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Published in:
Journal of Thrombosis and Haemostasis

DOI:
[10.1046/j.1538-7836.2003.t01-2-00177.x](https://doi.org/10.1046/j.1538-7836.2003.t01-2-00177.x)

2003

[Link to publication](#)

Citation for published version (APA):
Lindqvist, P. G., Rosing, J., Malmquist, A., & Hillarp, A. (2003). Etonogestrel implant use is not related to hypercoagulable changes in anticoagulant system. *Journal of Thrombosis and Haemostasis*, 1(3), 601-602. <https://doi.org/10.1046/j.1538-7836.2003.t01-2-00177.x>

Total number of authors:
4

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LETTERS TO THE EDITORS

Choice of replacement therapy for hemophilia

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Dear Sir,

In the February issue of the Journal, Giangrande and Mannucci ostensibly take opposite sides on the following subject: 'Recombinant factors only? Yes or no?' [1,2]. In a careful reading of their contributions, it is interesting to note that both prefer to use recombinant products for their patients, but both also believe that plasma-derived products should continue to be produced. Giangrande makes the argument that if physicians in developed countries use recombinant clotting-factor concentrates then plasma-derived products might well become cheaper and made available to patients in developing or underdeveloped countries. Mannucci makes the same argument in a slightly different fashion. He points out that since the developing world cannot afford recombinant products, plasma-derived products should be available for the treatment of hemophilic patients in undeveloped areas of the world.

I have long been persuaded that the best choice for treatment of hemophilic patients is recombinant products. This preference is based largely on the belief that clotting factor concentrates prepared by recombinant technology are probably safer than plasma-derived products because they are manufactured under more controlled conditions and are not reliant on donor plasma from several thousand individuals. Both Mannucci and Giangrande imply that there is always the possibility that as yet unknown transmissible agents may contaminate the blood supply and not be inactivated by current technology. This is probably the legacy of the AIDS crisis when the hemophilic population was exposed to a transmissible agent that resulted in an incurable disease with exceedingly high morbidity and mortality. None of us wishes to see a repeat of such a colossal tragedy, and, for this reason, many of us are sensitized to the possibility, no matter how small, of an AIDS-like tragedy in the future.

Even though many physicians, including myself, prefer recombinant clotting-factor concentrates for the treatment of patients with hemophilia, rigid dogmatism that insists upon the sole use of recombinant products may be very unwise since dogmatism precludes a rational approach to future choice of therapy for hemophilic patients.

Some potential developments in plasma fractionation that offer increased yields of cryoprecipitate and factor (F)VIII from plasma is promising, especially since some might be adaptable to local blood bank technology. Dr Ed Shanbrom and his colleagues have recently described a method for obtaining 'supercryo' [3]. He has found that the yield of cryoprecipitate and FVIII can be increased to approximately 100% by increasing the citrate concentration of the starting plasma. More than that, the FVIII can be easily extracted from the cryoprecipitate, leaving most of the fibrinogen and von Willebrand factor to be used as a source of fibrin glue. Moreover, it seems feasible that an iodine column developed by his group to remove infectious agents might also be adaptable for use in local blood banks to remove infectious particles from FVIII preparations. If such procedures are confirmed, they may permit the accessibility of FVIII concentrates in those parts of the world that cannot afford recombinant products. It is also interesting that an economic model sponsored by Baxter Bioscience but developed independently by Evans and colleagues suggests that plasma-derived FVIII and IX concentrates may, in the long run, be economically feasible and safer than currently available cryoprecipitate fractions [4]. The model is 'evidenced based' and predicts that 'screened' cryoprecipitate could be contaminated by infectious agents not detectable in the window period by currently available screening techniques, including hepatitis viruses, HIV, and other infectious agents. Thus, the patients exposed over a lifetime to screened cryoprecipitate could be infected by such agents.

The debate on the source of clotting-factor concentrates for the treatment of hemophilia, highlighted by Giangrande and Mannucci, emphasizes the need to make safe and effective clotting factor concentrates available to all hemophilia patients, whatever their geographic location or economic status.

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Dear Sir,

Two hemophilia treaters debated the use of recombinant vs. human-derived plasma products for hemophiliacs [1,2]. There should be no debate for the world. The issues addressed mainly deal with developed nations which have made choices based on psychosocial rather than scientific issues. Treatment decisions present multifaceted problems to which there are no simple solutions.

Safety is a primary concern for all patients who have access to therapy. However, both human and recombinant factors carry with them the potential for transmission of infectious agents. Continued incremental removal of all human proteins has been costly and has not been scientifically proven to add safety to recombinant products. More viruses are eliminated from the donor pool for human-derived products as a result of improved donor testing and fractionation technology. The use of gamma irradiation has demonstrated substantial incremental eradication of viruses and bacteria from biologics [3]. No product can currently guarantee complete safety.

We now know that not all recombinant products are equivalent to human-derived ones. For example, recombinant B domain deleted factor (F)VIII and recombinant FIX do not have as good recoveries in children as do human-derived products. It remains unclear whether there are differences in inhibitor induction or in accomplishing immune tolerance. B domain deleted recombinant FVIII has had an increased number of previously treated patients who developed inhibitors. The preliminary data from the German prospective study of previously untreated patients suggest that recombinant therapy may induce more inhibitors than human-derived factors. In

addition, recombinant as well as human-derived FIX are equally implicated in both anaphylaxis and in producing the nephrotic syndrome in patients with FIX deficiency and inhibitors. Currently, there are two human-derived products and one recombinant product available for the management of bleeding in inhibitor patients. Their relative efficacy and adverse reactions are still to be determined. Severe von Willebrand's disease continues to depend upon human-derived factor replacement for therapy. Therefore, it is evident at this time that both types of products need to be available for optimal therapy.

On a more global view, costs and supply were briefly touched upon. They are not inconsequential issues. Where therapy is available, the skyrocketing costs of hemophilia care are under careful scrutiny. Supply issues are not new, have occurred in the past [4], and no doubt will recur.

This debate is an intellectual exercise rather than a practical one. Our real challenge is to reach the large proportion of hemophilia and von Willebrand's patients who now go either undiagnosed and/or untreated. Our job is to allay the misconceptions; therapy with human-derived products is safe and effective.

Both products MUST remain in the therapeutic armamentarium. Working together will help to ensure that optimal technology is applied to make products as safe and affordable as possible, thereby ensuring that all patients receive therapy.

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Choice of low molecular weight heparins

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Dear Sir,

On the question whether or not low-molecular-weight heparins (LMWHs) are interchangeable, Nenci [1] and Prandoni [2] take opposite positions even though their starting points are similar:

LMWHs are different chemical entities, with different activity profiles, and the effectiveness of the various compounds has hardly ever been compared directly. Thus there is no evidence-based information on which to answer the question, so either position is valid, in the absence of real proof.

In actual fact an evidence-based answer could be formulated, but we must go back over the history of the development of these compounds. In the late 1980s, with the first clinical trials of LMWHs for the prevention of venous thromboembolism

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(VTE) in general and orthopedic surgery and subsequently for the treatment of VTE, several compounds became available virtually at the same time. These molecules had different pharmacological profiles and each was tested at specific dosages, different for each drug, and compared with unfractionated heparin (UFH) or placebo. Nobody posed the question on whether or not it was ethical to let some of the patients without effective treatment. The meta-analyses of the early 1990s cleared up this question, enabling us to pass judgement: all the LMWHs in use at that time came through the test.

Things changed with later studies. From the very first trials of extended prophylaxis with LMWH after hip replacement it was clear that these drugs offered superior activity over VHF; the extent of this superiority was confirmed in subsequent trials, though some of these – mostly the earliest – were still designed and conducted with a placebo comparator, leaving an evident ethical doubt. Now that the role of the LMWHs seems well established we could no longer repeat a trial such as the MEDENOX [3] for prophylaxis in medical patients, or Lassen's study [4] of the prevention of VTE after leg injury requiring immobilization. The LMWHs used, at the doses selected in those trials, are now considered the standard. It is unthinkable to repeat these trials using other LMWHs in comparison with placebo, and it would be wrong to use other LMWHs in the same model in clinical practice, but at arbitrarily different doses that have no basis in evidence. As Prandoni [2] rightly notes, there is no point in organizing new comparative trials using different LMWHs tête-à-tête.

There are, however, other fields where questions are still unanswered: what about prophylaxis in laparoscopic or arthroscopic surgery, where a single, well-conducted trial with a LMWH could well establish a standard? The role of the LMWHs in the treatment of acute coronary syndromes (ACS) also merits a specific comment. About 5 years ago Collins, Peto, Baigent and Sleight signed a review article in the *New England Journal of Medicine* [5] on the role of aspirin, heparin and fibrinolytic therapy in suspected acute myocardial infarction. They mentioned a meta-analysis [6] done by themselves and others including Yusuf, and concluded, in relation to suspected acute myocardial infarction: 'nor is there good evidence that either intravenous or subcutaneous heparin produces any worthwhile improvement in outcome'. They added: 'among patients with unstable angina, there is also very little evidence of improvement in major clinical outcomes with the addition of intravenous heparin to aspirin either in individual trials or (despite its title) in a formal meta-analysis of those trials'. This last meta-

analysis comprised six trials in patients with unstable angina, and concluded that the relative risk of myocardial infarction or death in patients given heparin as well as aspirin was 0.67, with 95% confidence interval (CI) 0.44–1.02 [7]. Prandoni now cites a later meta-analysis by Eikelboom, including Yusuf too [8], which reviewed exactly the same six trials on unstable angina, but this time their conclusion is that the 'pooled analysis shows a nominally significant 33% reduction in the risk of death and myocardial infarction during the first week of treatment with unfractionated heparin, OR 0.67, 95% CI 0.45–0.99, $P = 0.045$. This looks like a somewhat casual, even ideological way to use a meta-analysis if completely opposite conclusions can be drawn from the same data. Prandoni mentions these conclusions on the roles of the different LMWHs in comparison with UFH in ACS as suggesting that they are substantially interchangeable in this indication. However, in this meta-analysis the end-points and their detection times are different from those established by the investigators in the single trials of LMWHs. This inevitably casts doubt on the credibility and the clinical implications of a large-scale analysis where the pooled data have been so totally and visibly manipulated.

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Dear Sir,

Of the advances in antithrombotic therapy in the last 20 years, the introduction of low molecular weight heparins (LMWHs) has truly changed clinical practice. Their efficacy, safety and practicality have led to their wide adoption in the prevention and treatment of venous thromboembolism and in the management of acute coronary syndromes. In the last issue of the Journal, two experts debated whether or not the various LMWHs are interchangeable. Both have emphasized the pharmacological and pharmacokinetic similarities and differences among the LMWHs but the conclusions regarding the interchangeability in practice differ. Nenci argues forcibly that based on clinical trial data, there is no evidence that there are important differences clinically among the LMWHs [1] while Prandoni argues equally forcibly that there are [2].

The interchangeability of LMWHs remains a topic of controversy and ongoing debate. What is the physician to do? There is no doubt that LMWHs offer practical advantages over standard heparin and in almost every indication for acute anticoagulation, are the agents of choice. Is there any evidence that one LMWH should be preferred over another? As pointed out by the experts, there have been no large-scale direct comparisons between the LMWHs so a decision has to be made based on indirect comparisons and perhaps on pharmacological differences. There is little to choose among the LMWHs in the prevention and treatment of venous thromboembolism and the decision regarding the most appropriate agent should be based on local preference and on regulatory approval. However, in the management of acute coronary syndromes, although all the LMWHs are effective, only one, enoxaparin, has been shown to

be more effective than unfractionated heparin where there are now five randomized clinical trials demonstrating its superiority. The results of one study could be a chance finding, but when five studies show the same superiority over heparin, there can be little doubt. Why have the other LMWHs not shown similar superiority in this setting? It could very well be that difference in study design and the patient populations contributed to the differences in outcomes. Be that as it may, until such time as the other LMWHs have been shown to be superior to heparin, it seems reasonable for the clinician to choose enoxaparin in this setting. Indeed the most recent guidelines by the American College of Cardiology and the American Heart Association, enoxaparin is recommended as a preferred treatment over unfractionated heparin in the management of patients with unstable coronary syndromes without ST segment elevation [3].

New data continues to support the use of low molecular weight heparins with broadened indications in the management of venous thromboembolism. Important new studies in the prevention of thrombosis in medically ill patients and in the management of patients with malignancy recently reported at the American Society of Hematology provide further evidence of the clinical importance of this group of antithrombotic drugs with trial data specific to individual LMWHs.

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Dear Sir,

For decades, the question, 'are low-molecular weight heparins (LMWHs) interchangeable?' has been posed by clinical investigators, manufacturers, regulatory authorities and the users/

purchasers of LMWHs. It has not been answered to the satisfaction of interested parties, because, with few exceptions, the question has not been investigated in appropriately designed clinical trials. The question can be posed at different levels: structural, pharmacological and clinical. As pointed out by one 'dualist' [1] LMWHs differ in their chemical structure, molecular weight distribution, pharmacokinetics, antifactors Xa and IIa ratios, ability to stimulate tissue factor pathway inhibitor release and in other properties [1]. The other 'dualist' contends that these differences are unimportant from a clinical perspective.

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tive [2]. What are the facts? The efficacy and safety of different LMWHs have been compared directly for the prevention of venous thromboembolism and indirectly for the treatment of venous thromboembolism and of acute coronary syndromes. The indirect comparisons used unfractionated heparin as the (anchor) control arm. The results of the direct comparisons are much easier to interpret than the indirect comparisons.

In two separate studies performed by Planès [3,4] the efficacy and safety of enoxaparin was compared with either revaparin or tinzaparin for the prevention of venous thrombosis after total hip replacement. The studies were well designed and showed that the efficacy and safety of enoxaparin was unlikely to be different than those of the other two LMWHs.

In their meta-analysis of the efficacy and safety of different LMWHs for the treatment of venous thrombosis, van der Heijden *et al.* [5] stated that there is no conclusive evidence that the LMWHs have different efficacy and safety profiles. Furthermore, in a preliminary report, no difference in the efficacy or safety was observed between tinzaparin and dalteparin in the treatment of about 500 patients with venous thrombosis or pulmonary embolism (P.S. Wells, personal communication). Because none of the LMWHs in the meta-analysis were compared directly with each other, and the only direct comparison is a preliminary report, any conclusions about the relative efficacy and safety of the various LMWHs for the treatment of venous thromboembolism have to be considered tentative.

Finally in randomized trials in patients with unstable angina, dalteparin was not superior to heparin, whereas enoxaparin was reported to be more effective [6]. However, the validity of the difference between enoxaparin and heparin has been questioned because enoxaparin was administered for a longer period of time than heparin. And, on re-analysis, there was no difference in the composite of death and MI, when the comparison was

performed during the truncated period that both enoxaparin and heparin were being administered [7].

In summary, at a clinical level, there is no convincing evidence that there are differences among the LMWH, but it cannot be concluded that they are identical. The issue has important financial implications to the manufacturers of LMWHs. Its resolution is quite straightforward, and requires comparisons of one LMWH with another in randomized double blind clinical trials in which both anticoagulants are administered in the same manner. Until such trials are performed the debate will continue to be fueled by commercial interests, and in many hospitals, the choice of agent will be driven by cost considerations.

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M. M. SAMAMA

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Dear Sir,

The 20-year-old low molecular weight heparins (LMWHs) are an attractive alternative treatment to UFH and the question raised by the experts, which has been disputed unsuccessfully in some meetings is of great practical importance.

A definite answer acceptable to the medical community is still lacking. Nevertheless, it should be recalled that various authors have included clinical trials conducted with different LMWHeparins in their meta-analyses.

The debate has led the two experts, Prandoni [1] and Nenci [2], to analyze the main characteristics related to the chemical

and pharmacological properties of the various available pharmaceutical preparations. There is no doubt for both experts that several differences can be identified (mode of preparation, pharmacokinetics and anti-Xa/anti-IIa ratio, for instance).

However, the rare direct head to head comparisons of the results of different clinical trials have not been able to demonstrate a superiority of one preparation over another regarding the efficacy/safety ratio [3,4].

The dispute is still going on especially regarding the use of a LMWH preparation in patients with unstable coronary artery disease, where enoxaparin led clearly to better results than some other preparations studied in the same indication. In contrast, a prolonged treatment with dalteparin was efficacious while enoxaparin did not improve the clinical results. It has been argued that bias in the selection of patients may have influenced the obtained clinical results and that indirect comparison of clinical trials may be not fully reliable. Moreover,

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the lack of standardized testing procedures for unfractionated heparin serving as a comparator to LMWH has been emphasized [5–7].

There are still two significant considerations when trying to give an answer to the important question related to the clinical differences of the various LMWHs.

Firstly a dose-efficacy ranging has not been performed for most preparations.

Moreover, in the treatment of venous thromboembolic episodes, it is surprising that the same dosages expressed in anti-Xa international units have been used for different LMWHs preparations and led to equally good clinical results. This observation suggests that LMWHs preparations are interchangeable at least in the treatment of deep venous thrombosis.

The second limitation is related to the mechanisms of action of LMWHs which is incompletely understood at least in part because of the multitargeted activity of these drugs. The good clinical results obtained with fondaparinux have underlined the importance of the anti-Xa activity which is an essential characteristic of the different LMWH preparations [8]. However, it is intriguing that no correlation has been clearly shown between the anti-Xa activity of LMWHs and their clinical effectiveness.

When considering again LMWHs in the treatment of DVT, it is clear that the same dosages of different LMWH preparations expressed in anti-Xa IU induce in the plasma a significantly different anti-Xa activity since the peak values obtained vary from 0.8 to 1.5 IU, while equivalent clinical results are obtained.

In addition, it is clear that administration of different LMWH preparations at equivalent doses in terms of anti-Xa activity, results in significantly different anti-IIa activities in plasma. Some other differences such as the amount of TFPI release have been evidenced. However, the clinical relevance of these differences is obscure.

Interestingly, although an opposite answer has been given by the two experts in the title, they both agree that each LMWH preparation should be administered 'at the dosage recommended' or at 'the relevant dosage' for each indication according to the results of the available clinical trials. This is a wise and indisputable conclusion of the debate. One may add that the selection of a LMWH preparation for each indication should take also into account the number, the size and the quality of the methodology of these trials.

For the reasons noted above, no definite answer can be given to the question at the present time. The best way to answer the question is to perform more direct comparisons in well designed controlled trials at least in some selected indications of these successful drugs.

In total, yes the LMWH preparations differ by their method of preparation, different chemical or enzymatic process as being used. They have different pharmacokinetics, a different anti-Xa/anti-IIa ratio and their capacity of releasing TFPI may vary from one preparation to another.

In contrast, they are used in the same clinical indications and the results obtained regarding their efficacy and safety in clinical trials showed comparable results with rare exceptions, the still disputed question being the choice of a LMWH in unstable angina. Another difference relies in the number and size of the clinical trials performed in each indication which is variable and should be taken into consideration when selecting a LMWHs preparations.

Finally, attention should be also given to the appropriate use and misuses of LMWHs which is of great importance although it is not related to their interesting debate.

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Etonogestrel implant use is not related to hypercoagulable changes in anticoagulant system

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Dear Sir,

Since the discovery of thrombotic complications related to the use of combined oral contraceptives (COC) in the 1960s, the dose of estrogen has gradually been decreased from 100 to 150 µg, to 20–30 µg ethinylestradiol. There have been expectations that the thrombosis risk would decline with the lower doses. However, a decrease of the thrombosis risk with lowered estrogen has been challenged by Rosendaal and coworkers, who also have questioned the impact of the gestagenic part of the COC [1]. Since 1995 several independent studies with public funding have reported a 2-fold increased risk of venous thrombosis among third generation progestogens (containing desogestrel or gestodene) COC users, as compared to users of second generation COC containing levonorgestrel. This is in contrast to studies without public funding, where no differences in thrombotic risk have been reported [1]. Thus, there is an unexplained heterogeneity in results regarding the thrombogenic potential of third generation COC and there are no independent studies evaluating the hemostatic changes with etonogestrel only. The motive for this study was to evaluate if implants with etonogestrel, a third generation gestagen, give rise to prothrombotic changes in the hemostasis system.

All women scheduled for etonogestrel implant between June 2001 and November 2001, without hormonal treatment during the last 2 months, and without a history of venous thromboembolism, were approached to participate in the study. In total, 31 women accepted to be included in the study. Blood samples for phenotypic hemostatic assays were collected as appropriate. The first blood sample (baseline) was collected after 15 min of rest, just before insertion of the implant. The second blood sample was scheduled to after exactly 1 month. Of the 31 invited women, 20 showed up for the second sample collection and were included in the study. The study was approved by the local Ethics Committee, Lund University, and informed written consent was obtained from all the subjects.

The response of plasma to APC was determined by three different APC resistance tests: two APTT-based assays, the Coatest APC Resistance, and the Coatest APC Resistance V (Chromogenix, Milan, Italy) and a test that quantifies the effect of APC on thrombin generation initiated in plasma via the extrinsic coagulation pathway [2,3]. Other hemostatic variables measured were prothrombin, factor (F) V, VII and VIII, protein S, protein C and antithrombin. Prothrombin was determined after activation with ecarin [4]. FV and VII were determined with one-stage clotting assays with Thromborel S (Dade-Behring, Liederbach, Germany) as source of thromboplastin. Factor VIII was quantitated using a chromogenic assay (Coatest Factor VIII, Chromogenix). Protein C and antithrombin were quantitated with the Berichrom Protein C and Berichrom antithrombin III assays, respectively (Dade-Behring). Free protein S was determined with an enhanced latex immuno assay (Instrumentation Laboratory, Milan, Italy). The presence of the FV Leiden mutation was determined by DNA analysis as described [5]. Student's *t*-test for paired data was used for comparison of the influence of the implant on the hemostatic variables, with *P*-values <0.05 regarded as significant. Analysis was performed with the SPSS software (SPSS Corporation, Chicago, IL, USA). It was calculated that the inclusion of 20 individuals would allow us to detect a 25% change or more of the ETP-based APC-sensitivity test, using a 0.05 two-sided significance level, with a 75% power.

The 20 volunteers were all of Caucasian descent, their mean age was 26 years (SD = 7), and 10 were nulliparous. In the study group as a whole (*n* = 20), the etonogestrel implant did not cause changes in APC sensitivity-ratios in none of the three ways we measured it. However, after insertion of the implant there was a significant increase in free plasma protein S, antithrombin, and prothrombin levels, and a significant decline in protein C level (Table 1).

This study show that etonogestrel implants have a significant effect on the plasma levels of protein S, protein C, prothrombin, and antithrombin, although the observed changes are small in terms of absolute difference. None of the three tests for plasma response to APC was significantly affected by the etonogestrel implant. A surprising finding was that the direction of changes for protein S, protein C and antithrombin was opposite to what has been found in users of desogestrel containing COC [2,6,7]. The only significantly affected parameter that is in line with the observations in COC users was an increase of prothrombin. Some of the changes of the parameters we studied are similar to those in a commercially sponsored study by Egberg *et al.* [8]. They reported increased antithrombin values (+0.04 U mL⁻¹,

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Received 30 May 2002, revised 22 October 2002, accepted 19 November 2002

Table 1 Hemostatic values before and after insertion of etonogestrel implants

	Before insertion mean \pm SD (n = 20)		After insertion mean \pm SD (n = 20)		Difference mean \pm SD (n = 20)	P-value	Interindividual CV% ⁴	Difference %	Normalized diff. (%) ⁵
APC-ETP ¹	2.84	1.59	2.73	1.43	-0.11	0.21	56	4	7
APC Resistance ²	2.68	0.48	2.67	0.47	-0.01	0.86	18	<1	2
APC-FV ³	2.04	0.27	2.01	0.24	-0.03	0.32	13	2	11
APT-time	33.67	2.4	33.51	2.1	-0.16	0.66	7	<1	7
Protein C	1.02	0.14	0.96	0.14	-0.06	0.02	14	6	42
Protein S	0.82	0.12	0.92	0.16	0.09	<0.001	15	12	81
Antithrombin	1.00	0.08	1.05	0.08	0.05	<0.001	8	5	62
Prothrombin	1.02	0.14	1.11	0.13	0.09	0.001	14	9	63
FV	0.90	0.13	0.91	0.13	0.01	0.95	14	1	8
FVII	0.87	0.24	0.82	0.22	-0.04	0.136	28	6	20
FVIII	1.00	0.36	0.98	0.30	-0.02	0.71	36	2	6

¹APC sensitivity ratio based on endogenous thrombin potential²APC resistance ratio based on prolongation of APT-time³As (2) above with predilution of sample in FV-deficient plasma⁴As calculated by (SD/mean) \times 100. CV, coefficient of variation⁵The difference in percent normalized by dividing with the interindividual coefficient of variation

$P = 0.001$) protein S free antigen (0.03 U mL^{-1} , $P = 0.001$), and decreased protein C activity (-0.04 U mL^{-1} , $P = 0.001$), which are similar to our results, both in term of significance and absolute differences. In their study they also observed a significant decrease of FVII (-0.07 U mL^{-1} , $P < 0.001$), which we could not reproduce although we did observe an overall decrease in the level of FVII.

Since we did not observe a prothrombotic pattern of hemostasis variables after etonogestrel implant it is tempting to speculate that the small effects of etonogestrel, compared to COCs, also reflects a lower thrombotic risk.

Acknowledgments

The study was supported by research funds of Malmö University Hospital.

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Factor X Leicester: Ile411Phe associated with a low antigen level and a disproportionately low functional activity of factor X

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Dear Sir,

Factor X (FX) is a vitamin K-dependent serine protease of central importance in the blood clotting cascade [1]. We report a novel Ile411Phe mutation in factor X (FX) which results in a severe bleeding tendency. The index case is of Gujarati Indian origin whose parents are first cousins. She has suffered from severe bleeding including menorrhagia and muscle hematomas and hemarthroses. On presentation, FX activity (ACL 3000, IL Instruments, Milan, Italy) was <1 iu dL⁻¹ (reference range 50–150 iu dL⁻¹), APTT was 93.7 s (control 40.2 s) as measured by the KCCT method and PT was 36.2 s (control 12.0 s) using Diagen thromboplastin (Diagen Ltd, Thame, UK). The FX antigen level was measured by ELISA using a polyclonal anti-FX antibody (Dako, High Wycombe, Bucks, UK) and was 8 iu dL⁻¹ (reference range 50–150 iu dL⁻¹).

The gene coding for FX consists of 27 Kb of nucleotide sequence containing 8 exons [2]. The exons and splice junctions in the FX gene were amplified as previously described [3] and sequenced using an ABI 310 DNA sequencer (P.E.Biosystems, Warrington, UK). The only mutation found was a homozygous single point mutation ATC to TTC in exon 8 causing an Ile411Phe substitution. No other member of this family has any history of a bleeding disorder. DNA from the patient's mother, brother and one of her sons was found to be heterozygous for this mutation. Their FX activity levels were intermediate. These results, together with the fact of consanguinity suggest an autosomal recessive mode of inheritance for this mutation.

The interpretation of missense mutations is facilitated by molecular modeling [3–5]. The Ile411Phe mutation was investigated using multiple sequence alignments of FX from seven species and also for 74 human serine proteinase sequences identified in the SWISSPROT and TREMBL databases using PSI-BLAST [6]. MEGALIGN (DNASTAR, Madison, USA) was used for alignments using the CLUSTAL V algorithm [7]. Residue 411 was found to be isoleucine or valine in all but one of the 81 sequences. Both Ile and Val are branched aliphatic and

hydrophobic. The single exception was the unexpected occurrence of Phe562 at the equivalent position in human thrombin.

The crystal structure of FXa is closely similar to that of thrombin [8,9]. The two structures (PDB codes 1xka and 1ppb) were compared using INSIGHT 98.0 (Biosym/MSI, San Diego, USA) on Silicon Graphics workstations with Crystal Eyes stereoglasses in order to account for the detrimental effect of Phe411 in FX but not when Phe562 is incorporated in thrombin. The quantitative secondary structure analysis using DSSP [10] showed that Ile411 occurred in β -strand O [4], and the solvent accessibility calculation using COMPARE with a water molecule radius of 1.4 Å as probe [11,12] showed that this is completely buried within the protein core [4]. Both Ile411/Phe562 are adjacent to a conserved Trp399/Trp550 residue in identical conformations (Fig. 1a,b), which is adjacent to the catalytic triad of the active site of FX (not shown). On the opposite side of Ile411/Phe562, a conserved disulfide bridge (Cys350-Cys364 in FX; Cys496-Cys510 in thrombin) shows two different conformations in FX and thrombin. The bridge points towards Ile411 in FX, but away from Phe562 in thrombin in order to accommodate the bulkier aromatic ring of Phe562 in thrombin. Figure 1(a and b) also indicate that the bridge is closely packed against a surface loop of residues Tyr367-Gln371 between α -strands L and M in FX. This loop is three residues shorter than the corresponding loop Tyr513-Arg520 in thrombin. None of the 70 other human serine proteinase sequences shows a loop of the same length as found in thrombin. This suggests that the longer loop in thrombin determines whether or not a Phe residue can be successfully incorporated in the protein structure.

Energy minimization was performed to see why FX might not accommodate a Phe411 residue. The crystal structure of FXa was used as the starting model. Phe411 was built using the Builder module of INSIGHT II, then both the mutant and wild type structures were subjected to 4×300 rounds of global Polak conjugate gradient minimization with the AMBER forcefield, using the DISCOVER_3 module of INSIGHT II. Further rounds of localized energy minimization were carried out over Phe356-Asn361 and Ser398-Ile422 as these regions showed the most change during refinement. Refinement was ended when no further significant change in the protein conformation was observed. Figure 1(c) showed that the incorporation of Phe411 in FX resulted in a significant structural rearrangement in the Ala365-Asp373 surface loop. This protein loop includes two of the four residues, Tyr367 and Asp368 whose main-chain

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Received 12 August 2002, revised 25 September 2002, accepted 29 September 2002

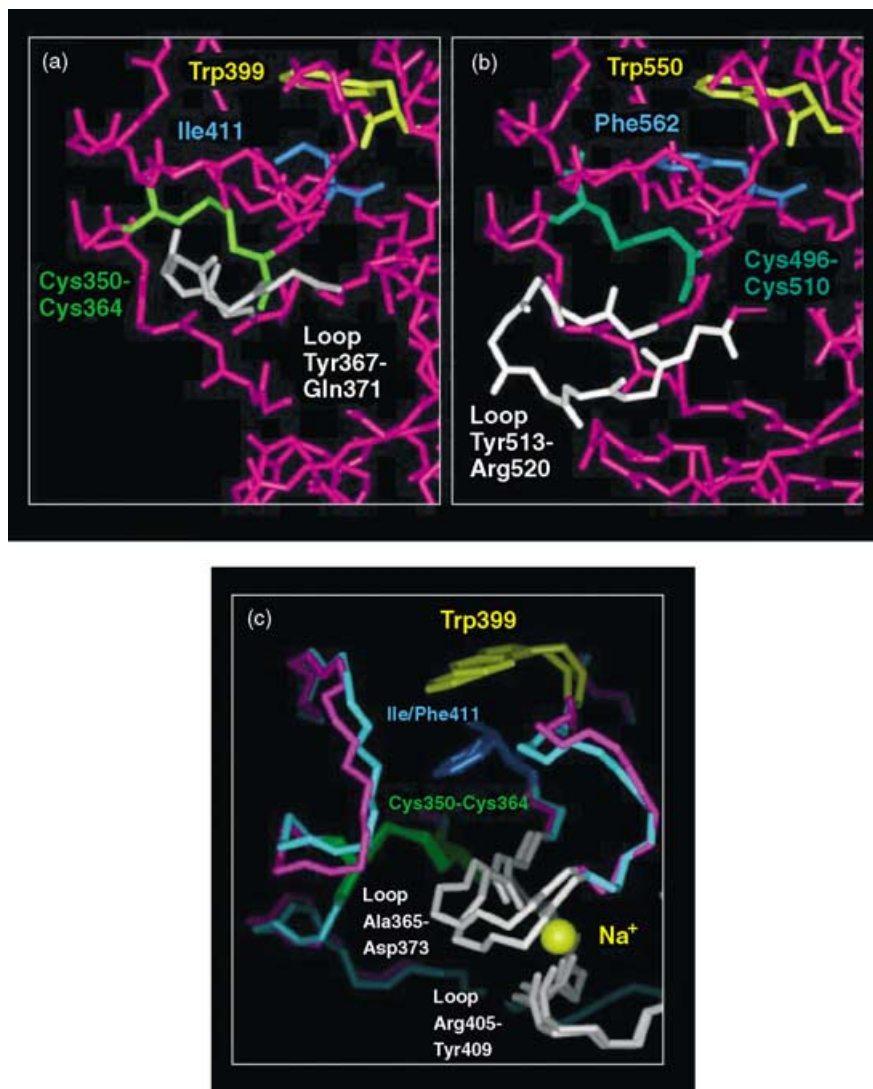


Fig. 1. Molecular views of Ile411 in FXa and Phe562 in thrombin. (a) The serine proteinase domain of FXa is depicted with Trp399 in yellow, Ile411 in blue, the Cys350-Cys364 disulfide bridge in green, and the four-residue surface loop Tyr367-Gln371 in gray. Ile411 is fully buried and is packed against the Cys350-Cys364 disulfide bridge which is twisted towards that of Ile411 because of the short Tyr367-Gln371 loop. (b) The equivalent view of the serine proteinase domain of thrombin is shown. The larger eight-residue loop enables the Cys496-Cys510 disulfide bridge to adopt a different conformation pointing away from Phe562 so that it can be packed within the protein core. (c) Outcome of energy minimization analyses for wild-type FXa and its Ile411Phe mutant. The two fully minimized structures were superimposed using all the backbone atoms in order to highlight the conformational differences between the two structures. The mainchain trace views without carbonyl atoms of the two structures are shown (wild-type: blue; mutant: purple). The residue coloring from (a) and (b) is used again here. The inclusion of the second sodium-binding loop Arg405-Tyr409 is shown in gray and the sodium ion is shown in yellow, together with the four carbonyl oxygen atoms of residues 367, 368, 405 and 408 that form ionic contacts with the sodium ion.

carbonyl oxygen atoms form ligands of a sodium ion site in FX. Hence it is possible that the Ile411Phe mutation has affected the strength of sodium binding to FX. Sodium binding is involved in the conformational transition of FXa from an enzymatically slow form (with no Na^+ bound) to a fast one (with Na^+ bound). The sodium binding loop is further implicated in allosteric interactions with a Ca^{2+} binding site at Asp250 and Glu260 in FX [13]. A Glu552Ala mutation in prothrombin is detrimental to its function, being most likely to result from modification of the sodium ion binding site [14].

Our molecular modeling analysis was uniquely assisted by the fortuitous occurrence of Phe562 in thrombin, for which the crystal structure clarified the likely consequence of the substitution. Our analysis suggests that the Ile411Phe mutation results in protein misfolding and disrupts sodium binding. This is consistent with our antigen result showing that some FX protein is still present in plasma, and the very low level of activity implies that this small amount of FX is functionally impaired. The verification of these molecular modeling ana-

lyses by studies of recombinant FX incorporating this mutation will be required.

Acknowledgments

We thank Dr V. Mitchell of the Department of Haematology, Leicester Royal Infirmary, and Dr B. Myers of the Department of Haematology, Queen's Medical Center, Nottingham for providing patient samples and clinical data.

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Prostacyclin is a platelet activator when protein kinase A is inhibited

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Dear Sir,

Prostacyclin released from endothelial cells is one of the most potent platelet antagonists. It binds predominantly to the IP₂-receptor, thereby activating the GTP-binding protein G_s, that stimulates adenylyl cyclase and raises cAMP. The next step is activation of cAMP-dependent kinase or protein kinase A. Activated protein kinase A inhibits platelet activation at multiple steps in the activating pathways that trigger a rise in cytosolic Ca²⁺, [Ca²⁺]_i, which is a key step in the initiation of aggregation, secretion and the generation of a procoagulant surface.

We recently reported that human hematopoietic stem cells respond to stimulation by the stable prostacyclin analog iloprost with a rise in cAMP [1]. Unexpectedly, the rise in cAMP was accompanied by a rise in [Ca²⁺]_i. Thus, stem cells differ from platelets in that they respond to prostacyclin with a rise in [Ca²⁺]_i which is not inhibited by cAMP.

Maturation of stem cells to megakaryocytes is accompanied by a gradual shift in the response to prostacyclin. The early appearance of glycoprotein IIIa (CD61) and late appearance of glycoprotein Ib (CD42b) is accompanied by a loss of Ca²⁺ stimulation and gain of Ca²⁺ inhibition. The onset of Ca²⁺ inhibition occurs in a phase in which the expression of protein kinase A subunits changes profoundly. The protein kinase A complex consists of two regulatory subunits bound to two catalytic subunits. Binding of four molecules of cAMP to the two regulatory subunits releases the catalytic subunits which become active. Diversity in this mechanism is obtained by the differences in subunit composition. There are at least four different regulatory subunits (types Iα, Iβ, IIα and IIβ) ranging in molecular weights between 49 and 55 kDa, and three different catalytic subunits (types α, β and γ) ranging between 39 and 50 kDa. In addition, different A-kinase anchoring proteins (AKAP's) localize the protein kinase A complex to different subcellular compartments.

There is a sharp increase in the inhibition of Ca²⁺ by prostacyclin at a late stage of megakaryocyte maturation, shortly before platelets are formed. This gain of function is accompanied by a strong up-regulation of protein kinase A-catalytic subunits. This observation raises the possibility that the concentration of catalytic subunits determines the capacity of prostacyclin to inhibit rises in [Ca²⁺]_i. Indeed, when the megakaryocytic cell line CHRF-288–11 is transfected with a construct of the catalytic subunit α, the inhibition by prostacyclin increases two-fold [2].

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Received 19 August 2002, revised 30 September 2002, accepted 30 September 2002

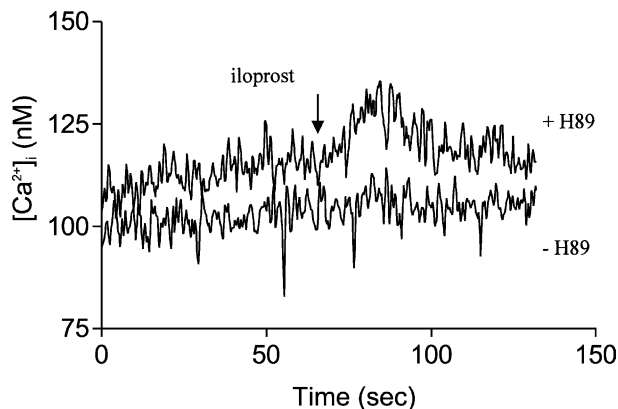


Fig. 1. The stable prostacyclin analog iloprost raises $[Ca^{2+}]_i$ in platelets when protein kinase A is inhibited by H89.

These findings raise the question whether the Ca^{2+} response induced by prostacyclin really disappears during maturation of megakaryocytes and platelet shedding or is merely suppressed at more mature maturation stages by the up regulation of protein kinase A catalytic subunits. Studies with the protein kinase A inhibitor H89, show that both possibilities might exist. There is a substantial down-regulation of prostacyclin-induced Ca^{2+} increases during megakaryocyte maturation, but blockade of protein kinase A by H89 restores part of the Ca^{2+} increasing capacity.

To address the question whether the down-regulation of prostacyclin-induced Ca^{2+} increases is complete before plate-

lets are formed, we measured the mobilization of Ca^{2+} in Fura-2 loaded platelets in 15 healthy volunteers (with informed consent). In 3 subjects we found a slight, but consistent increase in $[Ca^{2+}]_i$ ranging between 15 and 20 nM when platelets were pretreated with H89 but not in the absence of the inhibitor (Fig. 1). The other 12 subjects did not show this response. Thus, at least some individuals have platelets for which prostacyclin is a Ca^{2+} raising agonist, a property that under normal conditions is suppressed by protein kinase A. Theoretically, abnormalities in protein kinase A would unmask this unexpected platelet property. Whether or not such a condition occurs in certain disease states remains to be investigated.

Acknowledgments

Supported by the Netherlands Heart Foundation no. 97. 142 and the Netherlands Thrombosis Foundation.

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Successful use of recombinant activated factor VII in controlling upper gastrointestinal bleeding in a patient with relapsed acute myeloid leukemia

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Dear Sir,

Recombinant activated factor (F)VII is used mainly for the treatment of hemophilia patients with inhibitors [1]. Its unique mechanism of action, activation of FX by forming a complex with tissue factor (TF) at the site of active bleeding [2], has laid

the basis for the treatment of various coagulopathies and severe bleeding episodes. This report demonstrates for the first time the beneficial effect of recombinant factor VIIa (rFVIIa) in controlling life-threatening upper gastrointestinal (UGI) bleeding in a patient with acute myeloid leukemia in the presence of thrombocytopenia. The formation of thrombus at the site of bleeding stomach mucosa is objectively documented.

Case report

In May 2001, a 32-year-old male was diagnosed as having acute myeloid leukemia. Complete remission was achieved following 7 days treatment with cytarabine $100 \text{ mg m}^{-2} \text{ day}^{-1}$ and 3 days of daunorubicin 60 mg m^{-2} (7 : 3 regimen), following which he received two additional courses of consolidation chemotherapy

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Received 9 September 2002, accepted 4 November 2002

with high-dose cytarabine, etoposide and mitoxantrone. He subsequently underwent autologous peripheral stem cell transplantation in September 2001. He was in complete remission until he relapsed in December 2001 with a white blood count of $9600 \mu\text{L}^{-1}$ and 38% blasts.

He was then treated with anti-CD33 conjugated with calicheamicin (gemtuzumab ozogamicin), which resulted in severe pancytopenia. The blood count and coagulation studies at this point were as follows: hemoglobin 7 g dL^{-1} , white blood cells $0.2 \times 10^9 \text{ L}^{-1}$, platelet count $30 \times 10^9 \text{ L}^{-1}$. Prothrombin time 12 min (normal range 11–14 min) activated partial thromboplastin time (APTT) 29 min (27–40 min) and the fibrinogen

level was 546 mg dL^{-1} (normal range 200–400 mg dL^{-1}). His liver function tests which included bilirubin, transaminases and alkaline phosphatase levels were within normal limits. One week after completing the gemtuzumab ozogamicin regimen, severe melena appeared. At this point the hemoglobin level was 8.5 g dL^{-1} , platelet count $22 \times 10^9 \text{ L}^{-1}$, prothrombin time 12 min and APTT 30 min. The liver function tests remained normal. An intensive transfusion with packed red cells ($2\text{--}3 \text{ U day}^{-1}$), platelets 12 U day^{-1} , and fresh frozen plasma 8 U every other day was started. In addition, the patient was treated with an intravenous proton pump inhibitor, tranexamic acid 3 g day^{-1} and aprotinin. Despite treatment the patient continue

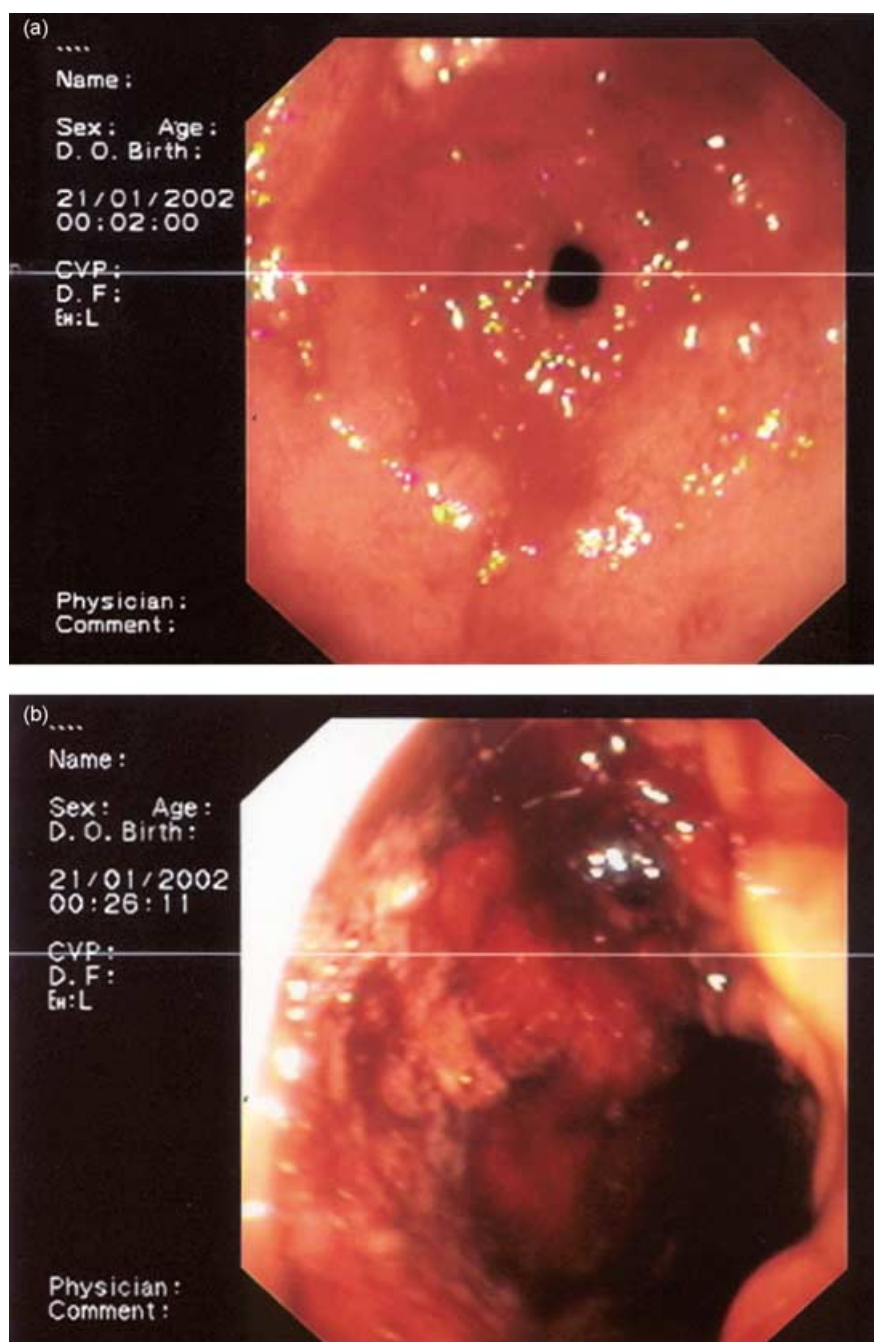


Fig. 1. (a) Distal body of the stomach showing active bleeding before rFVIIa administration. (b) Clot formation (upper left) 20 min after rFVIIa administration.

to bleed and his hemoglobin level did not rise beyond 8.5 g dL^{-1} with platelets count of $20 \times 10^9 \text{ L}^{-1}$. Because of the active bleeding, which was uncontrolled by conventional measures, gastroscopy was performed and disclosed fresh blood in his stomach due to diffuse erosive gastritis, mainly in the distal body of the stomach (Fig. 1a). Therefore, rFVIIa (NovoSeven[®], Novo Nordisk A/S, Bagsvaerd, Denmark) $90 \mu\text{g kg}^{-1}$ was immediately administered. Twenty minutes after the infusion, a repeat gastroscopy demonstrated a significant reduction in the amount of gastric hemorrhage and formation of clots (Fig. 1b). Thereafter, two additional doses of rFVIIa $90 \mu\text{g kg}^{-1}$ were administered, 4 and 8 h after the first dose. The coagulation studies after rFVIIa infusion showed prothrombin time value shortening to 8 min, APTT did not change and FVII level of 1000%.

The patient remained stable for 2 days after the rFVIIa infusion with hemoglobin level of 10 g dL^{-1} with reduction in melena and with no need for further red cell transfusion, although he continued to receive platelet transfusions but without significant increment in platelet count, which remained below $25 \times 10^9 \text{ L}^{-1}$. After this period, rectal bleeding reappeared with a repeated drop in his hemoglobin level, and at this time the prothrombin time was 10 min and the APTT 36 min. A colonoscopy revealed diffuse bleeding ulcerations throughout the colon. He was treated with blood and platelet units until bleeding subsided when his platelet count recovered.

Discussion

Recombinant FVIIa was developed primarily for the treatment of hemophilia A or B with inhibitors. In recent years, the use of rFVIIa has been widely extended to treat patients with bleeding diatheses of various causes [3]. This can be attributed to the unique mode of action of rFVIIa in achieving hemostasis. It has been shown that rFVIIa can induce hemostasis in the absence of FVIII or FIX by forming complexes with TF and by binding to activated platelet surfaces independently of TF, thereby inducing the thrombin burst needed for propagation of coagulation.

NovoSeven[®] has been used in a diverse clinical bleeding situations such as platelet disorders [4], trauma [5], liver disease [6] and uncontrolled coagulopathy, when all other therapeutic options have failed [7]. In the present report, a patient with relapsed refractory acute myeloid leukemia developed severe UGI bleeding in the presence of severe thrombocytopenia induced by anti-CD33. Despite intensive conventional treatment, the bleeding worsened. The next step recommended by the surgeon was gastrectomy, which could have been a dangerous procedure for this particular patient who was also severely thrombocytopenic and neutropenic. To avoid the surgical procedure, rFVIIa was administered. After a single dose of rFVIIa, the patient improved clinically and the bleeding decreased following formation of local fibrin clots at the sites of gastric erosions, as demonstrated by gastroscopy. However, bleeding occurred again 2 days later from diffuse colonic ulceration. If the source of bleeding had been localized only to the stomach it is likely that a longer period of controlled hemostasis could have been achieved. The use of rFVIIa has been described in

two acute myeloid leukemia patients following bone marrow transplantation complicated by diffuse lower gastrointestinal bleeding. In one patient, massive gastrointestinal bleeding developed in the face of ongoing rFVIIa treatment, and the other had transient stabilization of her lower gastrointestinal bleeding, but she experienced a renewed profound upper gastrointestinal bleeding [8]. Another report [9] describes a female with acute lymphoid leukemia who developed severe thrombocytopenia secondary to chemotherapy. Massive gastrointestinal bleeding appeared, following which rFVIIa $90 \mu\text{g kg}^{-1}$ was administered, resulting in cessation of bleeding.

Vlot *et al.* [10] described a 59-year-old man without malignancy who bled massively from a large duodenal ulcer. Despite intensive treatment including surgery, the bleeding continued and the patient was in danger of exsanguination. The patient condition stabilized after administration of rFVIIa $90 \mu\text{g kg}^{-1}$ every 2 h for 21 h.

To the best of our knowledge this is the only case report which describes and objectively documents successful hemostasis of UGI bleeding in the setting of acute myeloid leukemia associated with thrombocytopenia. It also emphasizes the potential for management of life-threatening hemorrhage in a severely pancytopenic patient.

As rFVIIa half-life is in the range of 2–3 h, it is advisable to perform immediate endoscopy after rFVIIa treatment to evaluate the efficacy and potential need for further therapy.

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