In vitro Procoagulant Activity Induced in Endothelial Cells by Chemotherapy and Antiangiogenic Drug Combinations: Modulation by Lower-Dose Chemotherapy

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Abstract

One of the emerging problems concerning the use of antiangiogenic drugs, when used in combination with certain chemotherapy regimens, is enhanced rates and severity of adverse clotting events. For as yet unknown reasons, certain drugs and particular combinations can induce an elevated incidence of thromboembolic events in treated cancer patients [e.g., SU5416, a vascular endothelial cell growth factor receptor-2 (VEGFR-2) antagonist, when combined with gemcitabine and cisplatin (CDDP)]. Such results highlight the need to develop assays capturing the essence of enhanced clot formation under such combination treatment and which may have predictive potential as well. Here, we report the possibility of such an assay (i.e., the ratio of tissue factor over tissue factor pathway inhibitor expression or activity in cultured human endothelial cells calculated as a coagulation index). A marked increase in coagulation index was observed after exposure to SU5416 and the CDDP/gemcitabine chemotherapy combination in contrast to either of these treatments used alone. Substitution of SU5416 with any one of ZD6474, SU6668, IMC-1121, a monoclonal antibody to VEGFR-2, or an antibody to VEGF (bevacizumab) did not cause a marked increase in the coagulation index, nor did the combination of SU5416 with 5-fluorouracil and leucovorin. Finally, we note that reducing the concentrations of gemcitabine and CDDP (i.e., use of “metronomic dosing” in vitro) significantly attenuated the coagulation index increase induced by these drugs, suggesting that use of low-dose chemotherapy regimens might be an approach to consider for reducing the incidence of adverse clotting events associated with chemotherapy alone or in conjunction with antiangiogenic drug combination therapies.

(Cancer Res 2005; 65(12): 5365-73)

Introduction

Significant increases in thromboembolic events, which can sometimes be lethal, are associated with malignancy (1–3). There are several reasons for this, including the well-known procoagulant nature of many types of cancer (1–3), extensive bed rest, the use of treatment devices (such as inline catheters), and the procoagulant effects of certain cancer treatments, including surgery and chemotherapy (1–3). With respect to chemotherapy, the nature of the procoagulant effect seems to vary with different chemotherapeutic drugs and regimens, the extent of which can be exacerbated by using certain combinations of chemotherapeutic drugs or combining chemotherapeutic drugs with other types of anticancer therapeutic agents (4). An example of the latter is the use of certain antiangiogenic drugs (5) as illustrated by a recent clinical report of a highly significant increase in thromboembolic events in a phase 1 clinical trial of advanced solid tumor patients treated with gemcitabine and cisplatin (CDDP) in combination with SU5416, a small-molecule receptor tyrosine kinase inhibitor of the type 2 receptor (KDR/Flk-1) for vascular endothelial cell growth factor (VEGF; ref. 5). Eight of 19 (42%) patients experienced significant adverse thromboembolic or other vascular (arterial) events in this trial (5). In striking contrast, other trials, in which either SU5416 was tested alone or when CDDP plus gemcitabine was used, have generally shown very low incidences of thromboembolic events in the range of 0% to 2% (6–8). Although such results are particularly alarming, there are indications that other antiangiogenic agents combined with particular types of chemotherapeutic agents may, albeit to varying degrees and possibly through different mechanisms, also adversely affect the integrity of the hemostatic system. Thus, thalidomide used as an antiangiogenic agent is usually not in itself associated with a significant increase in thromboembolic events (9) but can be when used in combination with chemotherapeutic drugs (10). More recently, there was an announcement of a 2-fold increase of potentially serious clot-related adverse events, primarily arterial clots, in colorectal patients receiving 5-fluorouracil (5-FU) with bevacizumab (Avastin), the humanized anti-VEGF antibody compared with the chemotherapy alone control arm, although this risk remains low overall (i.e., 5%). This increased risk was primarily seen in patients with preexisting vascular risk factors, such as age >70 years and prior myocardial infarction or stroke. Bevacizumab treatment was nevertheless associated with a marked overall survival for these patients due to its antitumor/antiangiogenic effects. Taken together, the aforementioned clinical findings suggest that it is the interaction of antiangiogenic drugs with chemotherapy rather than antiangiogenic drugs per se that, in general, are responsible for the increased clotting events observed in these clinical circumstances. The specific mechanisms that can cause exacerbation of prothrombotic events in cancer patients in response to specific combinations of antiangiogenic drugs and chemotherapy agents are unknown and are being analyzed (11). They are undoubtedly

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complex and multifactorial. As such, this clearly highlights the need to understand the constituents of such thrombosis-causing circumstances and establish criteria as well as assays to predict (and prevent) their occurrence. With this in mind, we set out to determine whether an in vitro assay involving human vascular endothelial cells could be developed for such a purpose.

In regards to drug-induced coagulopathy, the hemostatic state of endothelial cells is of particular interest for at least two reasons. First, endothelial cells normally serve as an "anticoagulant barrier" that separates the coagulation proenzymes of blood plasma from the "hemostatic envelope" of perivascular tissues (12). At the same time, endothelial cells are targets of both antiangiogenic and anticancer/cytotoxic agents (including SU5416 and quite possibly gemcitabine/CDDP as well; refs. 4, 13–15), whereby their integrity and function (including anticoagulant properties) could be compromised. Indeed, endothelial cells have a potential to express, in a regulatable manner, at least two major opposing influences that control the activity of the coagulation system [i.e., tissue factor (12) and tissue factor pathway inhibitor (TFPI; ref. 16)].

The former (tissue factor) is a transmembrane protein, which acts as a receptor for the coagulation factor VII (VII) and its activated form—fVIIa. As traces of the latter are present in normal plasma, contact between blood and tissue factor in the extravascular compartment (e.g., on injury) or presented from within the intravascular compartment (e.g., by monocytes or altered/damaged endothelium) precipitates formation of the tissue factor/fVIIa complexes, which activate coagulation factor X (X) to fXa. This leads to conversion of small quantities of prothrombin to thrombin (factor IIa), and the latter step is further amplified through involvement of platelets and coagulation factors fVa, fVIIIa, and fXa resulting in burst of thrombin activity and formation of the fibrin clot (17). The central role of tissue factor and tissue factor/fVIIa complex in this process is opposed by TFPI, a Kunitz-type antithrombin that binds to fX and prevents formation of the active tissue factor/fVIIa/Xa complex. The main sources of TFPI are endothelial cells, the surfaces of which also serve as the reservoir of 80% to 85% of this inhibitor (16). Thus, endothelial lining of quiescent blood vessels contains an abundance of TFPI in the virtual absence of tissue factor expression. This anticoagulant balance (high TFPI/tissue factor ratio) can, however, be altered on endothelial cell exposure to stimuli of proangiogenic (e.g., VEGF) vs. antiangiogenic (e.g., tumor necrosis factor) nature, as they often trigger marked up-regulation of tissue factor expression and activity (18). This could lead to reduction/reversal of the TFPI/tissue factor ratio and cause procoagulant conversion of activated endothelial cells.

The major purpose of this study was to examine how the hemostatic balance between tissue factor and TFPI may be affected by pathologic/pharmacologic rather than activating conditions, specifically in this case as a result of exposure of endothelial cells in vitro to antiangiogenic drug/chemotherapy treatment protocols and whether such induced changes might have the potential as an assay predictive of certain adverse cloting events in vivo. Two different scenarios were considered, including testing in vitro the SU5416 VEGF receptor-2 (VEGFR-2) inhibitor in combination with either high or low doses of certain chemotherapeutic drugs (especially gemcitabine and CDDP). Our results show that the former regimen triggered a procoagulant conversion of endothelial cells in vitro as measured by dramatic increase in their surface tissue factor/TFPI expression/activity ratio (i.e., coagulation index).

Interestingly, this seemed to be uniquely amplified by SU5416 but not by various other inhibitors of the VEGF/VEGFR pathway, a finding strikingly consistent with previously reported clinical observations (5) and hence suggestive of being potentially predictive of other drug combinations having a similar and significant procoagulant activity. In contrast, low ("metronomic") concentrations of the same agents triggered antiendothelial effects without dramatic increases in the tissue factor/TFPI index. These results suggest that prothrombotic side effects of these anticancer and antiangiogenic agents are not inevitable and might be ameliorated by procedures such as metronomic low-dose chemotherapy rather than traditional maximum tolerated dose chemotherapy regimens.

Materials and Methods

Cell cultures. Gemcitabine was purchased from Eli Lilly Canada, Inc. (Toronto, Ontario, Canada). CDDP was from Faulding Canada, Inc. (Vaudreuil, Quebec, Canada); IMC-1121 is an anti-VEGFR-2 blocking antibody (19) from ImClone Systems, Inc. (New York, NY); SU5416 (20) and SU6668 (21) were originally obtained from SUGEN (South San Francisco, CA) and ZD6474 (22) was obtained from AstraZeneca (Cheshire, United Kingdom); all three were developed primarily as small-molecule VEGFR-2 antagonists (23). Bevacizumab (Avastin), the humanized anti-human VEGF antibody, was obtained from Genentech (South San Francisco, CA; ref. 24).

Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and maintained in MCD131 medium supplemented with 10% of FCS, 10 IU/mL heparin, 5 ng/mL basic fibroblast growth factor, 10 ng/mL epidermal growth factor, and 10 ng/mL VEGF on gelatin-coated dishes. The cells were maintained under standard cell culture conditions at 37°C and 5% CO2 in a humid environment. Medium was changed once every 2 days.

Drug treatments. Cells were seeded onto plates or dishes, allowed to adhere for 24 hours, and then treated with various concentrations of CDDP, gemcitabine, or SU5416 for 18 hours. Cell proliferation, DNA fragmentation, tissue factor protein/activity, and TFPI protein/activity were assessed. In the experiments where a combination treatment was tested, the medium was replaced with growth factor–free medium for 3 hours. Thereafter cells were treated with SU5416 (10 μmol/L) 30 minutes before the treatments with CDDP, gemcitabine, or CDDP plus gemcitabine in the MCD131 medium with 2% serum and 10 ng/mL VEGF for 18 hours. Again, cell proliferation, DNA fragmentation, endothelial cell–associated tissue factor, and TFPI activity and protein expression were assessed. In experiments involving SU6668 (14 μmol/L) and ZD6474 (1 μmol/L), VEGF/KDR tyrosine kinase inhibitors, IMC-1121 (10 nmol/L), or bevacizumab (10 μg/mL), cells were cultured with these reagents 30 minutes before chemotherapy drug treatments.

Some comments on the various drug concentrations we used for our experiments need to be stressed. The respective doses of targeted agents were selected based on their activity against endothelial cells in vitro and pharmacokinetic considerations in humans. As reported in the clinical trial involving combination therapy of SU5416 with CDDP and gemcitabine, the maximal blood concentrations of CDDP and gemcitabine were 12 to 15 and 50 to 70 μmol/L, respectively (5). Based on this, dose responses of the drugs on endothelial cell proliferation, apoptosis, endothelial cell–associated tissue factor/TFPI activity, and expression were tested at concentrations ranging from 0.1 to 100 μmol/L. With respect to SU5416, a concentration of 10 μmol/L was chosen based on its inhibitory effects on endothelial cell proliferation in both HUVECs and HMVECs. The 10 μmol/L concentration caused 50% to 65% growth inhibition. A concentration of 14 μmol/L was used for SU6668 as an equivalent "dose" to SU5416. ZD6474 (1 μmol/L) was selected in our study based on its IC50 against VEGFR-2/KDR (40 nmol/L) and its IC50 in inhibiting VEGF-stimulated HUVEC proliferation (60 nmol/L; ref. 22).
In addition, IMC-1121 (10 mmol/L) was tested according to its IC_{50} (1 mmol/L) in blocking VEGF/KDR interaction (19).

**Cell proliferation and DNA fragmentation.** The [3H]thymidine incorporation assay was employed to determine cell proliferation. Briefly, cells were seeded onto 96-well plates at 5,000 per well. [3H]Thymidine (2 μCi/well) was added 6 hours before the end of incubation with the treatments mentioned above. Cell proliferation is represented by the rate of [3H]thymidine incorporation.

DNA fragmentation assay was done using a Cell Death ELISA kit (Roche, Mannheim, Germany). Cells were seeded onto 24-well plates at 2 × 10^4 per well, allowed to adhere, and then treated with CDDP, gemcitabine, or CDDP plus gemcitabine with or without SU5416 for 18 hours as mentioned above. At the end of the incubation, both floating and adhering cells were lysed with the buffer provided with the kit. The level of DNA fragmentation in the lysate was measured according to the manufacturer's instructions.

**Cell surface–associated tissue factor and tissue factor pathway inhibitor activity.** Cells on 60-mm dishes treated with CDDP, gemcitabine, or CDDP plus gemcitabine with or without SU5416 for 18 hours were collected at the end of the incubation, treated with 4 mmol/L EDTA, and subsequently washed twice with PBS. Cells were then resuspended in assay buffer at a concentration of 1 × 10^5 cells/mL. Cell suspension (20 or 25 μL) was applied for measuring cell surface tissue factor or TFPI activity, respectively, by using commercially available kits Actichrome tissue factor and TFPI (American Diagnostica, Inc., Greenwich, CT) based on the formation or inactivation of fXa. The values of tissue factor and TFPI activity are presented as pmol/L or μg/U^10^5 cells, respectively.

**Tissue factor and tissue factor pathway inhibitor protein expression.** Cells were lysed using lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 1% Triton X-100, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 100 μg/mL phenylmethylsulfonyl fluoride) at the end of treatments. Tissue factor and TFPI protein levels were measured using commercial ELISA kits (American Diagnostica). Total protein levels in cell lysates were measured with Bio-Rad Protein Assay Solution (Bio-Rad Laboratories, Hercules, CA). The values of tissue factor and TFPI protein are presented as ng/mg.

**Western blot analysis.** Cell lysates separated by SDS-PAGE were transferred electrophoretically to Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). The membranes were probed with antibodies against p44/42, phospho-p44/42, p38, or phospho-p38 mitogen-activated protein kinase (MAPK; Cell Signaling Technology, Inc., Beverly, MA) and followed by incubation with a peroxidase-conjugated anti-mouse or anti-rabbit antibody (Promega, Madison, WI). Blots were then visualized by using the enhanced chemiluminescence kit (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, United Kingdom).

**RNA isolation and Northern blot analysis.** Total RNA was prepared from treated cells using the Trizol reagent according to the manufacturer's instruction (Life Technologies, Burlington, Ontario, Canada). Total RNA (20 μg) was size fractionated by gel electrophoresis in 1% agarose gel and then transferred to nitrocellulose membrane by a capillary transfer. Northern blots were hybridized using 32P-labeled tissue factor or TFPI cDNA probes. The tissue factor probe was a 0.8-kb cDNA fragment digested from PUC18 containing a 1.0-kb tissue factor gene insert (American Type Culture Collection, Rockville, MD) by EcoRI digestion. The TFPI probe was obtained as a 283-bp reverse transcription-PCR product using the following primers: forward 5'-CTACGTGTTCAGTGATCACGAGTCA-3' and reverse 5'-CAGTGCAATAATTGCAGTACG-3'.

**Statistical analysis.** All data are expressed as mean ± SE, with sample sizes of three to eight per group. All experiments were done in triplicate. Comparisons of data between groups were done with one-way ANOVA followed by Student's-Newman-Keuls' test. An associated probability (P) of <5% was considered significant.

**Results**

**Effects of cisplatin and gemcitabine on endothelial cell surface tissue factor and tissue factor pathway inhibitor activities.** Incubation of either HUVECs or HMVECs with CDDP or gemcitabine at various concentrations for 18 hours showed a significant and dose-dependent increase in endothelial cell surface tissue factor activity and a decrease in TFPI activity in both cell types (Fig. 1A and B). CDDP seems more potent of the two with respect to the up-regulation of cell surface tissue factor activity (4- to 5-fold) compared with gemcitabine treatment (which only caused ~2-fold increase). Both CDDP and gemcitabine (0.1-100 μmol/L) dose-dependently inhibited cell proliferation and increased DNA fragmentation as well, although the effects of gemcitabine were much stronger (Fig. 1C and D). The rationale for the drug concentrations used in this and other experiments is outlined in Materials and Methods (Drug treatments).

**Effects of combination of cisplatin and gemcitabine with SU5416 on endothelial cell surface tissue factor and tissue factor pathway inhibitor activities.** The concentrations of CDDP, gemcitabine, and SU5416 used here were chosen according to the results of proliferation and apoptosis experiments (causing 50-70% changes), which were also consistent with the plasma levels of the drugs involving the combination therapy as reported in a clinical trial (5).

As expected, SU5416, the VEGFR-2 small-molecule inhibitor, used at the concentration of 10 μmol/L inhibited endothelial cell proliferation and increased DNA fragmentation in the presence of 10 ng/ml VEGF in the medium (Fig. 2E and F). Cell surface tissue factor activity and total tissue factor expression were also inhibited by SU5416 under the same conditions (Fig. 2A and B), confirming that VEGF itself leads to the up-regulation of tissue factor as shown previously (25). CDDP (10 μmol/L) or gemcitabine (10 μmol/L) alone potentiated the in vitro "procoagulant" properties by further increasing both endothelial cell surface tissue factor expression and surface activity and decreasing TFPI expression and activity compared with the VEGF-containing medium groups (Fig. 2A-D). CDDP was more potent as far as up-regulation of tissue factor and down-regulation of TFPI activity is concerned. When CDDP (10 μmol/L) and gemcitabine (10 μmol/L) were used in combination, cell surface tissue factor activity was further increased compared with either drug alone, although the inhibition of TFPI protein levels and activities were not further altered. Surprisingly, SU5416 (10 μmol/L) significantly prevented the up-regulation of both tissue factor protein expression and endothelial cell surface tissue factor activity induced by CDDP. Because gemcitabine had only a moderate effect on tissue factor expression, the effect of SU5416 was minimal in this case. Interestingly, SU5416 did not reverse the pronounced stimulatory effect of the CDDP plus gemcitabine combination on tissue factor expression and activity, SU5416 also did not affect CDDP or gemcitabine (used alone)–induced down-regulation of TFPI protein expression and cell surface activity but dramatically enhanced the inhibition of both TFPI protein and activity when three drugs were used together (Fig. 2C and D).

When the net balance between endothelial cell tissue factor and TFPI was calculated as a coagulation index ratio [i.e., the ratio of cell surface activity (or total protein) of tissue factor to TFPI], the results clearly showed that when SU5416 was used together with both CDDP and gemcitabine a marked increase in coagulation index was observed but not with either drug alone (Fig. 3). In fact, SU5416 significantly attenuated the increase of coagulation index in CDDP (10 μmol/L)–treated endothelial cells (Fig. 3). Also of interest is the observation that when CDDP and gemcitabine were used together with SU6668 (a structurally similar small-molecule VEGF/KDR tyrosine kinase inhibitor to SU5416 but having a higher IC_{50} at 14 μmol/L, an equivalent dose to SU5416 at 10 μmol/L for inhibiting KDR, this combination actually decreased the coagulation index by
inhibiting tissue factor activity without affecting TFPI activity, indicating that KDR inhibition per se in all likelihood is not the mechanism mediating the imbalance between endothelial tissue factor and TFPI caused by the combination of SU5416 with CDDP and gemcitabine (Fig. 3). This was further confirmed by the experiments using ZD6474 (10 μmol/L), another VEGF/KDR tyrosine kinase inhibitor (22), or IMC-1121 (10 nmol/L), a very high affinity and specific neutralizing antibody against human VEGF/KDR (19); when either of these antiangiogenic VEGFR-2-inhibiting drugs was used together with CDDP plus gemcitabine, an increase in coagulation index was not observed; instead, ZD6474 and IMC-1211 actually decreased the coagulation index by inhibiting endothelial cell tissue factor activity without significantly affecting TFPI (Fig. 3). This was true as well for bevacizumab (10 μg/mL), the neutralizing anti-VEGF antibody (Fig. 3). Finally, when SU5416 was used together with other two different chemotherapeutic drugs, 5-FU (2.5 μmol/L) and leucovorin (5 μmol/L), no increase of coagulation index was observed (data not shown).

Although some of the data presented here were based on the results from HMVECs, all experiments were done in both HMVECs and HUVECs, and the results were similar (data not shown).

Preliminary analysis of possible mechanisms of the regulation of tissue factor and tissue factor pathway inhibitor induced by cisplatin and gemcitabine: role of mitogen-activated protein kinases. There are several studies implicating p44/p42 and p38 MAPKs in tissue factor expression in endothelial cells induced by VEGF or tumor necrosis factor-α (18, 26, 27). Hence, we investigated whether p44/p42 and/or p38 activation in endothelial cells were affected after 18-hour treatments with CDDP, gemcitabine, or CDDP plus gemcitabine in the presence or absence of SU5416 (10 μmol/L). Both p38 and p44/p42 phosphorylation were enhanced by CDDP (10 μmol/L), gemcitabine (10 μmol/L), and, most significantly, CDDP plus gemcitabine (Fig. 4A and B). Inhibition of p38 activity by SB203580 (10 μmol/L) reversed the increased endothelial cell–associated tissue factor induced by all these aforementioned treatments (Fig. 4C). However, inhibition of p44/42 phosphorylation showed diverse effects (e.g., attenuated CDDP-induced tissue factor up-regulation), had no effect on tissue factor in CDDP plus gemcitabine–treated group, but further enhanced gemcitabine-induced increases of tissue factor. Interestingly, SB203580 was able to antagonize the potentiating effect of PD98059 (50 μmol/L) on gemcitabine-induced tissue factor up-regulation (Fig. 4C). This indicates that the up-regulation of tissue factor activity/expression by CDDP and gemcitabine were different, with the former occurring via both p44/42 and p38 MAPK pathway, whereas the latter was p38 dependent. Clearly, the situation becomes more complicated when the two drugs are used together, especially if combined with certain kinase inhibitors. In the current study, inhibition of TFPI by chemotherapeutic drugs was not affected by either PD98059 or SB203580 (data not shown). As shown in Fig. 5, none of the chemotherapeutic drug treatment significantly affected tissue factor mRNA levels, suggesting that the up-regulation of tissue factor activity/expression by CDDP and gemcitabine were different, with the former occurring via both p44/42 and p38 MAPK pathway. In contrast, TFPI mRNA expression was inhibited by CDDP either alone or in the combination with gemcitabine and/or SU5416.

“Metronomic dosing” of chemotherapy in vitro lowers or eliminates the increase in coagulation index. Much lower concentrations of both CDDP and gemcitabine were still capable of inhibiting tissue factor activity without affecting TFPI activity, indicating that KDR inhibition per se in all likelihood is not the mechanism mediating the imbalance between endothelial tissue factor and TFPI caused by the combination of SU5416 with CDDP and gemcitabine (Fig. 3). This was further confirmed by the experiments using ZD6474 (10 μmol/L), another VEGF/KDR tyrosine kinase inhibitor (22), or IMC-1121 (10 nmol/L), a very high affinity and specific neutralizing antibody against human VEGF/KDR (19); when either of these antiangiogenic VEGFR-2-inhibiting drugs was used together with CDDP plus gemcitabine, an increase in coagulation index was not observed; instead, ZD6474 and IMC-1211 actually decreased the coagulation index by inhibiting endothelial cell tissue factor activity without significantly affecting TFPI (Fig. 3). This was true as well for bevacizumab (10 μg/mL), the neutralizing anti-VEGF antibody (Fig. 3). Finally, when SU5416 was used together with other two different chemotherapeutic drugs, 5-FU (2.5 μmol/L) and leucovorin (5 μmol/L), no increase of coagulation index was observed (data not shown).

Although some of the data presented here were based on the results from HMVECs, all experiments were done in both HMVECs and HUVECs, and the results were similar (data not shown).
inhibiting endothelial cell growth if applied over an extended period of time. Thus, when the cells were incubated with lower concentrations of CDDP (100 nmol/L) or gemcitabine (100 pmol/L-100 nmol/L) for 120 hours (instead of 18 hours), these treatments resulted in a dramatic inhibition of cell proliferation not observed during a shorter treatment (Fig. 6A). Interestingly, growth inhibition comparable with that achieved with acute treatment with higher doses of both agents did not translate, in this case, into similar increases in endothelial cell surface tissue factor activity. For instance, tissue factor levels were not affected in any of the gemcitabine-treated groups or were much less affected in CDDP-treated group at the concentration of 1 μmol/L compared with an 18-hour incubation of CDDP at higher concentrations (10-100 μmol/L), which caused similar extent of inhibition of endothelial cell proliferation (Figs. 1A and 6B). In addition, SU5416 did not cause a significant increase of coagulation index (Figs. 1A and 6B). Moreover, endothelial cell surface TFPI activities were not inhibited by this longer-term, low-dose treatment (Fig. 6C). Taken together, 'low-dose' chemotherapy drugs either alone or in the combination with SU5416 did not significantly affect coagulation index as shown in Fig. 6D. In contrast, the surviving endothelial cells (~2%) after 5-day treatments with high doses of CDDP, gemcitabine, CDDP plus gemcitabine, and CDDP plus gemcitabine plus the same concentration of SU5416 (10 μmol/L) showed increased tissue factor and decreased TFPI activity, especially in the CG+SU combination treatment, similar as observed in those with 18-hour exposure (Figs. 2A-D and 6B-D). In this case, the surviving cells also showed a significant change in cell size (~5-fold) and showed little proliferation and apoptosis activities. Taken together, these findings might indicate that chemotherapy drugs at lower doses given in a more protracted manner (i.e., metronomically) could conceivably have the advantage of causing a lower rate of thromboembolic events, assuming such in vitro results can be "translated" in vivo.

**Discussion**

Our results suggest the intriguing possibility of estimating an endothelial cell–associated coagulation index *in vitro*, based on the ratio of tissue factor/TFPI activity, and consideration of this as a possible predictive marker or assay for the potential procoagulant activity associated with treatment with anticancer drugs, in this case, chemotherapy alone (28), or in combination with antiangiogenic agents (5). Thus, an increase in the coagulation index was observed in VEGF-stimulated human endothelial cell cultures exposed to a combination of gemcitabine, CDDP, and SU5416 compared with either the chemotherapy drugs or SU5416 used alone. Strikingly, this seems to reflect the potent thrombogenic
effects of SU5416 and CDDP/gemcitabine combination observed in cancer patients treated in a clinical trial with these three drugs (5). Also of considerable interest is that this effect may be highly specific to this particular drug combination (at least in terms of its magnitude), because none of the other angiogenesis inhibitors we tested, either alone or in combination with CDDP/gemcitabine, caused such a dramatic increase in the tissue factor/TFPI coagulation index. These drugs included ZD6474 and SU6668, both small-molecule VEGFR-2 antagonists (although neither is completely specific for VEGFR-2), the latter being a member of the same structural class of receptor tyrosine kinase inhibitors, such as SU5416 (i.e., an indolinone; ref. 29). Similarly, use of humanized monoclonal antibodies specific for human VEGFR-2 (IMC-1121) or VEGF (bevacizumab) was not associated with an increase in the coagulation index. In addition, no increase was observed when SU5416 was used together with 5-FU/leucovorin, which seems to parallel the observation that no significant increase of thromboembolic events occurred in the phase I clinical trial in which SU5416 was used together with 5-FU and leucovorin in patients with colon cancer (6).

Also of interest was the finding that protracted treatment of endothelial cells using lower concentrations of CDDP and gemcitabine [i.e., “metronomic” dosing in vitro (30)] was associated with a significantly attenuated induction of the in vitro coagulation index. If this finding translates to the in vivo situation, it could be indicative of another potential toxicity/morbidity advantage of metronomic chemotherapy regimens (14) compared with maximum tolerated dose regimens, especially when chemotherapy is used in conjunction with an antiangiogenic agent. Indeed, it was of interest to note that the low-dose gemcitabine or CDDP did not alter or only affected slightly endothelial cell surface tissue factor (Fig. 6B and C). Although the causes of thromboembolic events in patients with cancer are complex and known to be multifactorial, numerous studies have implicated tissue factor as both a significant and a
common contributory factor and one that is now believed to possess a significant link with the angiogenic process as such (1, 31, 32). However, the development of a thromboembolic event depends on the balance between procoagulant and anticoagulant factors also in relation to the properties of the local endothelial cells (33, 34). Therefore, we felt it would be of considerable interest to investigate whether the balance between endothelial tissue factor and TFPI, the principal physiologic inhibitor of the tissue factor/fVII complex (35), was modified by combination treatments involving chemotherapy and antiangiogenic drugs.

In the current study, incubation of endothelial cells with CDDP and gemcitabine for 18 hours at concentrations up to the peak plasma levels of the drugs used in the clinic (5, 36, 37) dose-dependently increased endothelial cell surface tissue factor activity and decreased TFPI activity. CDDP showed more potent effects in increasing tissue factor and decreasing TFPI activity (Fig. LA and B), which may help explain why CDDP therapy is associated with a higher incidence of thrombotic complications in the clinic (38, 39).

In vitro studies have suggested that apoptotic cells might acquire procoagulant properties (40). In addition, two previous reports showed that endothelial cells have markedly increased cell surface tissue factor activity after induction of apoptosis regardless of the mechanisms causing such apoptosis (40, 41). In our studies, we found that both CDDP and gemcitabine dose-dependently induced endothelial cell apoptosis and correspondingly increased endothelial cell surface tissue factor activity, which is consistent with the aforementioned reports. However, the facts that SU5416 dramatically attenuated CDDP-induced up-regulation of cell surface tissue factor activity but did not affect the high rate of apoptosis in the CDDP group (Fig. 2A and F) and inhibited tissue factor activity but was still associated with an increase in apoptosis in VEGF containing medium group suggests that apoptosis is not a prerequisite for the up-regulation of tissue factor activity, at least in this case.

We have also obtained some limited evidence in the current study that the up-regulation of tissue factor by chemotherapeutic drugs may be attributed, at least in part, if not primarily, to the activation of p38 MAPK (Fig. 4). The combination treatment of the chemotherapeutic drugs (CDDP plus gemcitabine) with SU5416 (CG+SU) did not further enhance tissue factor activity/protein compared with CDDP plus gemcitabine, suggesting that the dramatic increase of coagulation index in this particular combination treatment was mainly due to the inhibition of TFPI.

Two obvious questions are raised by our results: (a) What is the reason why the coagulation index is so dramatically increased by the particular combination of CDDP/gemcitabine plus SU5416? and (b) What are the underlying mechanisms? Although definitive
answers are not clear at this time, we can state that vasculotoxic damage induced by chemotherapeutic drugs concurrent with diminution of VEGF, an endothelial cell survival factor (11), would seem an unlikely mechanism to explain the results, because substitution of SU5416 with a variety of other VEGFR-2 antagonists or antibodies, including SU6668 (which is structurally similar to SU5416), did not cause an increase in coagulation index when combined with gemcitabine and CDDP, nor did the bevactuzumab antibody. This implies that the underlying mechanisms may be complex, subtle, and agent specific (i.e., idiosyncratic). For example, in the case of SU5416, it may be that certain other receptor tyrosine kinases targeted by the drug (including unknown ones), and not VEGFR-2, are involved in the results we observed. Nevertheless, recent clinical observations suggest a more general concern regarding hemostatic consequences of anticancer treatment protocols containing a combination of cytotoxic chemotherapeutic and antiangiogenic drugs. For instance, up to 5% of arterial clotting events have been observed in colorectal carcinoma patients receiving bevactuzumab and infusional 5-FU.2 It is noteworthy that, as we have shown here, bevactuzumab, unlike SU5416, did not induce any appreciable increases in endothelial coagulation index, even when combined with CDDP/gemcitabine, under the conditions tested. This suggests that other elements of the hemostatic circuitry may be affected. One possibility in this regard is the fibrinolytic system, the function of which may affect the ability of the vasculature to cope with formation of microthrombi and macrothrombi. This is particularly relevant because VEGF-A, the prime target of bevactuzumab, and some structurally related growth factors [e.g., VEGF-B (42)] are known to alter (i.e., increase) expression and/or activity of elements of the fibrinolytic circuitry, such as plasminogen activators (tissue plasminogen activator and urokinase-type plasminogen activator) and urokinase receptor (urokinase-type plasminogen activator receptor; refs. 43–47). These findings highlight the inherent linkage between the hemostatic system and tumor microcirculation and hence the need to investigate and develop methods to analyze, anticipate, and prevent adverse clotting events in patients receiving particular chemotherapy and antiangiogenic drug combinations. Such methods include predictive assays/experimental models perhaps of the kind reported here, different dosing regimens for chemotherapy (e.g., low-dose metronomic chemotherapy), and use of anticoagulants, many of which interestingly (e.g., heparinoids) themselves might have antiangiogenic properties (48).

Acknowledgments

Received 9/1/2004; revised 3/28/2005; accepted 4/7/2005.

Grant support: Canadian Institutes for Health Research postdoctoral fellowship (L. Ma), NIH grant CA-41233 and National Cancer Institute of Canada (R.S. Kerbel), and National Cancer Institute of Canada (J. Rak). R.S. Kerbel is a Canada Research Chair in Molecular Medicine. J. Rak is a research scientist of the National Cancer Institute of Canada.

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We thank Cassandra Cheng for excellent secretarial assistance and Dr. Herbert Hurwitz for helpful suggestions.

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