Comparison of *ex vivo* exposure to

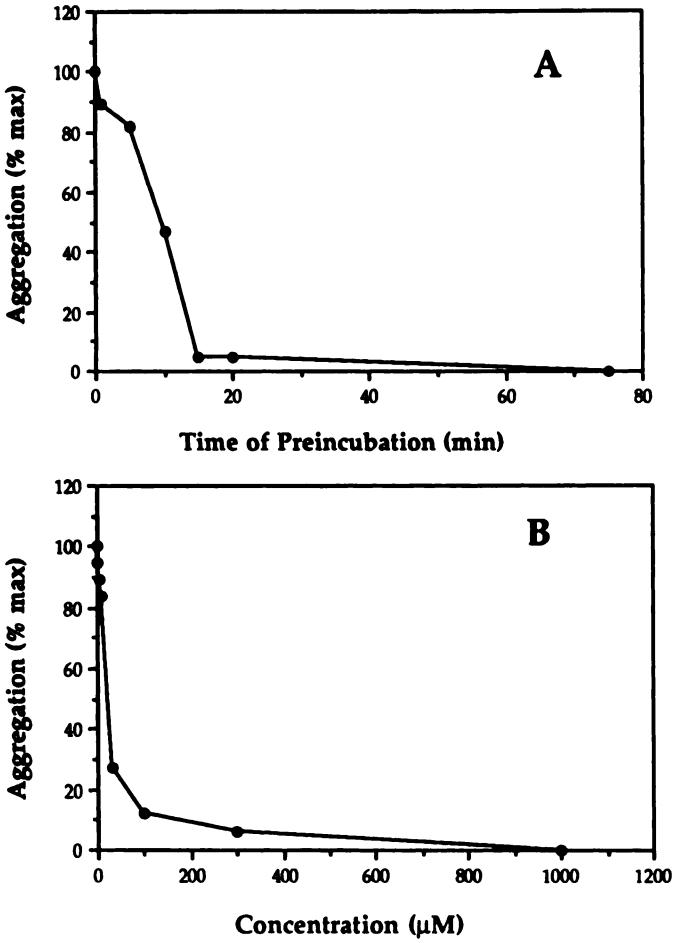

Fig. 6. Effects of acrolein on washed platelets. A, Washed platelets were preincubated with 30 μM acrolein for the indicated times and then stimulated with 8 nM γ-thrombin. B, Washed platelets were preincubated with the indicated concentrations of acrolein for 60 min and then stimulated with γ-thrombin (8 nM).

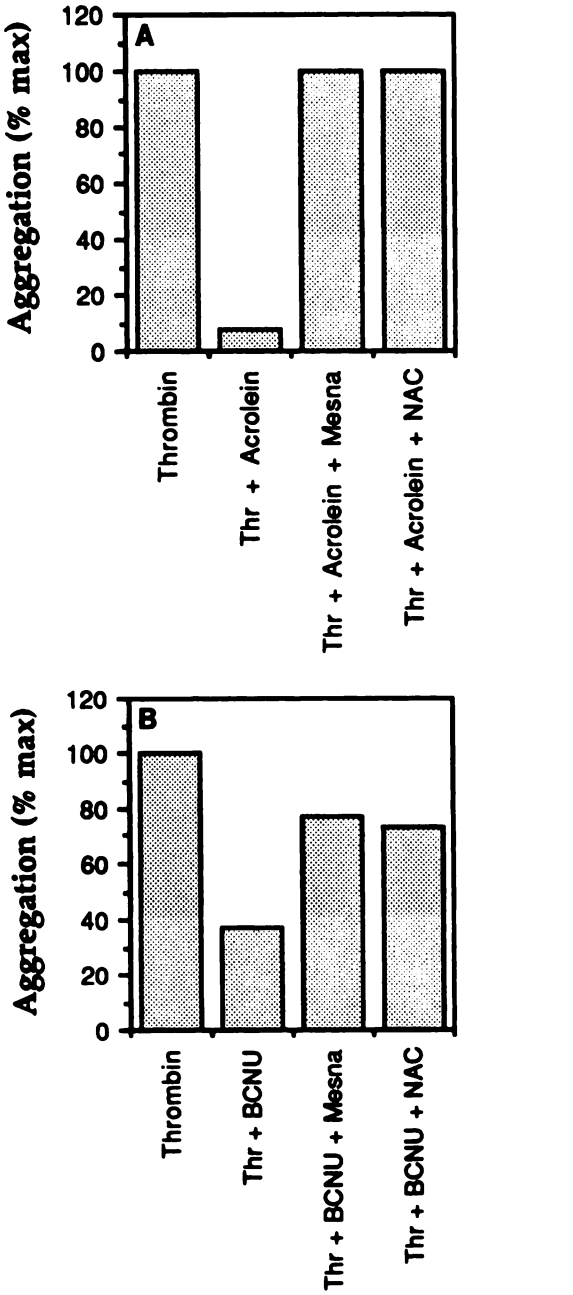

Fig. 7. Protective effects of MESNA and *N*-acetylcysteine. A, Washed platelets were preincubated with vehicle, 100 μM acrolein, acrolein plus 1 mM MESNA, or acrolein plus 100 μM *N*-acetylcysteine (*NAC*) for 1 hr and then stimulated with 20 nM thrombin (*Thr*). Aggregation was monitored and is expressed as percentage of maximal aggregation seen with thrombin alone. B, Same study with BCNU preincubation.

chemotherapeutic agents and clinical exposure is complicated by many features, including drug dose, drug schedule, and patient pharmacodynamics. During the treatment of patients under the high-dose protocol that prompted these studies, we performed pharmacokinetic measurements of the individual antineoplastic agents on a daily basis. The plasma levels of cyclophosphamide and BCNU achieved during combined treatment with high-dose cyclophosphamide, cisplatin, and BCNU varied between patients and days of treatment. For the parent cyclophosphamide, the median C_{max} measured during 1-hr intravenous infusions on days 1, 2, and 3 of treatment was 345 μM (standard deviation, 35 μM), 329 μM (standard deviation, 40 μM), and 307 μM (standard deviation, 51 μM), respectively, with a clearance of 47.7 ml/min/m^2, 73.7 ml/min/m^2, and 81.0 ml/min/m^2 on days 1, 2, and 3, respectively. Pharmacokinetic measurement of cyclophosphamide metabolites is technically difficult and unreliable, and thus methods for direct measurement of 4-HC and acrolein are not available. However, the high plasma levels of cyclophosphamide achieved suggest that its major metabolites may achieve concentrations sufficient to inhibit platelets.

For BCNU, the C_{max} in patients receiving 600 mg/m^2 over 2 hr was 24.5 μM (standard deviation, 14.5 μM), which is within the range of concentrations utilized in these studies. Thus, BCNU and metabolites of cyclophosphamide may mediate platelet inhibition *in vivo*.

Effects of acrolein and BCNU on signal transduction pathways. The preservation of dense and α granules in platelets after HDC (4) and the ability of the detergent Triton X-100 to fully release ATP from these platelets (data not shown) strongly suggested that HDC was interfering with the biochemical pathways involved in platelet activation in response to agonists. These effects could be at any level, ranging from agonist/receptor interaction to initial phases of signal trans-

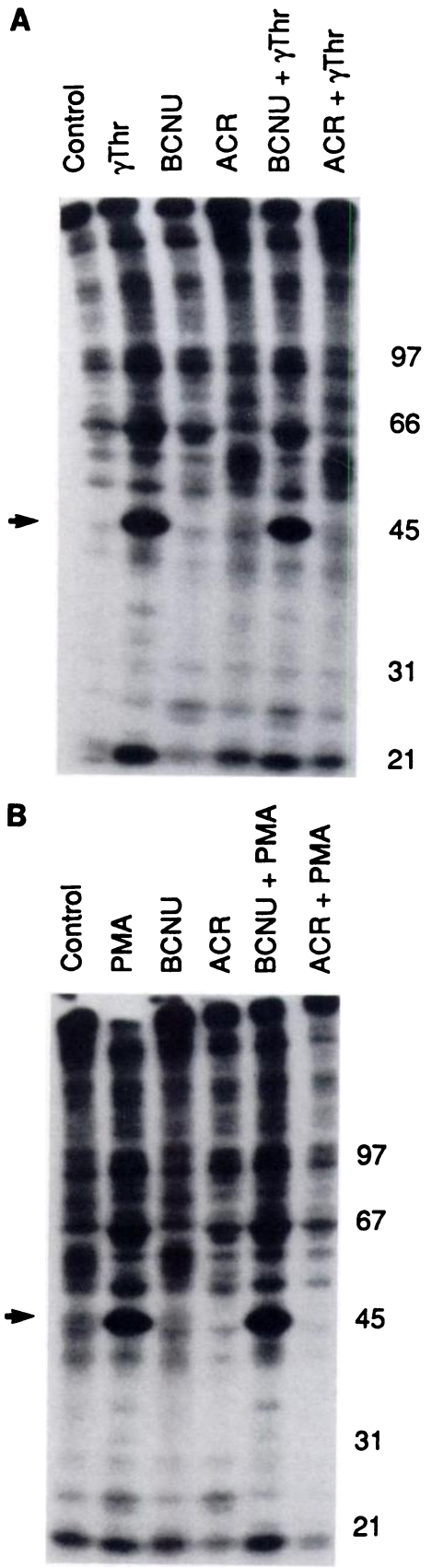

Fig. 8. Effects of BCNU and acrolein on phosphorylation of platelet proteins. A, Washed platelets were preincubated with either vehicle control, 100 μM BCNU, or 100 μM acrolein (*ACR*) for 1 hr and then stimulated with either vehicle or 30 nM γ-thrombin (*γThr*). B, Same experimental protocol but 100 nM PMA was used as the agonist instead of γ-thrombin.

duction such as phosphoinositide hydrolysis, inositol trisphosphate generation, calcium mobilization, diacylglycerol generation, or protein kinase C activation or more distal signaling events (13–16). Therefore, the effects of acrolein and BCNU on signal transduction mechanisms were investigated in platelets.

Examination of the effects of agonists and second messengers (or their analogs) on protein phosphorylation in intact platelets offers a well defined direct approach to evaluate whether the effects of inhibitors occur at the level of receptor, post-receptor activation of the calcium/myosin light chain kinase pathway or the diacylglycerol/protein kinase C pathway, or more distal signaling events. When intact washed platelets were stimulated with 30 nM γ-thrombin for 2 min, phosphorylation of multiple substrates occurred, predominant among which was the 40 (47)-kDa polypeptide that is a known substrate for protein kinase C *in vitro* and in intact platelets (17). The other prominent substrate was the 20-kDa myosin light chain, which is a substrate for both myosin light chain kinase (18) and protein kinase C (19). Preincubating platelets with 100 μM BCNU did not affect 40-kDa or 20-kDa protein phosphorylation in response to thrombin (Fig. 8A) but totally inhibited the aggregation response. These results strongly suggest that the initial interaction of thrombin with its receptor and subsequent signaling events resulting in activation of protein kinase C and myosin light chain kinase remain intact in the presence of BCNU.

Preincubation of platelets with acrolein (100 μM) totally inhibited 40-kDa protein phosphorylation in response to γ-thrombin and only partially inhibited 20 kDa protein phosphorylation (Fig. 8A). The effects of acrolein suggest inhibition of early components of the signal transduction pathway.

To evaluate further the level of inhibition of platelets by acrolein, phosphorylation studies were carried out in the presence of the phorbol ester PMA. Treatment of platelets with 100 nM PMA resulted in phosphorylation of a number of substrates, the most notable of which was the 40-kDa protein kinase C substrate (Fig. 8B). Pretreatment of platelets with 100 μM acrolein resulted in complete inhibition of PMA-induced phosphorylation (Fig. 8B). Because PMA bypasses initial receptor events and directly activates protein kinase C (20), these results strongly suggest that acrolein inhibits the cellular activity of protein kinase C and/or its interaction with its cellular substrates. BCNU did not inhibit PMA-induced phosphorylation, consistent with its inability to inhibit thrombin-induced phosphorylation (Fig. 8B).

Therefore, these results demonstrate that acrolein interferes with a specific phase of the signal transduction pathway of platelet activation at the level of cellular activation of protein kinase C. BCNU, on the other hand, appears to inhibit platelets at more distal components of the biochemical pathways involved in platelet activation.

Discussion

HDC results in an acquired platelet defect characterized by the absence of secondary aggregation (inhibition of irreversible aggregation and secretion). Morphologic studies disclosed the presence of intact dense and α granules in these platelets,

suggesting a defect in intracellular biochemical mechanisms that are involved in platelet activation (4). Determination of the mechanism by which HDC induces this platelet defect has been hampered by the lack of suitable *in vitro* models. The results from this study demonstrate that BCNU, one of the chemotherapeutic agents used in HDC, is capable of inhibiting platelet aggregation *in vitro* in a concentration-dependent manner. The effective concentrations of BCNU are achievable in plasma of patients undergoing HDC. Neither *cis*-platinum nor cyclophosphamide, the other two main chemotherapeutic agents in HDC, were able to inhibit platelets under similar conditions. However, a major metabolite of cyclophosphamide, 4-HC, inhibited platelet aggregation, and this inhibition also required preincubation of platelets with this agent. Acrolein, another major metabolite of cyclophosphamide implicated in the pathogenesis of hemorrhagic cystitis, behaved similarly to BCNU and 4-HC in inhibiting platelet aggregation, with a requirement for preincubation. The ability of these agents to inhibit platelets was demonstrated against all tested agonists, including ADP, collagen, and thrombin. Thus, at least one of these agents (BCNU) along with the metabolites of cyclophosphamide are candidate mediators of the pathogenesis of the acquired platelet defect during HDC.

The ability of MESNA and *N*-acetylcysteine to prevent totally the effects of acrolein and to ameliorate significantly the effects of BCNU on platelet aggregation strongly suggests that the induction of this acquired platelet defect may be mediated through critical sulfhydryl groups on key cellular targets. This is consistent with previous studies that have examined the effects of sulfhydryl inhibitors on platelet function. It was shown that *para*-chloromercuribenzoate, *N*-ethylmaleimide, and methyl mercuric nitrate inhibited ADP-induced aggregation and clot retraction by interfering with cellular thiol groups of platelets (21–23).

The development of this *in vitro* model for the induction of a platelet defect with chemotherapeutic agents and their metabolites has allowed probing of the mechanisms involved in the pathogenesis of this disorder. Two important conclusions arise from the phosphorylation studies in intact platelets pretreated with either acrolein or BCNU. First, acrolein inhibits platelets by interfering with the cellular activation of protein kinase C. These results further corroborate the essential role of protein kinase C in mediating platelet aggregation and secretion (5, 17, 24). Second, BCNU acts by a mechanism distinct from that of acrolein and does not interfere with the initial phases of signal transduction but appears to inhibit more distal components of the biochemical pathways of platelet activation. The mechanism of action of 4-HC has not been evaluated in this study, but preliminary results suggest that 4-HC acts similarly to acrolein in inhibiting 40-kDa protein phosphorylation.

These results should be of great significance in further dissection of the molecular mechanisms of platelet activation and inhibition. For example, acrolein does not inhibit protein kinase C α or β (the predominant calcium-dependent isoenzymes in platelets) *in vitro*.[3] This suggests that the cellular effects of acrolein may be mediated 1) by inhibition of other, heretofore unidentified, isoenzymes of protein kinase C or 2) by interference with specific interactions of protein kinase C and its substrates in intact cells. On the other hand, BCNU may become a very useful tool in dissecting out important components in distal signal transduction pathways involved in platelet activation.

The ability of BCNU and acrolein to induce platelet dysfunction and the distinct mechanisms by which they operate may also extend to other cell systems. Notably, HDC results in significant endothelial cell dysfunction, resulting in venoocclusive disease and disturbances in hemostasis and thrombosis. Therefore, results obtained from platelet studies may have implications for other systems as well.

[3] W. Khan and Y. Hannun, unpublished observations.

Acknowledgments

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References

1. Peters, W. High dose chemotherapy and autologous bone marrow support in breast cancer, in *Important Advances in Oncology* (V. T. Devita, S. Hollman, and S. A. Rosenberg, eds.). J. B. Lippincott, New York, 135–150 (1991).
2. Lazo, J. S. Endothelial cell injury caused by antineoplastic agents. *Biochem. Pharmacol.* **35**:1919–1923 (1986).
3. Gottdiener, J. S., F. R. Applebaum, V. J. Ferrans, A. Deisseroth, and J. Ziegler. Cardiotoxicity associated with high-dose cyclophosphamide therapy. *Arch. Intern. Med.* **141**:758–763 (1981).
4. Panella, T. J., W. Peters, J. G. White, Y. A. Hannun, and C. S. Greenberg. Platelets acquire a secretion defect after high-dose chemotherapy. *Cancer (Phila.)* **65**:1711–1716 (1990).
5. Werner, M. H., L. Senzel, A. Bielawska, W. Khan, and Y. A. Hannun. Diacylglycerol overcomes aspirin inhibition of platelets: evidence for a necessary role for diacylglycerol accumulation in platelet activation. *Mol. Pharmacol.* **39**:547–556 (1991).
6. Siess, W., F. Siegel, and E. G. Lapetina. Arachidonic acid stimulates the formation of 1,2-diacylglycerol and phosphatidic acid in human platelets. *J. Biol. Chem.* **258**:11236–11242 (1983).
7. Hannun, Y. A., C. R. Loomis, A. H. Merrill, Jr., and R. M. Bell. Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and human platelets. *J. Biol. Chem.* **261**:12604–12609 (1986).
8. El-Yazigi, A., and C. R. Martin. Improved analysis of cyclophosphamide by capillary gas chromatography with thermionic (nitrogen-phosphorus) specific detection and silica sample purification. *J. Chromatogr.* **374**:177–182 (1986).
9. Colvin, M. The alkylating agents, in *Pharmacologic Principles of Cancer Treatment* (B. Chabner, ed.). W. B. Saunders, Philadelphia, 276–308 (1982).
10. Cox, P. J. Cyclophosphamide cystitis: identification of acrolein as the causative agent. *Biochem. Pharmacol.* **28**:2045–2049 (1979).
11. Brock, N., J. Pohl, and J. Stekar. Studies on the urotoxicity of oxazaphosphorine cytostatics and its prevention. 2. Comparative study on the uroprotective efficacy of thiols and other sulfur compounds. *Eur. J. Cancer Clin. Oncol.* **17**:1155–1163 (1981).
12. Andriole, G. L., J. T. Sandlund, J. S. Miser, V. Arasi, M. Linehan, and I. T. Magrath. The efficacy of Mesna (2-mercaptoethane sodium sulfonate) as a uroprotectant in patients with hemorrhagic cystitis receiving further oxazaphosphorine chemotherapy. *J. Clin. Oncol.* **5**:799–803 (1987).
13. Huang, E. M., and T. C. Detwiler. Stimulus-response coupling mechanisms, in *Biochemistry of Platelets* (D. R. Phillips and M. A. Shuman, eds.). Academic Press, Orlando, 1–68 (1986).
14. Majerus, P. W., V. S. Bansal, D. L. Lips, T. A. Ross, C. A. Mitchell, K. K. Caldwell, and T. W. Cunningham. The phosphatidylinositol pathway of platelets and vascular cells. *Ann. N. Y. Acad. Sci.* **614**:44–50 (1991).
15. Nozawa, Y., S. Nakashima, and K. Nagata. Phospholipid-mediated signaling in receptor activation of human platelets. *Biochim. Biophys. Acta* **1082**:219–238 (1991).
16. Werner, M. H., L. Senzel, A. Bielawska, W. Khan, and Y. A. Hannun. Diacylglycerol overcomes aspirin inhibition of platelets: evidence for a necessary role for diacylglycerol accumulation in platelet activation. *Mol. Pharmacol.* **39**:547–556 (1991).
17. Sano, K., Y. Takai, J. Yamanishi, and Y. Nishizuka. A role of calcium-activated phospholipid-dependent protein kinase in human platelet activation. *J. Biol. Chem.* **258**:2010–2013 (1983).
18. Daniel, J. L., I. R. Molish, M. Rigmaiden, and G. Stewart. Evidence for a role of myosin phosphorylation in the initiation of the platelet shape change response. *J. Biol. Chem.* **259**:9826–9831 (1984).
19. Kawamoto, S., A. R. Bengur, J. R. Seleirs, and R. S. Adelstein. *In situ* phosphorylation of human platelet myosin heavy and light chains by protein kinase C. *J. Biol. Chem.* **264**:2258–2265 (1989).
20. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. Direct activation of calcium-activated, phospholipid-dependent protein ki-

nase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257**:7847–7851 (1982).
21. Robinson, C. W., Jr., R. G. Mason, and R. H. Wagner. Effect of sulfhydryl inhibitors on platelet agglutinability. *Proc. Soc. Exp. Biol. Med.* **113**:857–861 (1963).
22. Aledort, L. M., S. B. Troup, and R. I. Weed. Inhibition of sulfhydryl-dependent platelet functions by penetrating and non-penetrating analogues of *para*-chloromercuribenzene. *Blood* **31**:471–479 (1968).
23. Fantl, P. Thiol groups of blood platelets in relation to clot retraction. *Nature (Lond.)* **198**:95 (1963).
24. Hannun, Y. A., C. S. Greenberg, and R. M. Bell. Sphingosine inhibition of agonist dependent secretion and activation of human platelets implies that protein kinase C is a necessary and common event of the signal transduction pathway. *J. Biol. Chem.* **262**:13620–13626 (1987).

Send reprint requests to: Yusuf A. Hannun, Duke University Medical Center, Department of Medicine, Box 3355, Durham, NC 27710.