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Morphological studies on platelet function using a microfluidic chip. A proof of concept study

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SUMMARY

Platelets are blood cellular components involved in hemostatic processes and thrombus formation. Activation and inhibition of platelets result in an increase in morphological changes and a significant reduction in adhesion. There are several approaches towards the determination of the functional status of platelets, based on criteria such as cell adhesion, molecular changes at the cell surface, etc. In recent years, microfluidic devices have been introduced to mimic conditions proper to the vascular system, and so emulate thrombus formation in vivo. This study presents a microchip, the Thrombi Chip®, which is partially fitted with fluidic properties. This microchip has various types of micro-channels into which the platelets are inserted and, after drug treatment, the investigation is completed with the examination of the chip under an invert light microscope.

For microscopy, cells were labeled with FCDA (human platelets) and Rho6G (mouse platelets). Counts and morphometric measurements of the adhered cells were carried out using digital images. To validate the results obtained with the microchip, the fractions of mice platelets were investigated with flow cytometry as well. Scanning electron microscopy was used to examine the morphological changes related to activation and inhibition in human platelets.

The results show that, with this microchip, activation and inhibition of platelets can be detected. Flow cytometry studies largely confirm the microchip results. Certain variability in the results observed in human platelets is considered normal, as donors were randomized. In this respect the mouse platelets were much more uniform.

Measurements with the microchip require that the sample be divided into three groups: control, activated and inhibited, resulting in a set of data, which, after respective evaluation, provides activity profiles, giving information on the status and response capacity of a sample. Such profiles could have diagnostic relevance and therefore be useful in a clinical context, for example in the monitoring of the effects of short- and long-term treatment of patients, as well as to test new drugs.

Key words: Micro-fluidic chip – Platelet activation – Platelet inhibition – Cell adhesion – Cell shape – Thrombosis and hemostasis

INTRODUCTION

Platelets are enucleated blood cellular components, which are involved in hemostatic processes and in thrombus formation (Ware et al., 2013; Jurk and Kehrek, 2010). Platelets respond to activators with noticeable morphological and molecular cell

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surface modifications, becoming more adhesive than resting ones (Jagroop et al., 2000; Kamath et al., 2001; Karpman 2001). In this context, molecules such as CAM and integrins are relevant as activated platelets enhance their expression, being responsible for increased adhesion when they become translated into the cell membrane (Gupta and Reviakine, 2014; Popov et al., 2014).

The activation state of platelets can be established by methods based on the detection of such molecular changes. Other methods determine the status of adhesion and aggregation of the platelets. Specific antibodies, for instance against p-selectin (CD62p) and CD41/CD61 complex, enable the use of flow cytometry to identify platelets and determine whether they are activated, constituting a reliable method towards the assessment of their functional changes (Linden, 2013; Middelburg et al., 2013; Daskalakis et al., 2014).

Others, such as aggregometry and retention tests, evaluate the increased adhesion of activated platelets, determining a ratio between attached cells and those still afloat in the original suspension. Aggregometry is a well-established method, which finds wide application in clinical laboratories (Harrison, 2005). The following are examples of retention tests: glass beads retention test (Desnoyers and Verry, 1972; Czapek et al., 1978) and Homburg retention test (HRT) (Nickels et al., 2003; Weidig et al., 2004), as well as the platelet adhesion assay (PADA) (see discussion). In the first test, cells become attached to glass beads coated with proteins and arranged in columns. In the HRT the cells are retained in a filter of non-thrombogenic material, which is mounted in so-called Sysmex retention tubes (Sysmex Deutschland GmbH, Hamburg, Germany).

The idea of developing the micro-fluidic chip presented here (Giro, 2004; Mestres and Weber, 2006) arose from experience made with the spreading assay (Breddin and Bauke, 1965) and the HRT (Krischek et al., 2005). The microfluidic chip method concentrates on platelets, which are able to adhere. By counting them in three different situations (control, activated and inhibited) one obtains information on their functional state and reaction capacity; an approach not far removed from the HRT but one which offers the possibility of examining the microchip under the light microscope, enabling analysis of cell shape (morphometric parameters), and is thus closely related to the spreading assay.

This micro-fluidic chip, the Thrombi-Chip® (Mestres and Weber, 2006), is a prototype consisting of multiple channels with different designs, into which a small defined volume of platelet suspension is introduced. The aim of this study is to prove whether platelets vitally labeled with fluorescent stains and treated with agonist or antagonist drugs undergo changes (number of adhered cells and cell shape) detectable with the microchip, and whether this information could be relevant for the interpretation of functional state and platelet responsiveness.

MATERIALS AND METHODS

Cells

Platelets were obtained from 21 human healthy volunteers (17-19 years old) and from 36 C57BL/6 mice.

Blood from volunteers (one per test series) was collected by venipuncture in 5 ml anticoagulant citrate collection plastic tubes (BD). The blood donors gave their written consent.

The mice were kept and sacrificed according to EU and national regulations for animal experimentation. The ethic commissions of the institutions involved (URJC, Instituto Carlos III) authorized this project independently of each other.

10 ml of human venous blood was extracted by venipuncture with a 20-gauge cannula and transferred to two tubes, each containing 5 ml, to which was added 3 ml of solution I (citric acid 23 mM, sodium citrate 53 mM, PGE1 560 nM dissolved in PBS). This sample was then centrifuged at 130 g for 10 minutes at 4ºC. The supernatant, i.e. the fraction rich in platelets, was collected and transferred to new tubes containing 5.3 ml of solution II (citric acid 23 mM, sodium citrate 53 mM, PGE1 747 nM dissolved in PBS) and centrifuged at 1430 g for 10 minutes at 4ºC. The pellet formed after this second centrifugation was re-suspended in 1.6 ml PBS, and the presence of platelets was assessed by contrast phase microscopy. The platelets were counted with a cell counter (Coulter) and adjusted with PBS to a cell concentration of $17x10^5$/ ml. In the next step, cells were stained with FL-stain CFDA (5-carboxyfluorescein diacetate) in accordance with the staining protocol recommended by Molecular Probes® (http://www.lifetechnologies.com). Thereafter, the human platelet suspensions were distributed to three tubes: one for activation with 100µM adenosine-5´diphosphate sodium salt (ADP, Sigma-Aldrich. Ref. A2754), a second for inhibition with acetyl salicylic acid (ASA) (Sigma-Aldrich. Ref. A5376) 100-200 µg/ml, and a third tube, which remained untreated and served as a control.

The Thrombi-Chip channels were filled (10 µl per channel with a Gilson P20 pipette nº10 tip) with the above-mentioned cell suspensions, and the chips were incubated for 30 minutes at 37ºC to enable the cells to adhere. The ends of the channels were tightly sealed with Parafilm® in order to avoid evaporation. The microchips were studied under a fluorescence microscope (Nikon Eclipse 90i) equipped with a FITC filter (EX 465-495 DM 505 BA 515-555). A 20x zoom lens was used for pho-

tography.

In the studies with the mouse platelets, the blood sample was taken directly from the heart, in order to collect as high a blood volume as possible. In general, 1 ml blood per mouse was collected, using per experiment series at least three mice.

The procedure for the preparation of the mouse platelets' rich fraction was as follows: 1 ml blood was mixed with 1 ml PBS which contained heparin (40 UI/ml) and was kept on ice at 4ºC. For platelet isolation 1 part of Lympholite® was added to 1 part heparinized blood (thus avoiding turbulences in the solution) and kept at room temperature. The suspension was centrifuged at 800 g for 30 minutes at 20ºC. Platelets-rich plasma was washed with PBS and centrifuged at 1000 g for 10 minutes at 4ºC. The pellet was re-suspended and washed again as above. After this second washing the pellet was re-suspended in Tyrodes-HEPES solution, adjusted to a volume of 200 µl and the number of cells was determined with the flow cytometer (see later). Thereafter platelets were stained with Rho 6G, (Molecular Probes Inc.). The suspension of stained platelets was then distributed to 3 tubes, one remaining untreated and serving as a control, a second in which the cells were activated with ADP (70 µM), and the third batch of cells were treated in a first step with Apyrase (ATP di-phospho-hydrolase, EC 3.6.1.5) (100 UI/ml)., followed by a second step with ADP. The cell suspensions were injected with a Gilson P20 pipette (tip nº10) into the Thrombi-Chip channels (10 µl per channel) and incubated for 30 minutes at 37ºC. Imaging was performed with a confocal scanning laser microscope (Leica Espectral sp5) and using the 20X zoom lens.

Morphometry, image analysis and statistics

Digital images of cells attached to the chip were obtained using the 20X zoom lens either in fluorescence or confocal scanning microscopy. Pro count chamber 4 or more photographs were taken, so that pro channel and depending on the type, approx. 12-20 pictures were obtained. Counts and morphometric measurements of platelets were performed using these digital pictures.

In addition to cell counts (number of cells per area), the following cell form parameters were noted: size expressed in µm diameter, Feret max, Feret min, aspect ratio (AR), roundness, solidity, and circularity.

Image J (NIH) was used for image analysis and Origin 8.0 for the statistics.

Flow cytometry analysis

Mice platelets were studied using flow cytometry (FACS Calibur, Becton Dickinson) to determine the number of cells and the expression of integrin IIb/ß3 at the cell surface, a marker for activated platelets (Niiya et al., 1987). A specific monoclonal FITC-coupled antibody (Rat anti-mouse integrin IIb/ß3 active form, clone JON/A) was used. Three different platelets suspensions (untreated, activated with ADP, and thereafter inhibited with Apyrase) were incubated with saturating amounts of 1/100 diluted antibody for 15 minutes at 37ºC in a light-protected incubator. After incubation, the cells were washed in PBS in order to remove excess antibody, after which flow cytometry was immediately performed. The flow cytometer was calibrated with micro-beads (Calibrites FACS Calibur), and, in order to detect platelets, no size threshold was defined. Platelets were identified by their typical distribution by FSC/SSC on dot plot diagrams and the results were analyzed using the data handling software Flow Jo 6.3.4.

Thrombi-Chip

The Thrombi-Chip consists of a polymer called Topas® (thermoplastic cyclo-olefin copolymer = COC). In size terms, the chip has the same dimensions as a histology slide (Fig. 1). The chip used here is a test model incorporating several channel designs. There are four basic variants or designs: 1) linear, 2) linear with three rectangular counting chambers, 3) linear with 5 rectangular counting chambers and 4) a meandering type. The channels are built quadrangular in cross-section and in three different diameters (Fig. 1).

The channels were constructed so that they can be filled airtight through the input side with tips of different diameters (max. 1.2 mm). On the opposite side of each channel (output) there is a reservoir (V = 10 µl) for collecting excess sample liquid,

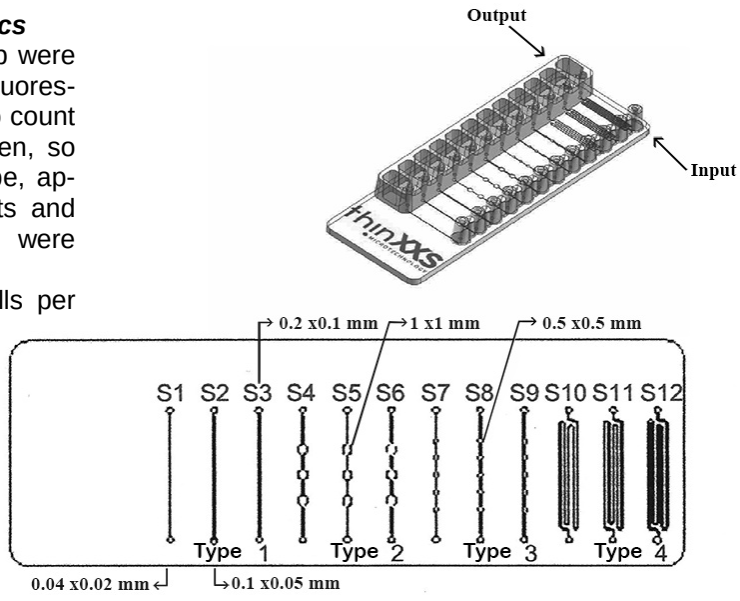

Fig. 1. 3D diagram of Thrombi-Chip showing the actual design used in the present study. The lower part of this figure shows Thrombi-Chip channel designs, with dimensions and shapes.

which, at the same time, prevents a drying-out of the channel during incubation. Finally, in a separate manufacturing step to close the quadrangular channel, the back of the chip was covered with a thin lamina, which interestingly is the only surface of the channel inner walls to which cells become attached. An invert microscope was used to look through this thin lamina, which is also manufactured by Topas® and is optically suitable for light and fluorescence microscopy. The main criterion in the assessment of the different channel designs in this microchip prototype was their suitability for the attachment of platelets.

Scanning electron microscopy

Human platelet-rich plasma prepared as above, was washed in PBS and incubated at 37ºC for 30 minutes in PBS containing ADP or ASA or without any drug (controls) and finally passed through a Sysmex retention tube (Sysmex GmbH, Hamburg). Cells became attached to the retention tube filter and were divided into 4 quadrants to facilitate their examination with scanning electron microscopy (Fig. 2 A).

The filters with cells were separated from the retention tubes and fixed by immersion in PBS containing 2.5 % of glutaraldehyde for 2 hours at +8ºC. After washing with PBS, they were fixed in 2% of osmium tetroxide in PBS for 2 hours at room temperature and protected from light, dehydrated in an ascending alcohol series, critical point-dried, using CO_2 as intermediary, and coated with platinum (2-4 nm) in a Emithec K550X sputter apparatus. The examination was performed with a CamScan S2 scanning electron microscope, obtaining SE-images of ROIs at 20 kV.

RESULTS

Scanning electron microscopy of activated and inactivated human platelets

Platelets attach very well to filter surfaces (Fig. 2 A). At high magnifications details of cell surface topography can be recognized, allowing the differentiation of several platelet forms. Basically three platelet forms were observed:

1) Round or spherical as well as others resembling a small discs,
2) Irregular in shape with numerous pseudopods, conferring platelets a rough appearance, and
3) Platelets with an extremely flattened shape, with a great width and close contact with substrate and numerous thin pseudopods emerging from the cell border.

Inhibited platelets display a smooth surface and are round in shape i.e., they correspond to the morphological type 1 mentioned above (Fig. 2 B). The presence of pseudopods is very unusual in inactivated platelets. The inhibited platelets show lesser adhesion ability, which is why the number of attached platelets of this type is, in general, rather low.

Activated platelets display a quite different and characteristic surface topography (Fig. 2 C). Numerous pseudopods protrude from the cell surface. Some of them appear to be in contact with the substrate or are slightly bent and free (Fig. 2

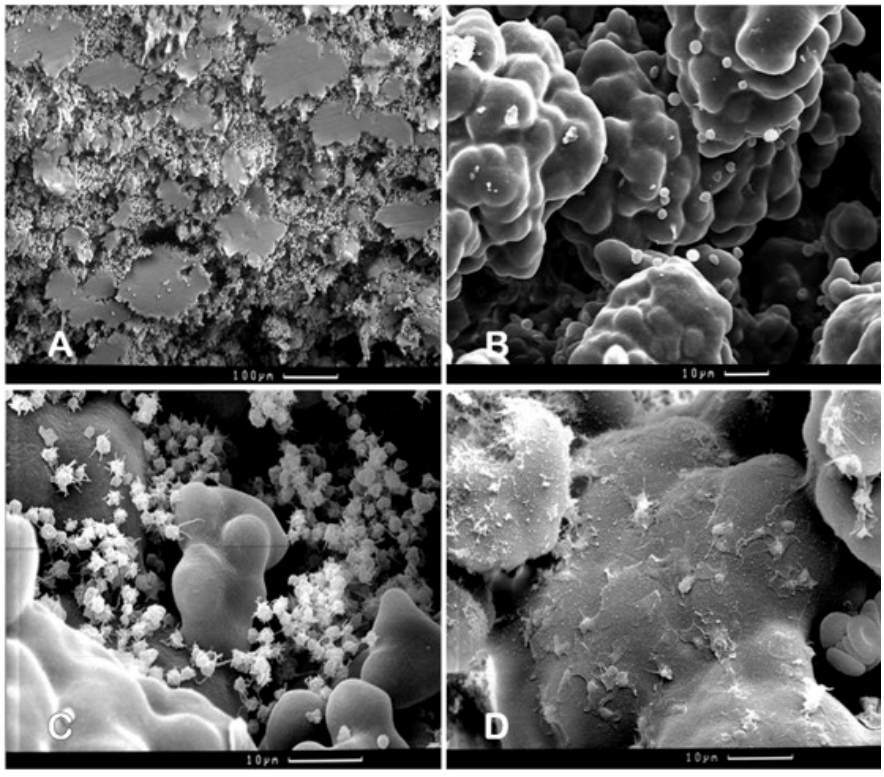

Fig. 2. Scanning electron microscopy of human platelets (SE image). **(A)** Surface of a sectioned filter with untreated platelets occupying surfaces of filter interstices; **(B)** ASA-inhibited platelets. Note their round shape and smooth surface; **(C)** ADP-activated platelets with numerous pseudopods; **(D)** ADP-activated platelets, presenting a very flattened appearance and a large surface of contact with the substrate. At cell borders thin pseudopods can be observed.

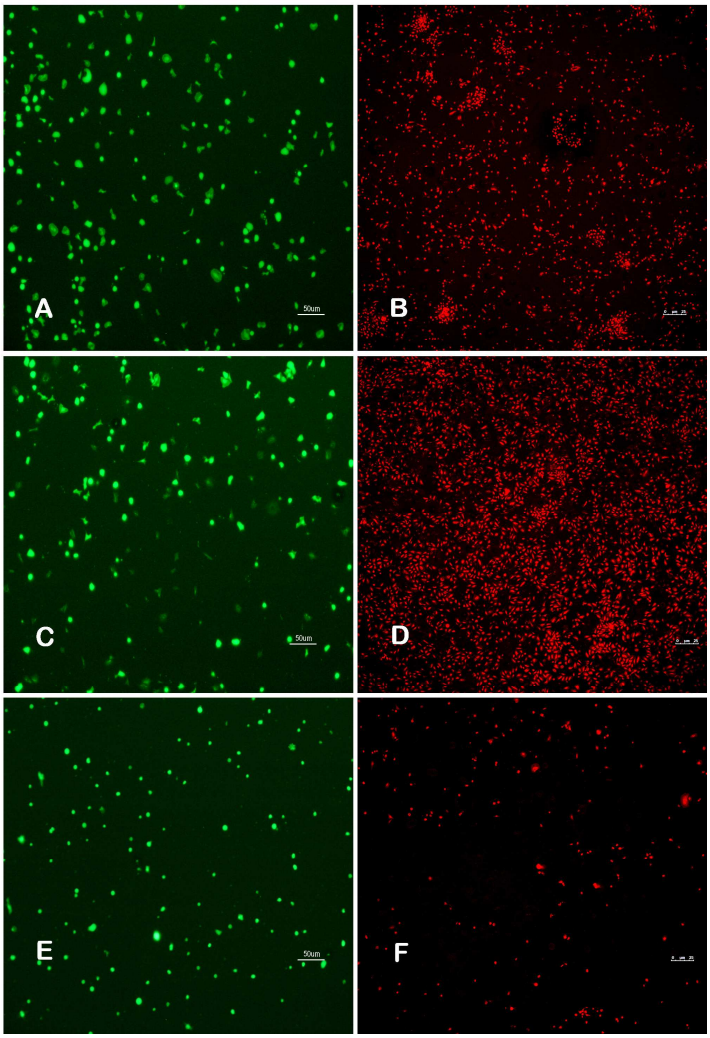

Fig. 3. Vital stained platelets with CFDA (human) and with Rho 6G (mice) for fluorescence microscopy. Human platelets **(A)** control, **(C)** activated with ADP and **(E)** inhibited with ASA. Mice platelets **(B)** control; **(D)** activated with ADP and **(F)** inhibited with Apyrase plus ADP.

C). In addition, small aggregates can occasionally be seen, in which cell processes appear closely intermingled. Although the present experiments were rather short, (30 minutes incubation time) small aggregates of platelets could already be observed in a few samples (Fig. 2 C). Additionally, the spreading out of highly activated platelets displaying a peculiar flattened shape, with a much extended contact area with the substrate could also be seen (Fig. 2 D).

Response of human platelets to ADP and ASA examined with the Thrombi-Chip®

The concentration of platelets was adjusted to $17x10^5$/ml in all samples investigated. 10 µl cell suspension pro channel was introduced as described. Cell counts were performed on digital images with an image field of $3.41x10^5$ µm^2.

Platelets underwent changes in size after ADP and ASA treatment (Fig. 3). The size of adhered platelets ranged between 4.22 and 9.04 µm diameter.

The fluorescence intensity was, in general, uniform over the entire cell body, but in some cases at the center of the cell, where organelles accumulate higher fluorescence intensity than in peripheral cytoplasm was observed (Fig. 3 A, C, E).

Platelets adhered to the surface of the chip channel chambers in the following channel types: S4, S5, S6, S7, S8 and S9 (see Fig. 1). In channels without counting chambers no relevant attachment of cells was observed.

The counts revealed that the number of adhered platelets in control samples varied between 160 and 640 platelets per area (Fig. 4 A). Practically no signs of aggregation were observed.

In samples exposed to ADP, large platelets seemed to be more frequent than small ones, with a rather homogenous distribution on the chip. Sometimes platelets appeared closely associated, probably forming small aggregates (Fig. 3 C).

With ASA, the platelets were smaller in size, appearing homogeneously distributed on the chip surface (Fig. 3 E).

No difference in the fluorescence intensity of the platelets was detected in the three experimental situations.

The number of adhered platelets, which had been treated with ADP increased 1.3 to 2.4 fold compared to the corresponding control (Fig. 4A). However, there were cases where the number of activated platelets increased discretely below 10%, in comparison with corresponding controls.

ASA treatment causes a decrease of 20% to 60% in the number of adhered platelets in comparison with the corresponding control (Fig. 4 A). In some experiments, however, the number of platelets (counts) was higher than the controls, reaching values up to 40% compared to the control, but remaining clearly below the values of the ADP samples (not shown).

Morphometric analysis revealed that both Feret diameters increase in platelets treated with ADP and become reduced in samples exposed to ASA (Fig. 4 C). Interestingly, there is no difference in the aspect ratio (AR) in the three groups investigated, i.e., control, ADP and ASA (Fig. 4 C). The parameters solidity, roundness and circularity show no significant changes either after activation or after inhibition (Fig. 4 C).

Response of mouse platelets to ADP and Apyrase examined with the Thrombi-Chip

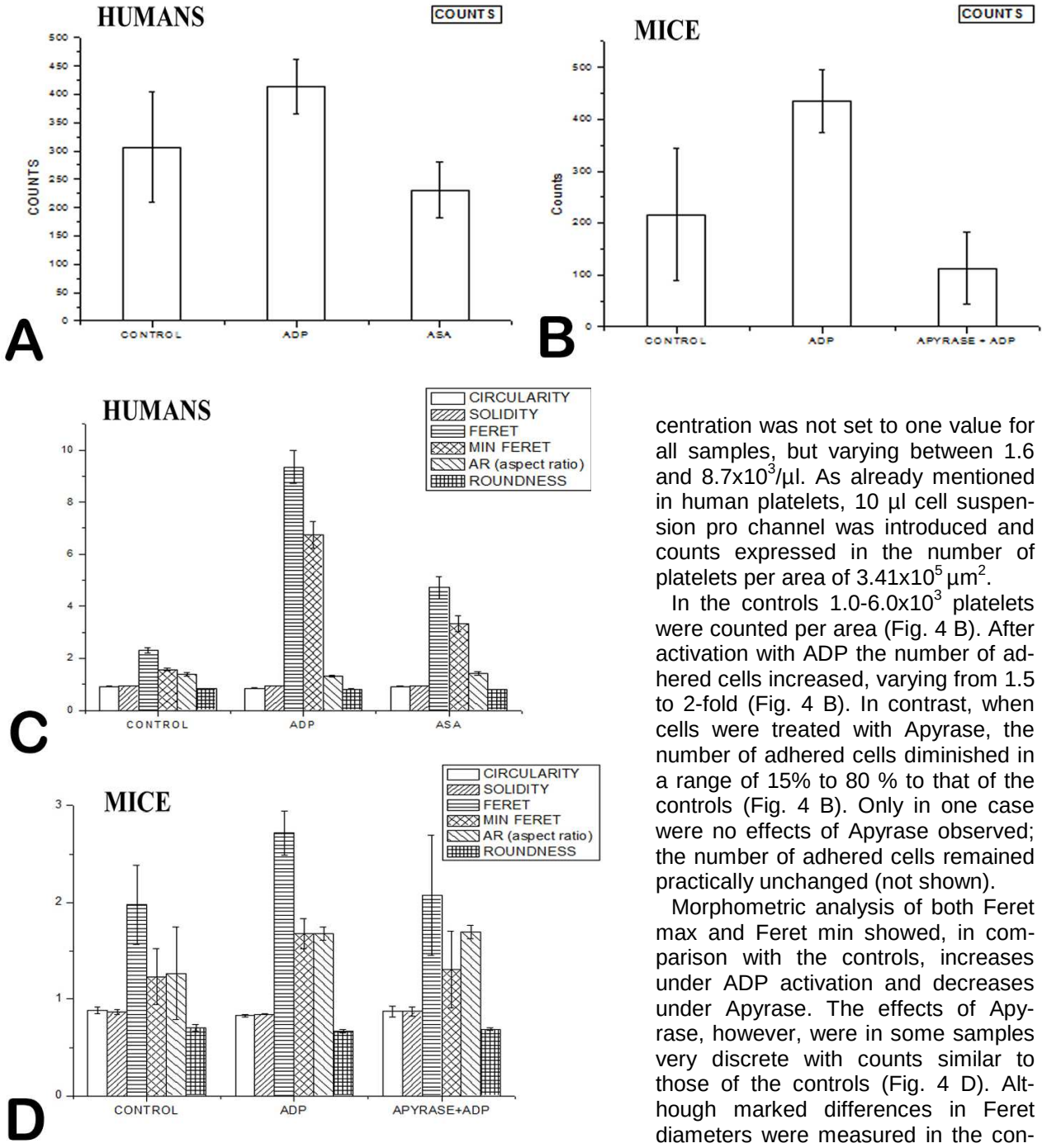

Fig. 4. Counts of platelets adhered to the Thrombi-Chip and morphometric analysis. **(A)** Counts of platelets under different functional conditions; **(B)** Counts of mice platelets under different functional conditions; **(C)** Morphometric analysis of human platelets in control, activated and inhibited state; both Ferets show differences in the three experimental situations, which can be interpreted as changes in cell size, with high values in activated platelets. AR indicates that cell shape does not change. **(D)** Morphometric analysis of mice platelets under control, activated and inhibited conditions. Also in mice platelets both Ferets undergo modifications, indicating changes in cell size but not in shape. In all other parameters no relevant changes were observed for both human and mice platelets.

Mice blood volumes collected per mouse were rather low and even varied, this being the reason why the platelet concentration was not set to one value for all samples, but varying between 1.6 and 8.7×10^3/µl. As already mentioned in human platelets, 10 µl cell suspension pro channel was introduced and counts expressed in the number of platelets per area of 3.41×10^5 µm^2.

In the controls 1.0-6.0x10^3 platelets were counted per area (Fig. 4 B). After activation with ADP the number of adhered cells increased, varying from 1.5 to 2-fold (Fig. 4 B). In contrast, when cells were treated with Apyrase, the number of adhered cells diminished in a range of 15% to 80 % to that of the controls (Fig. 4 B). Only in one case were no effects of Apyrase observed; the number of adhered cells remained practically unchanged (not shown).

Morphometric analysis of both Feret max and Feret min showed, in comparison with the controls, increases under ADP activation and decreases under Apyrase. The effects of Apyrase, however, were in some samples very discrete with counts similar to those of the controls (Fig. 4 D). Although marked differences in Feret diameters were measured in the controls, ADP-activated and Apyrase-inhibited platelets, the AR remains practically unchanged in all experimental situations (Fig. 4 D). The parameters solidity, circularity and roundness appeared unmodified in all three groups (Fig. 4 D).

To assess results achieved with the microchip, all mouse samples were analyzed by flow cytometry (Fig. 5). The study of the control samples showed that approx. 10% of cells expressed CD41/CD61 (Fig. 5 A). In samples treated with ADP the fraction of cells expressing CD41/CD61 in-

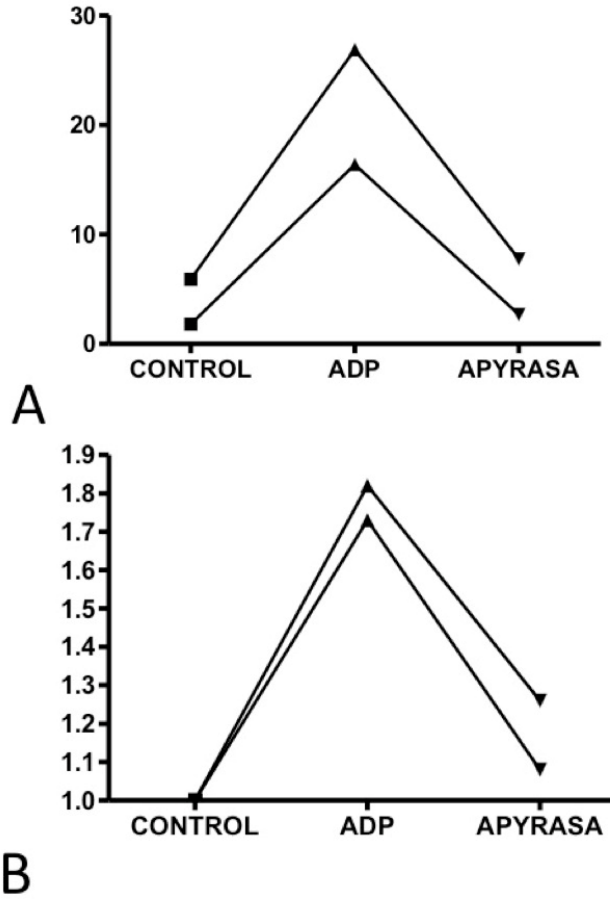

Fig. 5. Functional assay in resting and activated platelets. Platelets were collected, treated with adenosine diphosphate (ADP) or Apyrase plus ADP and analyzed by flow cytometry using JONA (CD41/CD61 active form) antibody. Platelets were gated in the low SSC/ FSC dot plot window. Frequency of JONA+ platelets (upper graph) and their intensity fluorescence (as an increase in the mean fluorescence intensity (MFI) relative to control, bottom graph) in the JONA channel are shown.

creased 2 to 5-fold, sometimes even more (Fig. 5A). Inhibition with Apyrase results in a remarkable reduction of labeled cells, approximately reaching levels similar to those measured in the controls (Fig. 5 A).

In a few cases the number of cells expressing CD41/CD61 treated with Apyrase remained high (not shown).

Mean Fluorescence Intensity (MFI), a parameter which indicates the binding of labeled antibodies and - indirectly- also the number of binding sites on the cell surface, reflects their up and down variations related to activation by ADP and Apyrase inhibition respectively (Fig. 5 B).

DISCUSSION

The activation of platelets is a process with a sequence of events such as changes in cell adhesion, cell shape and cell metabolism, whereby the expression of proteins involved in cell adhesion increases, with secretion and translocation of glycoproteins to the cell surface and aggregation with formation of a stable plug (Kamath et al., 2001; Brass, 2003).

The morphological changes of platelets related to their functional state as described in the present study agree with previous studies performed with light and electron microscopy (Zucker and Borrelli, 1954; White, 1968, Jagroop et al., 2000). It should be stressed that the formation of pseudopods, also called filopodia, a process which accompanies platelet activation, is essential in hemostasis (Milton and Frojmovic, 1979).

Furthermore, it is important to mention that platelets are involved in many pathological processes such as vascular diseases, atherosclerosis, heart infarct and others (Ware et al., 2013). They play an essential role in such processes, adhering to the inner surfaces of injured vessels, thereby increasing the synthesis of proteins such as integrins (IIa / IIIb), fibrinogen receptor, p-selectin (CD62p) and thus augmenting adhesiveness (Middelburg et al, 2013). The next event in the hemostasis sequence is the formation of platelet aggregates, through which a plug is formed to seal the injured vessel wall, facilitating the progress of coagulation (Harrison, 2005). The time scale and agent dose in our experiments was selected primarily to address the early stages of platelet reaction, i.e., prior to the onset of aggregation.

Assessment of the state of activation of platelets in vitro is a matter of great interest in clinical medicine and drug development. For this purpose there are a number of methods to test the activity of platelets which, in general terms, are based on four main lines of evidence:

1) Platelet aggregation, measured by light transmission (LTA=light transmission aggregometry) or by impedance, LTA undoubtedly being the most frequently used in clinical routine (Gurbel et al. 2007; Lordkipanidze et al. 2007),
2) Activation-dependent changes at the platelet surface combined with fluorescence-activated flow cytometry, which is probably the best method for studying membrane proteins and activated platelets (Middelburg et al., 2013),
3) Based on Platelet adhesion, for instance, the so-called retention and adhesion tests (Glass bead retention test: Pitney and Potter, 1967; Homburg retention test: Krischek et al., 2005, Platelet adhesion assay [PADA]: Schumann et al., 2005), and
4) Platelet morphology, the so-called spreading assay, with which it is possible to see changes in the shape and size of platelets under the microscope (Breddin and Bauke, 1965; Nickels et al., 2003).

In this paper preliminary evidence is presented

that the response of platelets to activation and inhibition can be measured with the aid of a microfluidic chip, using as parameters their adherence (number of cells attached) and the shape of platelets (morphometric parameters).

The platelets were stained with fluorescent vital stains, CFDA and Rho6G, neither of which interfere with cell dynamic processes (Fan et al. 2007; Tamura et al., 2013). An alternative to fluorescence is diaphorase-histochemistry, in which the reaction end product is dyed black as a result of mitochondrial activity (Mestres et al., 1999). The advantage of this technique lies in the possibility of substituting fluorescence by conventional light microscopy and also the fact that the chips are rendered storable, which is not possible with fluorescence- stained preparations. Although this alternative has been not used in the present study, it promises to be of aid in monitoring and in retrospective studies in clinical settings.

In the present experiments the morphological behavior of platelets was studied under three situations: platelets without any manipulation, i.e., controls, platelets responding to activation with ADP and to inhibition with a suitable antagonist, in our case acetyl salicylic acid (ASA) or Apyrase (EC 3.6.1.5). ADP was used as a platelet agonist, because it is a source of energy and a classic agent in this type of investigation (Born, 1962, Huang and Detwiler, 1981). ASA was used for platelet inhibition; as it inhibits the enzyme COX1 in an irreversible way and impedes the activation of platelets and secretions of alpha-granules, constituting the "gold standard" as an anti-platelet agent in the prevention of thrombosis (Parker and Gralnick, 1989; Schrör, 1997). In experiments with mouse platelets, inhibition was carried out with Apyrase, an enzyme that degrades ADP and ATP, both energy sources for platelets (Pilla et al., 1996; Rossatto et al., 2003).

Cell counts performed on the microchip permit differentiation between the three experimental situations under consideration. In control samples the number of platelets adhered to the microchip has been considered as an indicator of the base activity state of each sample. ADP activates platelets and, consequently, the number of adhered cells increases, when compared to the controls. Interestingly, in humans cases were observed in which untreated platelet fractions already show very high counts (adherent cells) and, in such cases, ADP does not significantly increase any further the number of adherent cells, probably because the cell population was already activated prior to commencement of the experiment.

Mice platelet samples have shown a similar response to ADP, but, in contrast to humans, no high activation before the experiment was ever observed, indicating that mice platelets show a more homogeneous activity status than human platelets, probably due to the living conditions.

Inhibition with ASA renders lower numbers of adhered human platelets to the microchip. However, in a few cases, only weak inhibition or even a complete absence of it were observed, i.e., platelet counts were rather high despite inhibition. Whether these results represent ASA–resistance, a somewhat controversial issue, requires additional investigation (Puri, 2007; Schrör, 2009).

The experiments with mouse platelets have, moreover, revealed that a set cell concentration is not required when working with the microchip, enabling the use of variable cell concentrations without any disadvantageous effect on the results.

Morphometric analysis has shown that, under activation, platelets tend to increase in size, as indicated by the Feret diameters, but the aspect ratio (AR) suggests that the shape of the cells remains unchanged. Parameters such as circularity, roughness and solidity do not reveal any significant differences between the experimental situations. However, the fact that current microchip geometry impedes the use of lenses higher than 20X does make this statement questionable.

The results obtained by flow cytometry confirm activation and inhibition of mouse platelets with ADP and Apyrase respectively, correlating well with the measurements performed with the microchip. Under the effects of ADP, platelets are activated and express the active form for the receptor complex CD41/CD61 in their membrane, this being, in turn, evidence for platelet activation (Michelson, 2006). Since Apyrase hydrolyzes energy-rich phosphate compounds such as ADP and ATP, thus decreasing the energy available for metabolism and for the synthesis of proteins, the diminution of labeled cells counts can only be interpreted as a sign of inhibition (Molnar and Lorant, 1961; Pilla et al., 1996). In our experiments, inhibition by Apyrase was not detected in all cases, or at least not to the same extent. Apyrase is a reliable inhibitor of platelets, but discrepancies have been reported, indicating that, in order to obtain the desired effect in some cases, the enzyme dose should be modified according to the specimen and/ or experimental situation (Maloney et al., 2010).

In recent years numerous micro-fluidic chips have been developed, which, in general, have found application in studies on fluids in motion (Maloney et al., 2010; Li et al., 2012). In research, micro-fluidic chips found applications mimicking flow conditions of blood vessels, for instance, in studies on coagulation and thrombus formation in vitro (Muthar and Diamond, 2013; Li and Diamond, 2014). In our case, the chip enables us to operate with small sample volumes and examination with the light microscope. Topas®, the material used in our microchip prototype, is new in this field. Previous studies have already demonstrated that Topas® is a non-thrombogenic material (Giro, 2004). In our case, once the channels were filled with the cell suspension there was no flow or

movement of fluids. In view of the working conditions in our experimental settings, fluidic properties have - in our case - only marginal significance. The cell suspension is still and the effect of gravity ensures that cells become deposited only on the bottom of the channel, establishing a firm contact with the channel wall during incubation at 37ºC, a circumstance facilitating examination with the inverted microscope. Moreover, the sealing of the in- and output channels with Parafilm or a similar medium, prevents fluid loss by evaporation during incubation of the microchip at 37ºC. Otherwise, currents or movement of the liquid might occur which could affect adherent cells, at least those weakly attached.

The chip prototype presented here contains different channel designs and, as already said; only those with so-called “counting chambers” yield acceptable results. In straight channels and those with meanders no reliable results were obtained, consequently they will be discarded in future Thrombi-Chips. Moreover, it is noteworthy that *thinXXS* technology is compatible with a coating of the channel inner surface with proteins, ligands and other compounds. Such surface functionalization presents new possibilities for the application of the micro-fluidic chip to other research issues (Weber, 2014).

Summarizing, these investigations show that with the aid of a micro-fluidic chip it is possible to detect changes in shape and adhesion of platelets in connection with treatment with agonist and antagonist agents, which correlates well with the results obtained with the microchip and by flow cytometry technology. The micro-fluidic chip, Thrombi-Chip®, constitutes a new development for two main reasons 1) the material used for its construction and 2) the design of its micro-channels.

Measurements with the microchip require that the sample be divided into the three groups: control, activated and uninhibited, resulting in a set of data which, accordingly assessed, can lead to elaboration of the activity profiles which could be very useful, for instance in following up the effects of drugs on platelets or disorders in these cells.

A preliminary appraisal of these results validates the microchip principle. However, prior to implementation of the microchip in clinical laboratory settings, additional studies with a re-designed micro-fluidic chip involving the preparation of dose-response curves and their application to relevant clinical samples are necessary.

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