Influence of cell-associated tissue factor concentration on the anticoagulant activity of dabigatran. A possible explanation for the reduced incidence of intracranial bleeding

The Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) trial demonstrated that, compared with warfarin, dabigatran offers superior protection against stroke and systemic embolism and reduces the risk of life-threatening and intracranial bleeding (Connolly *et al*, 2009). The study also revealed a higher rate of myocardial infarction in the dabigatran group, a finding confirmed by a recent meta-analysis (Uchino & Hernandez, 2012).

Thrombosis and haemostasis, while sharing several common mechanisms, present some important differences. During haemostasis, clotting is triggered by tissue factor (TF, also termed coagulation factor III, FIII) exposed by the vascular lesion. TF is constitutively expressed by the cells of the vessel wall, mainly adventitial fibroblasts, and is abundant in many extravascular tissues (Semeraro & Colucci, 1997). Thus, upon vessel rupture, blood coagulation is initiated by high concentrations of TF to produce a proper fibrin clot in due time. During the thrombotic process, the source and the amount of TF may vary remarkably, depending on the specific situation. In atrial fibrillation, for example, thrombus formation is believed to result from multiple prothrombotic alterations, which include stasis within the left atrium, wall abnormalities and the expression of TF by the atrial endocardium itself and/or by infiltrating mononuclear cells (reviewed in Choudhury & Lip, 2003). It is conceivable, therefore, that under these circumstances the local expression of TF is markedly lower than in a damaged vessel wall during haemostasis. Because the difference in TF expression might influence the response to anticoagulant drugs, we compared the anticoagulant activity of dabigatran and warfarin in the presence of different concentrations of cell-associated TF.

Blood was obtained from patients with atrial fibrillation who had been on warfarin for more than three months [International Normalized Ratio (INR) 2·4 ± 0·3, $n = 4$) or on dabigatran treatment (150 mg bid, $n = 4$). All patients gave informed consent. In warfarin-treated patients, blood was collected between 9·00 and 10·00 a.m. while in dabigatran-treated patients it was taken 2–3 h after the morning dose (peak) and the day after, just before the next dose (trough). Plasma was prepared by double centrifugation (15 min at 1000 ***g***, then 10 min at 12 000 ***g***) and stored at −80°C until tested. Dabigatran concentration was assayed by a modified thrombin time (van Ryn *et al*, 2010) and was 236 ± 31 ng/ml in peak samples and 115 ± 41 ng/ml in trough samples. A pooled plasma from 10 healthy subjects was used as control.

Anticoagulant activity of patients' plasma was measured by a cell-based clotting assay as follows. Washed human mononuclear cells (10^7/ml) were prepared from the blood of healthy donors as reported (Colucci *et al*, 2001) and incubated overnight at 37°C with 1 μg/ml lipopolysaccharide (LPS, *E. coli* 0111:B4; Sigma, Milan, Italy) to induce maximal TF expression. After stimulation, the cells were centrifuged for 10 min at 1000 ***g*** and suspended at 20×10^6/ml in RPMI medium containing 10 μg/ml phospholipids. The cells were then disrupted by sonication and serially diluted (up to 1:625) in the same buffer. The concentration of TF in undiluted cell preparations, as assessed by enzyme-linked immunosorbent assay (Imubind TF; American Diagnostica, Greenwich, CT, USA), was 156 ± 70·2 pmol/l. The anticoagulant activity of patients' plasma was evaluated by a single-stage clotting assay (Colucci *et al*, 2001). Briefly, 100 μl cell suspension was added to a test tube containing 100 μl of test plasma and incubated for 2 min at 37°C, after which clot formation was initiated by the addition of 100 μl of 20 mmol/l $CaCl_2$. The clotting time was evaluated manually, and the anticoagulant activity at each cell concentration was calculated as the ratio between the clotting time of patient plasma and the clotting time of control plasma.

Warfarin plasma displayed a similar anticoagulant activity at all cell concentrations (average clotting ratio of 2·42) (Fig 1). On the contrary, the anticoagulant activity of dabigatran plasma was strongly influenced by cell number. In peak samples the clotting ratio decreased from >4 to 1·8 moving from the lowest to the highest concentration of TF-positive cells whereas in trough samples it decreased from 3·5 to 1·45. Qualitatively similar results were obtained when the cells were replaced by thromboplastin (Recombiplastin; Instrumentation Laboratory, Milan, Italy) (not shown), indicating that the change in TF activity was indeed the cause of the different response of dabigatran plasma.

This observation might help to explain some clinical findings. The fact that warfarin displays the same anticoagulant activity regardless of TF concentration suggests that it will be able to inhibit the clotting process equally well during haemostasis and thrombosis. In contrast, the efficiency of dabigatran may vary depending on the local availability of TF. In patients with atrial fibrillation, because thrombus formation within the atrial appendage is most likely to be caused by low TF combined with stasis and other prothrombotic alterations (Choudhury & Lip, 2003), both peak and trough concentrations of

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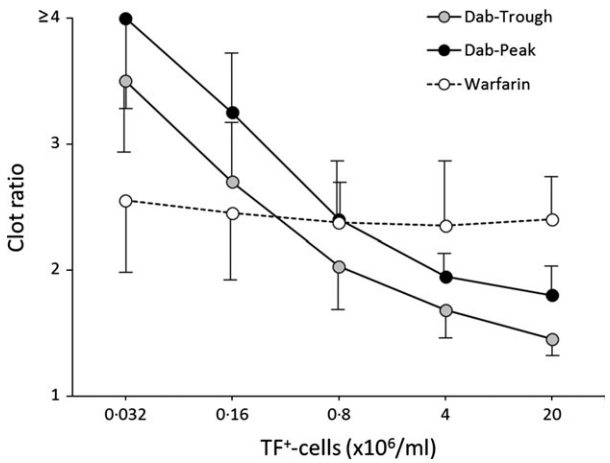

Fig 1. Influence of tissue factor-expressing mononuclear cells on the anticoagulant activity of plasma from patients under warfarin or dabigatran treatment. Clot formation in patient plasma was triggered by increasing concentrations of TF^+-cells, and the anticoagulant activity at each cell concentration was calculated as the ratio of the clotting time of test plasma and the clotting time of pooled normal plasma. Data are the mean ± standard deviation of four experiments performed on samples from different patients. Dab-Trough and Dab-Peak denote samples taken from dabigatran-treated patients at peak and trough concentration of drug, respectively. TF, tissue factor.

dabigatran are likely to efficiently inhibit the thrombotic process. On the other hand, dabigatran might have a lower impact on the formation of a haemostatic plug within tissues, such as the brain, where the concentration of TF is extremely high (Hoffman & Monroe, 2009), providing a plausible explanation for the lower incidence of intracranial haemorrhage in dabigatran-treated patients (Connolly *et al*, 2009; Uchino & Hernandez, 2012). However, the TF-dependent efficiency of dabigatran might also be behind the increased incidence of myocardial infarction seen in the RE-LY study. In fact, thrombosis within a coronary artery is triggered by the rupture of an atherosclerotic plaque, which is rich in TF-bearing cells, such as activated macrophages and smooth muscle cells, and cell-derived TF-positive microparticles (ten Cate, 2012). Moreover, our data might partly explain why dabigatran is less effective than warfarin in preventing early thromboembolic complications in patients with mechanical heart valves (Eikelboom *et al*, 2013). In these patients, coagulation can be induced by TF derived from damaged tissue, inflammatory cells and dysfunctional endothelial cells. This mechanism, coupled with platelet and contact pathway activation by the artificial surface of the valve (Becker *et al*, 2001; Eikelboom *et al*, 2013) will probably result in huge thrombin generation that overwhelms local levels of dabigatran.

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Authors' contributions

CTA performed the experiments and analysed the data; FZ and FV performed the experiments; ST provided the plasma samples and contributed helpful comments; NS contributed helpful comments and critically revised the manuscript, MC designed and supervised the research, analysed the data, and wrote the manuscript.

Conflict of interests

MC reports grants from Boehringer Ingelheim, outside the submitted work; the other authors have no conflict of interests to declare.

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