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# Inhibition of collagen-induced platelet reactivity by DGEA peptide✪

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**Direct interactions between collagen, the most thrombogenic component of the extracellular matrix, and platelet surface membrane receptors mediate platelet adhesion and induce platelet activation and aggregation. In this process two glycoproteins are crucial: integrin $\alpha_2\beta_1$, an adhesive receptor, and GPVI, which is especially responsible for signal transduction. Specific antagonists of the collagen receptors are useful tools for investigating the complexity of platelet-collagen interactions. In this work we assessed the usefulness of DGEA peptide (Asp-Gly-Glu-Ala), the shortest collagen type I-derived motif recognised by the collagen-binding integrin $\alpha_2\beta_1$, as a potential antagonist of collagen receptors. We examined platelet function using several methods including platelet adhesion under static conditions, platelet function analyser PFA-100™, whole blood electric impedance aggregometry (WBEA) and flow cytometry. We found that DGEA significantly inhibited adhesion, aggregation and release reaction of collagen activated blood platelets. The inhibitory effect of DGEA on static platelet adhesion reached sub-maximal values at millimolar inhibitor concentrations, whereas the specific blocker of $\alpha_2\beta_1$ — monoclonal antibodies Gi9, when used at saturating concentrations, had only a moderate inhibitory effect on platelet adhesion.**

**Considering that 25–30% of total collagen binding to $\alpha_2\beta_1$ is specific, we conclude that DGEA is a strong antagonist interfering with a variety of collagen-platelet interactions, and it can be recognised not only by the primary platelet adhesion receptor $\alpha_2\beta_1$ but also by other collagen receptors.**

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**Abbreviations:** ACD, acid citrate dextrose; BSA, bovine serum albumin; GPIaIIa, glycoprotein IaIIa; GPIIbIIIa, glycoprotein IIbIIIa; GPVI, glycoprotein VI; GPIV, glycoprotein IV; MoAb, monoclonal antibodies; PBS, phosphate-buffered saline; PFA, platelet function analyser; TIIICBP, type III collagen-binding protein; WBEA, whole blood electric aggregometry.

A number of agents inhibiting platelet adhesion to collagen *via* integrin $\alpha_2\beta_1$ have been identified. They include snake venom proteins (crovidisin, catrocollastatin, jaracetin, jararhagin) (De Luca *et al.*, 1995; Kamiguti *et al.*, 1997; Clemetson, 1999), monoclonal antibodies (Gi9, 6F1) (Nakamura *et al.*, 1998; Moroi *et al.*, 2000) and synthetic peptides (DGEA, GFOGER) (Staatz *et al.*, 1991; Knight *et al.*, 2000). Undoubtedly, depending on their specificity, the antagonists of platelet receptors are not only useful tools in model studies on the molecular mechanisms of platelet function, but also potential antiplatelet drugs to be used in antithrombotic therapy. Studies of platelet adhesion are complicated by the fact that several collagen receptors may be involved in this process which implies a 'multisite multistep' model of the platelet-collagen interaction (Watson *et al.*, 2000). In this model, the primary receptor for platelet-collagen adhesion $\alpha_2\beta_1$ is engaged in the initial direct contact with collagen and is responsible for the arrest of platelets on the sub-endothelial collagen surface (Di Paola *et al.*, 1999; Siljander & Lassila, 1999, Inoue *et al.*, 2003). This allows another collagen receptor molecule, glycoprotein VI (GPVI), to further activate blood platelets and transmit the signal through the GPVI-FcR $\gamma$-chain pathway. GPVI is considered as the major signalling receptor involved in collagen-induced aggregation (Perret *et al.*, 2003) and it can evoke the high-affinity state of $\alpha_2\beta_1$ (Jung & Moroi, 2000). Other receptors, like GPIV, TIIICBP (type III collagen-binding protein) or p65, may be involved in very early platelet-collagen interactions, but their roles are minor.

Staatz *et al.* (1991) were the first to describe a series of peptides derived from the $\alpha$1(I)-CB3 fragment of type I collagen, which are recognised by $\alpha_2\beta_1$ and have the ability to inhibit cell adhesion to collagen. In these studies the minimal active recognition sequence Asp-Gly-Glu-Ala (DGEA), corresponding to residues 435–438 of type I collagen sequence, was identified. Since DGEA-containing peptides effectively inhibited the $\alpha_2\beta_1$-mediated $Mg^{2+}$-dependent platelet adhesion to collagen, this tetrapeptide was considered a specific blocker of $\alpha_2\beta_1$-dependent functions. DGEA was frequently used in studies on the interactions between collagen and the $\alpha_2\beta_1$ receptor on various cells, including osteoblasts, breast carcinoma cells and arterial smooth muscle cells (Yamamoto *et al.*, 1995; McCann *et al.*, 1998; Mizuno *et al.*, 2000), although the inhibiting properties of DGEA were questioned by some other investigators (Barnes *et al.*, 1996). There are few papers reporting the use of DGEA in studies on blood platelets (Staatz *et al.*, 1991, Knight *et al.*, 1998).

In the present work we attempted to assess the usefulness of the DGEA peptide in the inhibition of blood platelets activated with fibrillar collagen. We hypothesized that DGEA may bind not only to $\alpha_2\beta_1$, but that this sequence may also be recognised by other collagen receptors, resulting in an overall profound inhibition of collagen-induced platelet functions.

## MATERIALS AND METHODS

***Blood samples and donors.*** Blood was collected from healthy donors (74 individuals; 35 men and 39 women), mean age 29.6 ± 10.4 years, under the guidelines of the Helsinki Declaration for human research and the studies were approved by the committee on the Ethics of Research in Human Experimentation at Medical University of Łódź. For the present study we selected only donors showing haematocrit of 38–45% and platelet count 150 000–350 000/$\mu$L at the time of enrolment. All the enrolled donors were non-smokers and none of them had taken aspirin or other drugs affecting platelet function for at least 10 days prior blood collection or had a history suggestive of underlying haemostatic disorders. Blood was withdrawn with special caution to avoid undesirable activation of cir-

culating platelets. All platelet reactivity measurements were performed within two hours after blood withdrawal.

In the studies with the use of PFA-100™ and whole blood aggregometry we employed buffered 0.105 M sodium citrate as an anticoagulant (blood:anticoagulant ratio 9:1, v/v). For adhesion assay and flow cytometry studies ACD (acid-citrate-dextrose, 98 mM sodium citrate, 71 mM citric acid, 111 mM glucose) at a blood:anticoagulant ratio 9:1 (v/v) for cytometry and 6.67:1 (v/v) for adhesion assay were used.

***Chemicals***. Insoluble equine tendon collagen type I was from Chrono-Log Corp. (Havertown, PA, U.S.A.), *p*-nitrophenyl phosphate, adenosine diphosphate (ADP), bovine albumin fraction V and other chemicals were from Sigma (St. Louis, MO, U.S.A.), phosphate-buffered saline (PBS) from Biomed (Lublin, Poland). Sterile 96-well microtiter platelets with flat-bottom wells were from Medlab Products (Raszyn, Poland). Collagen/ADP cartridges for PFA-100™ closure time measurements and bovine thrombin (Thrombin Test) were from Dade Behring (Deerfield, IL, U.S.A.). Fluorolabelled monoclonal antibodies: anti-CD61/PerCp, anti-CD62/PE, PAC-1/FITC and other chemicals for flow cytometry were from Becton Dickinson (San Diego, CA, U.S.A.). DGEA (Asp-Gly-Glu-Ala) peptide inhibitor for GPIaIIa was from AnaSpec (San Jose, CA, U.S.A.) and Gi9 antibodies anti-GPIaIIa were from Immunotech (Marseille, France).

***Incubation with antagonists***. Whole blood (for PFA-100, whole aggregometry and flow cytometry studies) or isolated platelet suspension (in adhesion studies, see below) were incubated with 0–10 mM DGEA or 10 $\mu$g/ml Gi9 antibodies for 10 min at room temperature. The stock solutions were prepared in distilled water (DGEA) or saline (Gi9) and added to platelet samples in a volume proportion up to 1:50.

***Platelet adhesion assay***. Platelet-rich plasma was obtained by centrifugation of whole blood at 190 × ***g*** for 12 min. Isolation of blood platelets and measuring of adhesion of control platelets or platelets pretreated with inhibitors was performed according to Bellavite *et al.* (1994). Briefly, wells of a 96-microtiter dish were filled with 50 $\mu$l of 40 $\mu$g/ml collagen dissolved in 0.05% acetic acid and left at 37°C overnight. After incubation, the well contents were aspirated, wells treated with 200 $\mu$l PBS containing 2 mg/ml BSA for 1 h, and then washed three times with PBS. Immediately after washing, the wells were supplemented with 25 $\mu$l of the tested platelet agonists: thrombin (final concentration 0.2 U/ml) or ADP (final concentration 20 $\mu$M) dissolved in a buffer containing 145 mM NaCl, 5 mM KCl, 10 mM Hepes, 0.5 mM $Na_2HPO_4$, 6 mM glucose, 0.2% BSA, 3 mM $CaCl_2$ and 3 mM $MgCl_2$, pH 7.4. After incubation with antagonists, 50 $\mu$l aliquots of platelet suspension ($1 \times 10^8$ cells/ml) were transferred to wells containing relevant agonists and then platelet adhesion was measured using a microplate reader (Benchmark, BioRad, Germany) according to the standard method (Bellavite *et al.*, 1994).

***PFA-100™ closure time (CT).*** The flow analyser PFA-100™ (Dade-Behring) allows the study of the overall platelet-derived primary haemostatic capacity in whole blood (Favaloro, 2001; 2002). Blood samples are aspirated under steady-flow conditions through a small aperture cut in a diaphragm coated with collagen type I and ADP. Platelets, when becoming activated, attach to the area surrounding the aperture, form a platelet plug and occlude the aperture, while the instrument records the time necessary for the occlusion (collagen/ADP closure time, $CT_{CADP}$), which is regarded as an indicator of platelet function (Bock *et al.*, 1999). In all samples the concentrations of vWF antigen in blood plasma of all the examined subjects were determined by enzyme-linked immunosorbent assay (Bartlett *et al.*, 1976). According to our results of PFA-100™ closure time collected for a large group of healthy individuals, we

considered the values of 66 and 106 s as the lower and upper limits for normal $CT_{CADP}$ and the values of $CT_{CADP} < 66$ s as indicative for platelet hypersensitivity.

***Whole blood electric impedance aggregometry (WBEA).*** Platelet aggregation in whole blood in the presence of collagen (2 $\mu$g/ml) was studied by the impedance method using a Whole Blood Aggregometer Chrono-Log 592 (Chrono-Log Corp.). The measurements were taken according to the Chrono-Log protocol (Ray *et al.*, 1994). Shortly, whole blood diluted 1:1 with 0.85% (w/v) saline was incubated with the respective antagonist, then supplemented with collagen, and the impedance value reflecting the extent of platelet aggregation was read after additional 6 min. According to our determinations for a group of healthy donors, we accepted the values of impedance of 14 Ω and 22 Ω as the lower and upper normal limits for collagen-induced whole blood platelet aggregation.

***Flow cytometry studies.*** In flow cytometric analysis both preparation and staining were performed according to the modified (Rozalski *et al.*, 2001) Becton Dickinson Procedure for Flow Cytometric Analysis of Platelets. In order to analyse of surface membrane antigens expression in circulating (resting) platelets or after *in vitro* platelet activation with collagen (final concentration 50 $\mu$g/ml, incubation for 1 min without stirring at room temperature), 10 $\mu$l aliquots of whole blood were diluted 10 × in PBS and the cell suspensions were immediately transferred to a mixture of staining antibodies (either CD61/PerCP and $IgG_1$/FITC or CD61/PerCP, PAC-1/FITC and CD62/PE). The labelling reaction was allowed to proceed for 20 min in the dark and was subsequently stopped by adding CellFix solution. Fluorescence of 2000–5000 platelets was measured with a FACSCalibur instrument (Becton-Dickinson, U.S.A.). The percentage of the specific fluorescence-positive platelets was obtained after subtraction of non-specific mouse $IgG_1$ binding. While the percent fraction of the antigen-positive platelets reflects the abundance of the subpopulation of activated platelets is, the relative fluorescence intensity reflects how abundant is any given antigen represented on platelet surface membranes.

***Statistical analysis.*** Means ±S.D. are given for all parameters. Shapiro-Wilk test was used to verify whether the data were normally distributed. Student *t*-test and Wilcoxon's signed rank test were used to estimate the significance of differences between the groups. Non-linear estimation (quasi-Newton algorithm) was used to calculate the $IC_{50}$ values for DGEA. The ROC (receiver operating characteristic curve) method was used to determine the cut-off points in the analysis of results of PFA-100™ closure time.

The inhibition values for DGEA and Gi9 were determined *vs.* control samples (without antagonists).

## RESULTS

In this work we applied two antagonists of the $\alpha_2\beta_1$ receptor: The DGEA peptide and monoclonal antibodies Gi9. We determined the inhibitory effects of both inhibitors on platelet adhesion to surface coated with type I fibrillar collagen under static and high shear stress conditions, as well as their effects on aggregation and the expression of activated GPIIbIIIa complex (monitored as the binding of PAC-1 MoAb) and P-selectin (monitored as expression of CD62 antigen) in collagen-activated whole blood platelets.

Under static conditions and in the presence of $Mg^{2+}$, the spontaneous platelet adhesion to fibrillar collagen (with no thrombin or ADP added) amounted to 21.0 ± 5.0%; upon induction with thrombin (0.2 U/ml) or ADP (20 $\mu$M) the values were 28.3 ± 6.3% and 23.0 ± 3.5%, respectively (data expressed as mean ±S.D., n = 30). We found that 5 mM DGEA completely inhibited both spontaneous and ADP-induced adhesion ($P<0.01$) (Fig. 1). As regards the thrombin-evoked platelet adhe-

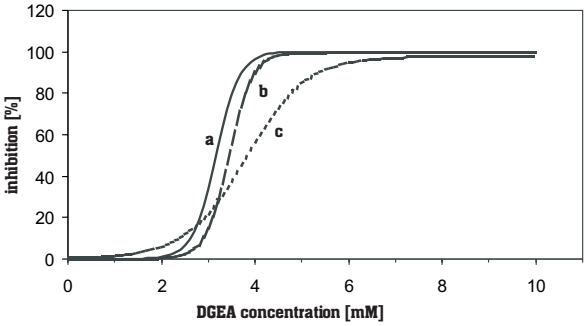


**Figure 1. Inhibition of platelet adhesion to collagen by DGEA under static conditions: a, control (spontaneous) adhesion with no agonist added; b, adhesion after stimulation with ADP; c, adhesion after stimulation with thrombin.**

Data presented as inhibition curves fitted to the equations calculated based on raw data.

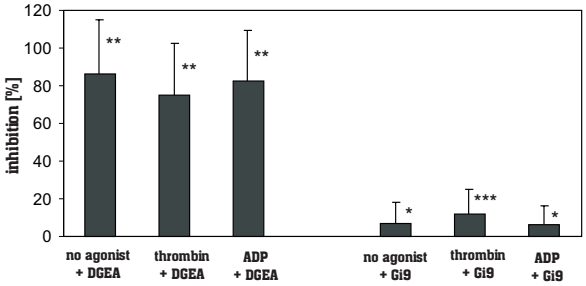


**Figure 2. Inhibition of platelet adhesion by DGEA and Gi9 without agonist and upon stimulation with thrombin or ADP.**

Data shown as a mean ±S.D., $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

sion, complete inhibition was observed at DGEA concentration of 10 mM ($P < 0.01$). The determined $IC_{50}$ value was the lowest for spontaneous adhesion (3.03 ± 0.42 mM) whereas the $IC_{50}$ values for ADP- and thrombin-induced adhesion were only slightly and non-significantly elevated: 3.12 ± 0.5 mM and 3.58 ± 0.62 mM, respectively. No significant differences between the $IC_{50}$ values were observed. According to these findings, we selected the highest tested concentration of the blocker (10 mM) for the purpose of further studies on the DGEA inhibitory effect of the fibrillar collagen-mediated platelet reactivity. We assumed that at this concentration all $\alpha_2\beta_1$ receptors and other collagen receptors recognising this sequence on the platelet surface should be occupied with the abundant DGEA molecules.

The influence of Gi9 monoclonal antibodies (10 $\mu$g/ml) on $\alpha_2\beta_1$-mediated platelet adhesion resembled that of DGEA, although the inhibition was less profound. Gi9 MoAbs significantly blocked the spontaneous ($P < 0.05$), as well as thrombin- ($P < 0.001$) and ADP-induced ($P < 0.05$) platelet adhesion to fibrillar collagen (Fig. 2).

In the next step of the studies we assessed the rate of platelet adhesion/aggregation in whole blood under conditions of high shear stress using PFA-100™ (collagen/ADP cassettes). The mean closure time in the group of examined donors (n = 27) was 93.4 ± 21.2 s. We showed that 10 mM DGEA and 10 $\mu$g/ml Gi9 remarkably prolonged the closure time ($P < 0.001$ for DGEA and $P < 0.01$ for Gi9) (Fig. 3). As the PFA-100™ closure time may fluctuate along with the alterations in plasma

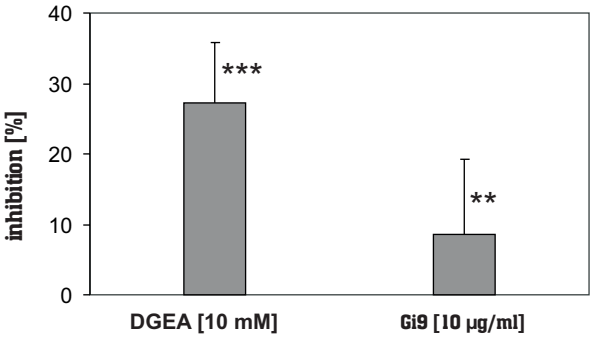


**Figure 3. DGEA- and Gi9-mediated inhibition of platelets' ability to occlude the PFA-100 capillary.**

The inhibition rates calculated from closure times standardized for plasma vWF concentration. Data for Gi9 shown as a mean ±S.D.; data for DGEA shown as a lower quartile-median-upper quartile, $P < 0.01$ (**), $P < 0.001$ (***).

vWF concentration, the data of closure time, recorded either prior to or upon the addition of a given antagonist, were routinely standardized for vWF concentration in all but seven DGEA-treated cases (44%). In those seven donors DGEA completely inhibited the

platelets' ability to occlude the PFA-100 capillary (closure time was above 300 s which is the upper limit of the instrument detection range). Based on these findings, the cut-off point for plasma vWF, estimated by means of the ROC (receiver operating characteristic) curves analysis was 70.8% (with 86% sensitivity and 100% specificity). It means that in the individuals with plasma vWF < 70.8% and in the presence of 10 mM DGEA, we might reasonably expect to observe the PFA-100™ closure times exceeding 300 s.

As regards the aggregation studies, the collagen (2 $\mu$g/ml)-induced platelet aggregation in whole blood (15.6 ± 1.4 Ω, n = 7) became significantly inhibited by DGEA ($P < 0.001$) but not by Gi9 (Fig. 4a).

The DGEA-mediated inhibition of platelet release reaction was monitored by flow cytometry as the altered expression of CD62 antigen of P-selectin and the expression of activated GPIIb-IIIa complex (monitored as the bound PAC-1 MoAb). Platelet activation with collagen (50 $\mu$g/ml) resulted in a significantly increased P-selectin (CD62) expression (2.84 ± 1.7% in resting platelets *vs.* 59.5 ± 13.9% after activation; $P < 0.01$) and PAC-1 expression (2.7 ± 2.2% in resting platelets *vs.* 59.5 ± 9.9% after activation; $P < 0.001$). Such an activation became remarkably attenuated in the presence of 10 mM DGEA, $P < 0.001$ (Fig. 4b).

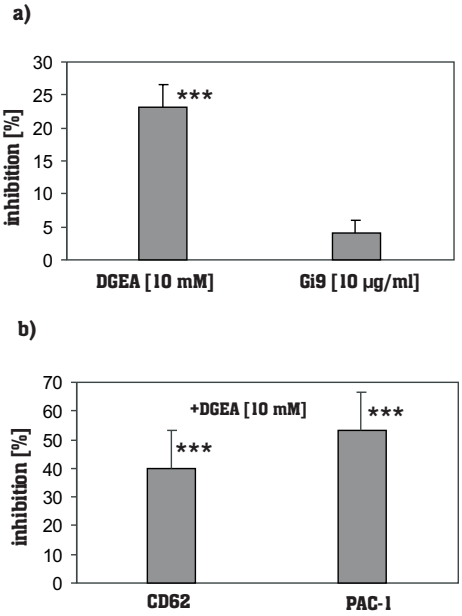


**Figure 4. Inhibition of collagen-induced platelet reactivity by DGEA and Gi9: a, in whole blood platelet aggregation; b, in flow cytometry studies.**

Data shown as a mean ±S.D., $P < 0.001$(***). Inhibition of platelet release reaction monitored as CD62 expression; inhibition of GPIIbIIIa activation monitored as PAC-1 expression.

## DISCUSSION

In our work we investigated the efficacy of the short peptide DGEA in the inhibition of platelet response following collagen stimulation. Since a few receptors are engaged in different stages of the platelet activation evoked by collagen, we monitored the effect of DGEA on platelet adhesion, aggregation and $\alpha$ granule release reaction.

DGEA was found to be a very potent blocker of the platelet reactivity in response to collagen. First, we demonstrated that DGEA, at millimolar concentrations, strongly or even completely inhibited the spontaneous and agonist-induced platelet adhesion to collagen in the presence of $Mg^{2+}$ under static conditions. On the other hand, we found that the specific blocker of $\alpha_2\beta_1$ – the monoclonal antibodies Gi9 only partly inhibited platelet adhesion. In our opinion, this finding is in favour of our suggestion that DGEA may be efficient enough to block the various platelet surface membrane receptors interacting with collagen. Compounding arguments for such a reasoning come from the available literature reports. First, collagen binding to $\alpha_2\beta_1$ has been estimated to comprise only up to 25–30% of total collagen binding to various platelet membrane receptors in the presence of $Mg^{2+}$ (Jung & Moroi, 1998). Second, it has been demonstrated that the maximal inhibition of platelet adhesion can be achieved by the simultaneous use of antagonists against $\alpha_2\beta_1$, GPVI and GPIV (Nakamura *et al.*, 1998; 1999). Therefore, since we demonstrated in

this study that high DGEA concentrations were efficient to block completely platelet adhesion, we further argue that the blocker is likely to interact with various collagen receptors and is not specific for $\alpha_2\beta_1$.

Furthermore, both DGEA and Gi9 MoAbs significantly inhibited the platelets' ability to form a plug in the PFA-100™ flow system. Whereas DGEA was found to have a strong antagonistic effect on the platelet function monitored with PFA-100™, the effect of the monoclonal antibodies Gi9 that specifically interact with platelet $\alpha_2\beta_1$ was only moderate. Under these haemodynamic conditions (shear stress about 5000–6000 $s^{-1}$) platelets are believed to both adhere and aggregate on the collagen surface (Kundu *et al.*, 1995; Favaloro, 2002; Salat *et al.*, 2002). Initially, the most important role in flowing blood is played by vWF, which binds to collagen surface and interacts with platelets (Wu *et al.*, 2000). In the second phase, platelets firmly bind to collagen *via* receptors $\alpha_2\beta_1$ and GVI, resulting in even more enhanced platelet adhesion and aggregation. We believe that both $\alpha_2\beta_1$ and GPVI would be necessary for the full activation of platelets under such flow conditions, because GPVI-deficient platelets can adhere to the collagen surface but cannot form aggregates (Moroi *et al.*, 1996; 1997). The process of adhesion is mediated especially by $\alpha_2\beta_1$ (Sixma *et al.*, 1997). In the PFA-100 studies with the use of Gi9, we observed that the $\alpha_2\beta_1$-dependent adhesion contributed in only several percent to the total platelet reactivity as measured by the closure time. Our finding that DGEA significantly and almost completely reduced platelet adhesion/aggregation in the PFA-100™ system further supports the experimental evidence provided herein that collagen receptors other than $\alpha_2\beta_1$ significantly contribute to platelet-collagen interactions under flow conditions.

We further showed that DGEA significantly inhibited the collagen-induced platelet aggregation measured in whole blood. On the other hand, antibodies Gi9 only slightly reduced the aggregation. The role of $\alpha_2\beta_1$ in platelet aggregation after collagen stimulation is recently a matter of debate. Some authors postulated a major participation of $\alpha_2\beta_1$ in aggregation, but others reported that $\alpha_2\beta_1$ may be only one of the contributors to this process. Such an inconsistent array of findings goes even further. It was found that anti-$\alpha_2\beta_1$ monoclonal antibodies (6F1 or Gi9) either did inhibit platelet aggregation completely (Coller *et al.*, 1989; Monnet *et al.*, 2000) or only slightly reduced the collagen-induced aggregation (Moroi *et al.*, 2000). Besides, controversial results regarding the role of $\alpha_2\beta_1$ in platelet aggregation were reported in studies using snake venom proteins (rhodocytin, trimucytin, jararhagin) (Kamiguti *et al.*, 1996; Inoue *et al.*, 1999; Suzuki-Inoue *et al.*, 2001; Liu *et al.*, 2002). Based on our data, which demonstrated that a specific $\alpha_2\beta_1$ blocker – Gi9 – insignificantly reduced platelet aggregation, it appears that $\alpha_2\beta_1$ plays a minor role in the aggregation. The stronger inhibitory effect of DGEA on aggregation could be attributed to the fact that it blocked other collagen receptors involved in this process, e.g. glycoprotein VI (Watson, 1999; Jarvis *et al.*, 2002).

Platelet activation by collagen results in intracellular signal transduction and a release of intraplatelet granule contents. In flow cytometry studies we evaluated the efficiency of DGEA in the inhibition of the activation of fibrinogen receptor GPIIbIIIa and platelet release reaction reflected by the extent of P-selectin (CD62) expression. At the concentration of DGEA which was efficient for the inhibition of platelet adhesion and aggregation, the release of P-selectin and the activation of GPIIbIIIa complex after stimulation by collagen became significantly reduced. Nakamura and co-workers found that in the presence of $Mg^{2+}$, platelet adhesion to fibrillar collagen induced the activation of GPIIbIIIa complex, and anti-$\alpha_2\beta_1$ antibodies inhibited this activation by about 40% (Nakamura *et al.*, 1999). On the other hand, it was observed that fibrillar collagen induced

platelet release reaction independently of the presence of $Mg^{2+}$ (Nakamura *et al.*, 1998). These reports and our results suggest that $\alpha_2\beta_1$ integrin, beside other collagen receptors, is responsible for conformational changes in GPIIbIIIa complex as well as platelet release in the course of fibrillar collagen-induced platelet activation.

Taken together, our data clearly demonstrate the complex nature of the platelet-collagen interactions. The DGEA peptide is likely to be a non-specific blocker of $\alpha_2\beta_1$-dependent platelet function. Probably, DGEA can be also recognised by other collagen receptors, like GPVI or p65, which are engaged in the initial and/or final stages of platelet activation. Further studies are necessary to examine the role of DGEA in the inhibition of collagen receptors and overall collagen-dependent platelet function.

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