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Transformed rat arterial smooth muscle cells induce platelet aggregation

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Abstract

Atherogenesis is characterized by a proliferation of arterial smooth muscle cells that may be of transformed nature. Platelets are implicated in the progression of atherosclerotic lesions through thrombotic complications. The present study was designed to investigate whether transformed arterial smooth muscle cells (SMC) could specifically aggregate platelets. We used rat transformed arterial SMC lines, V6- and V8-lines, that we had previously established. Experiments were performed with an in vitro homologous rat system. Suspensions of SMC were added without any other aggregating agent to rat heparinized platelet-rich plasma (PRP) in a coagulo-aggregometer. The effect of transformed V6-line and V8-line SMC was compared to that of their normal parental counterparts, V6- and V8-parent cells. Suspensions of transformed SMC induced, in a dose-dependent manner, an immediate and reversible ADP-like platelet aggregation. The amplitude of platelet aggregation was much higher with addition of transformed cells than of the corresponding control SMC (7.39 ± 0.75 cm vs. 0.85 ± 0.62 cm with 2×10^6 SMC, V6-line vs. V6-parent cells, respectively). ADP-like aggregation did not significantly differ between the two transformed V6- and V8-lines. ADP-like platelet aggregation was also obtained with supernatants of transformed SMC suspensions, the amplitude being higher with supernatants than with cell suspensions (21.0 ± 3.64 cm vs. 6.8 ± 1.22 cm with 1.0×10^6 V8-line cells, supernatant vs. cell suspension, respectively). The transformed SMC-induced aggregation of platelets was inhibited by apyrase (125 μM) and iodoacetate (25mM) and thus was ascribable to ADP released by the SMC. In addition, all suspensions of SMC, normal or transformed, but not their supernatants, induced plasma clotting after variable coagulation times. Coagulation was inhibited by hirudin (25 to 100 U/ml) and phospholipase A2 (10 U/ml) indicating thrombin generation through activity of the SMC membrane tissue factor. The present results show that transformed arterial smooth muscle cells may directly aggregate platelets via a release of ADP and this could be of pathophysiological relevance for thrombosis associated with atherosclerosis.

Keywords: Atherosclerosis; Thrombosis; Coagulation; Smooth muscle cells; Platelet aggregation; ADP; Transformed cells

1. Introduction

Atherogenesis is a complex process which involves interactions between blood and arterial wall

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cells [1]. It has become widely accepted that a role of platelets at the initial steps of atherosclerosis deserves little attention in human atherosclerosis. On the other hand, it has been well established that platelets may play a major role in the progression of already organized atherosclerotic plaques [2–4]. Thrombosis, reported as rather frequent over intact plaques [5], becomes an obligatory consequence of plaque fissuring or rupture [6–8]. Thus, occlusive thrombosis could play the role of a precipitating factor which converts clinically silent to overtly manifest atherosclerosis [9]. The thrombus paradigm as a causal factor of myocardial infarction has reappeared [8].

The main cellular components of the fibrous cap of the plaque are the smooth muscle cells (SMC). In case of ulceration or rupture of the cap, there should be direct interactions between the deep components of the lesion, subendothelium but also SMC, and blood cells. Thus, interactions between platelets and arterial SMC could be of considerable importance. SMC proliferation is one of the major cellular events in atherogenesis [10], but the nature of this proliferation is still questioned. There are two main hypotheses, possibly not exclusive, the inflammatory process [1] or proliferation linked to a genetic mutation [11]. This second theory has been based on the observation of a monotypism of the human atherosclerotic plaques [12] and suggested a monoclonal origin as for benign tumors of other tissues. This point of view, reviewed in [13, 14] has been supported by experimental data on animal models [15–17] and by some genetic features of human atherosclerotic plaques. In fact, DNA of human lesions [18] and more precisely of SMC from lesions [19], gave rise to transformed foci when transfected into NIH-3T3 cells, thus suggesting oncogene activation. The monoclonal theory has stimulated research on establishment of in vitro models of transformed and virus infected [20–22] arterial SMC and on the study of their properties, and focused up to now on lipid metabolism [23].

We have previously established two transformed rat arterial SMC lines [24]. Their availability provided us with the opportunity of investigating if transformation of SMC could be accompanied by properties consistent with some features of atherosclerosis. Most tumor cell lines and tumor tissues have the capacity to induce platelet aggregation [25,26] or blood coagulation [27]. In the case of cancer, these mechanisms are thought to play a role in the process of metastatic dissemination [28]. The aim of the present work was to test whether transformed arterial SMC could specifically activate platelets by comparison with normal SMC. The experiments have been performed with suspensions of cells of the transformed rat V6- and V8-SMC lines and their normal parental counterparts [24]. We now show that transformed arterial SMC could express proaggregant activities due to a release of adenosine diphosphate (ADP). In addition, all normal and transformed SMC suspensions induced coagulation due to generation of thrombin through activation of the extrinsic coagulation pathway.

2. Materials and methods

2.1. Materials

Heparin sodium (Fournier, Dijon, France), Phospholipase A2 and Phospholipase D (Boehringer, Manheim, Germany), adenosine diphosphate (ADP), potato apyrase, hirudin grade IV from leeches, thrombin, ethylene diamine tetra acetic acid (EDTA), ethylene glycol-bis tetra acetic acid (EGTA), iodoacetic acid, bovine serum albumin (BSA) fraction V essentially fatty acid free, collagenase type I (Sigma Chemical Co., St Louis, MO, USA), minimum essential medium (MEM), newborn calf serum, glutamine, sodium bicarbonate, penicillin/streptomycin, trypsin, Hanks balanced salt solution (HBSS) (Biomérieux, Marcy l'Etoile, France) were purchased as indicated.

2.2. Cell culture

All SMC were obtained from adult rat arterial smooth muscle by the explant technique as routinely used [29]. The previously described V6- and V8-line SMC and their parental counterparts, V6- and V8-parent cells, were utilized in these studies [24]. Cells were routinely restarted from liquid nitrogen frozen stocks and subcultured at least once before experiments. Cells were grown in 25 cm^2 flasks (Falcon Plastics, Oxnard, CA, USA) and maintained in a humidified 5% CO_2 at-

mosphere at 37°C. The medium for all SMC consisted of MEM supplemented with 10% heat inactivated newborn calf serum. Medium was changed 3 times a week.

2.3. Cell suspensions and supernatants

In the standard procedure, SMC to be used in aggregation studies were from cultures in the exponential growth phase. Cells, washed in HBSS free of Ca^{2+} and Mg^{2+}, were harvested by a mild trypsin treatment (1 min at room temperature in 0.08% trypsin, aspiration of the trypsin solution, then 12 min incubation at 37°C). Variations of that procedure were utilized when specified. For some experiments, cells were cultured to confluency, while for others, cells were harvested after treatment with EGTA (5mM, 1 h incubation at 37°C). Cell suspensions were then essentially prepared as described [30]. The harvested cells were counted in a Coulter counter (Coultronics, Model ZB, Hialeah, Flo, USA), washed 3 times in HBSS containing 0.1% bovine serum albumin (BSA) and centrifuged (300 × g, 10 min, 4°C). The resulting pellets were resuspended under a small volume (10^6 cells/10 to 20 μl). Cell viability was determined around 92%–95% throughout the experiments, by both trypan blue exclusion and lactate dehydrogenase (LDH) release; no significant difference was found between the various cell lines. Cells were maintained 2 h at 37°C before use in platelet aggregation studies to reach maximal ADP release from the SMC [31]. Supernatants of cell suspensions were obtained by centrifugation (2000 × g, 10 min).

2.4. Platelet-rich and platelet-poor plasma

Blood was collected from jugular veins of 3 diethyl ether-anesthetized rats after overnight deprivation of food. Blood (5–6 ml) was drawn from each rat in plastic syringes containing heparin (5 U/ml final) as anticoagulant. Platelet-rich plasma (PRP) was prepared as described [32]. Platelets were separated from red and white cells by centrifugation (150 × g, 8 min, room temperature), and PRP was collected in plastic tubes by aspiration. The remaining blood was further centrifuged (1000 × g, 18 min) and platelet poor plasma (PPP) was collected. Platelets were counted in a Coulter counter (Coultronics, Hialeah, Flo, USA); PRP was adjusted to a platelet count of $3 \times 10^5/\mu l$ by dilution with PPP. PRP was aliquoted in 500 μl in disposable capped polystyrene cuvettes and 'rested' at 25°C for 30 min. Aggregation studies were performed within 3 h after PRP collection.

2.5. Aggregation and coagulation assays

Platelet aggregation and plasma coagulation were assayed with 0.5ml PRP or PPP in polystyrene cuvettes placed at 37°C under constant 1100 rev./min stirring in a Rubel-Renaud recording turbidimetric aggrego-coagulometer (US patent no. 4.1 16.564) [33]. Magnitude of platelet aggregation is classically evaluated by the change in white light transmission which is recorded [34], maximal increase in light transmission represents maximal platelet aggregation. In the same way, light transmission increases when plasma clots. Procoagulant activity, i.e. ability to induce coagulation, was estimated by the time before occurence of the clot. Calibration of the aggregometer was performed with PRP (0% light transmission) and PPP (100% transmission). A 15% increase in light transmission induced a 20 cm shift of the pen on the recording apparatus; unroll speed of the recording paper was 2 cm/min.

For SMC-induced platelet aggregation, aliquots of SMC suspensions or SMC supernatants were added to heparin anti-coagulated PRP without any other initiator of platelet aggregation. Aggregation was observed for a minimum of 15 min. Final SMC concentrations were 10^5 to 12 × 10^6/ml. For all experiments, platelet aggregation induced with ADP (0.18μM final) was used as a positive control. It was run separately from the experimental samples and assayed several times throughout the experiment to test platelet response.

To characterize the mechanism of SMC-induced platelet aggregation, the following inhibitors were used: hirudin, an antagonist of thrombin (3–100 U/ml final; 1 unit antagonizes platelet aggregation due to 1 NIH U thrombin at 37°C, activity expressed in antithrombin units); apyrase, an ADP scavenging system (grade I from potato, ADPase activity approximately 3.2 U/mg, 25 to 125 μg/ml

final); phospholipase D (from cabbage, 10 U/ml final, i.e. 100 μg/ml final) [30]; phospholipase A_2 (from snake venom, 10 U/ml, i.e. 7 μg/ml final) [30]; ethylene-diamine tetra acetic acid (EDTA) (1 to 5 mM final); a metabolic inhibitor (iodoacetic acid, 25mM final, as described [31]). Inhibitors were added to platelets immediately prior to addition of SMC with the exception of hirudin and EDTA (30 min and 1 h prior, respectively). The various combinations of SMC and inhibitors brought the final volume to 550 μl. When necessary, iodoacetate was added to the SMC during preparation of the cell suspension and throughout the 2 h incubation before use in aggregation. In all cases, drug treatments were compared to the corresponding controls with vehicle alone.

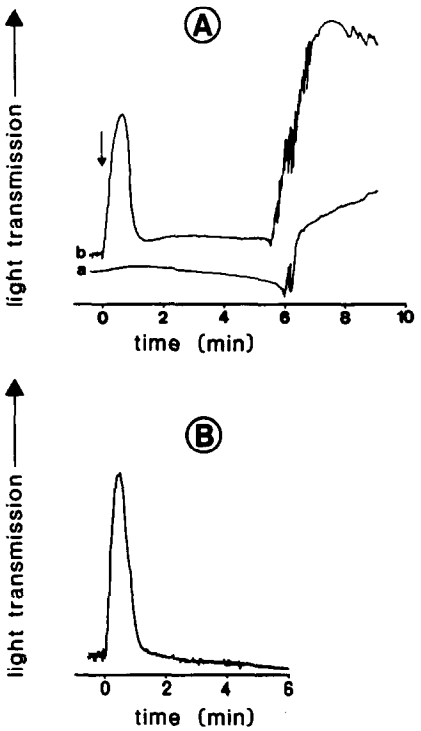

Fig. 1. Induction of ADP-like aggregation of rat platelets by addition of a suspension of transformed rat arterial SMC. As described in Materials and methods, platelet aggregation was induced by addition at time 0 min. (short arrow) of the aggregating agent to heparinized (5 U/ml) rat PRP. Aggregation was measured turbidimetrically with a coagulo-aggregometer as an increase in white light transmission through the aggregation cuvette and recorded over time (min). (A), Tracings of light transmission through PRP (b) or PPP (a) after the addition of a 10^6 V8-line SMC suspension as aggregating agent and whithout any other aggregating agent. Tracings are shown superimposed. (B), Control platelet aggregation tracing due to addition of ADP (0.18 μM final). Curves are representative of at least 3 experiments with similar results.

The aggregation tracings reported here are representative of at least 3 separate experiments with all assays performed in duplicate. Tracings are represented with their respective baselines superimposed. To quantitate the results, for the ADP-like aggregations, the maximal increase in light transmission (height of the peak), which refers to the maximal platelet aggregation, was measured and expressed in cm. Time before onset of coagulation was recorded and expressed in min. For inhibition experiments, results were expressed as percentage of the corresponding control value. Statistical analyses were conducted using Statview software on a Macintosh SE computer (Apple, Cupertino, CA). Means, standard deviations and paired Student's *t*-test for comparison of the means were calculated. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. PRP response to addition of a suspension of transformed rat arterial SMC (Figs. 1,2)

Induction of platelet aggregation by transformed rat SMC was tested in an homologous system of rat heparinized PRP. Fig. 1A (curve b) shows a typical curve of light transmission through PRP after addition of a suspension of transformed V8-line SMC. PRP response consisted of two phases: first, an immediate small reduction in light transmission, due to platelet shape change from discoidal to spherical [34], followed by a rapid and reversible increase in light transmission which resembled a classical ADP-induced rat platelet aggregation (Fig. 1B). Secondly, an irreversible phase began with a gradual increase in light transmission and was followed by erratic movements of the pen due to a blockade of the light path by a platelet/fibrin clot (Fig. 1A,b).

In order to clearly establish what was platelet aggregation or plasma coagulation, the effect of

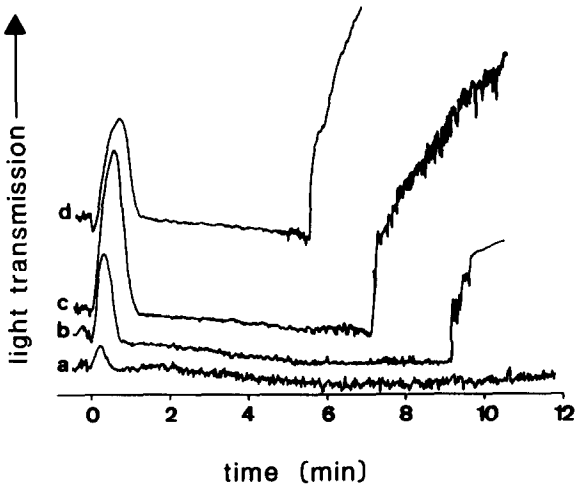

Fig. 2. Tracings of platelet aggregations were induced by addition of various numbers of V8-line SMC to heparinized (5 U/ml) rat PRP: (a) 0.5×10^6, (b) 1.0×10^6, (c) 2.0×10^6 and (d) 4.0×10^6 SMC. Tracings are representative of 3 experiments with similar results and are shown superimposed.

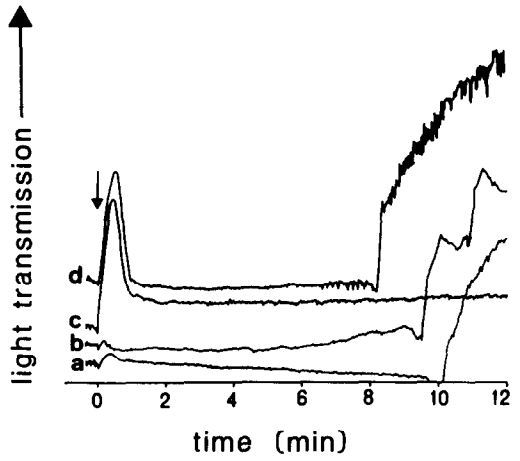

Fig. 3. Comparison of rat platelet aggregation tracings obtained by addition of normal or transformed rat arterial SMC (10^6cells) to heparinized rat PRP. Platelet aggregations were induced with normal, (a) V6-parent and (b) V8-parent, or transformed, (c) V6-line and (d) V8-line SMC. Tracings are representative of at least 3 experiments with similar results and are shown superimposed.

the V8-line suspension on PRP was compared to the effect on PPP (Fig. 1A, curves b, a, respectively). The first reversible PRP response was not observed in PPP, whereas a delayed irreversible response was maintained. This control confirmed the first wave as a platelet aggregation and the second as plasma clotting. PRP response depended on the number of transformed SMC added to the aggregation cuvette (Fig. 2); both the height of the aggregation peak and the coagulation time were dose-dependent.

3.2. Comparison of PRP responses to addition of normal or transformed SMC (Figs. 3,4)

Proaggregant activities of the transformed V6- and V8-line cells were compared to that of their respective normal parent cells, V6- and V8-parent, and examined in the same experiments. Typical aggregation curves are presented in Fig. 3. Quantifications of the height of ADP-like aggregation peaks and of the coagulation times are presented in Fig. 4A and B, respectively. Both transformed V6- and V8-line cells induced an immediate and reversible ADP-like platelet aggregation (Fig. 3, curves c, d). By contrast, no significant aggregation was induced by normal V6- and V8-parent cells (Fig. 2, curves a, b) or other normal SMC (not shown). Suspensions of both normal or transformed SMC induced coagulation. In the standard conditions used here, i.e. 10^6 trypsinized cells from exponential cultures added to 5 U/ml heparinized PRP, the V6-line profiles recorded over 15 min lacked the coagulation response observed with the other SMC. However, such a response has been observed with V6-line cells, but in other experimental conditions, i.e. confluent cultures or EGTA-harvested cells or 2.5 U/ml heparinized PRP (not shown). In Fig. 4A, the heights of ADP-like aggregation in response to the addition of transformed cells were significantly higher than in response to normal SMC. There was no significant difference between the aggregations induced by the two transformed V6- and V8-line cells. Concerning the procoagulant activities, no statistically significant difference could be discerned between the three sets of results for the V6-parents, V8-parents and V8-lines (Fig. 4B). By contrast, as already mentioned above (Fig. 3), the coagulation times observed after addition of V6-line cells were significantly higher than that

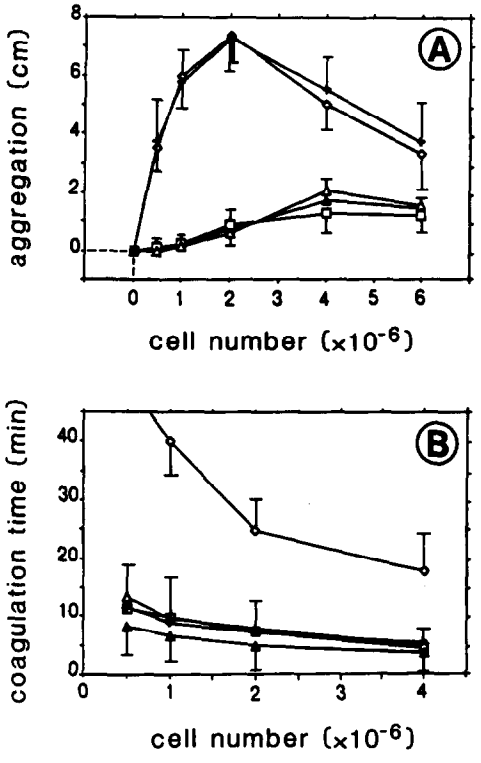

Fig. 4. Dose-dependent effect of normal or transformed SMC on induction of platelet aggregation and coagulation. SMC lines: (▲), control secondary SMC; (□), V6-parent; (Δ), V8-parent; (◇), V6-line and (+), V8-line SMC, in various numbers were added to heparinized (5U/ml) rat PRP. (A), height of the ADP-like aggregation peak. (B), coagulation time. Values represent the mean ± S.D. of at least 3 separate experiments.

observed with all the other SMC tested (Fig. 4B). This result indicates a weaker procoagulant activity of the V6-line. Thus, the transformed SMC lines specifically induced ADP-like aggregation of platelets, the two lines did not significantly differ in this proaggregant activity but differed in their procoagulant activities.

For a given cell line and for normal or transformed SMC, both the proaggregant and procoagulant activities depended on the number of SMC added to the platelet suspensions. For both transformed V6- and V8-line cells, the height of the ADP-like aggregation increased with the number of added SMC, up to 2×10^6 cells per assay, and decreased beyond (Fig. 4A). The coagulation time decreased with the number of added SMC (Fig. 4B).

3.3. PRP responses to supernatants of SMC suspensions (Fig.5)

In order to define if the SMC-induced PRP activations were due to soluble factors produced by the SMC or to membrane-linked activities, supernatants of SMC suspensions were prepared and tested on platelet aggregation. Supernatants of the

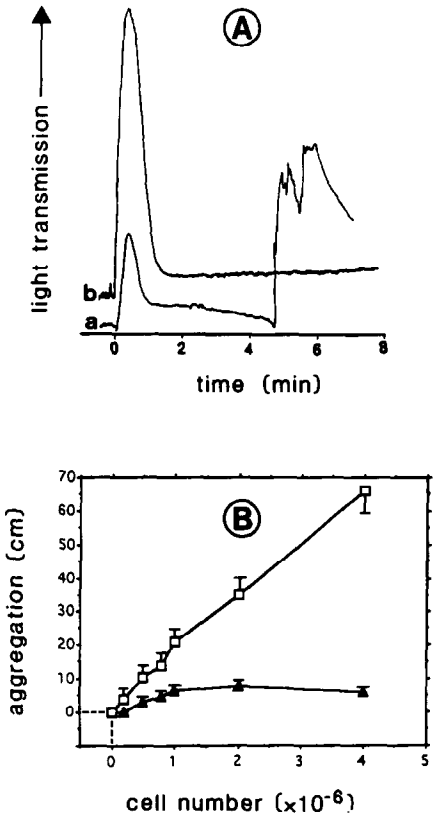

Fig. 5. Induction of rat platelet aggregation by suspensions or supernatants of suspensions of transformed V8-line SMC. (A), Representative platelet aggregation curves induced by (a), 10^6 V8-line SMC suspension or (b), its supernatant. Tracings are representative of 3 experiments with similar results. (B), height of ADP-like aggregations in function of the number of cells of the V8-line SMC suspension: (▲) suspensions or (□) supernatants of the cell suspension. Values represent the mean ± S.D. of at least 3 separate experiments.

transformed V6 and V8-line cell suspensions induced platelet aggregations of the same type as the cell suspensions (Fig. 5A). On the contrary, the supernatants alone did not induce plasma coagulation, even when tested over a 1 h period of time. No PRP response, either platelet aggregation or plasma coagulation, was observed with supernatants of normal SMC (not shown). Thus, the pro-aggregant activity observed with the transformed SMC suspensions can be reproduced by their supernatants, suggesting that this aggregation did not depend on the presence of SMC. These data indicate that the transformed SMC-induced platelet aggregation was due to a factor released by the SMC. On the other hand, the coagulation depended on a factor present on the SMC membrane, either normal or transformed.

Table 1
Effect of inhibitors on SMC-induced platelet aggregation and plasma coagulation

Inhibitor	ADP-like aggregation	Coagulation time
EDTA (mM)		
0	100	100
1	86.6 ± 5.4	
2	46.6 ± 3.2**	>1000**
4	0.0 ± 0.0**	>1000**
Apyrase (μg/ml)		
0	100	100
40	42.6 ± 7.4**	
125	6.8 ± 1.9**	124.1 ± 10.4*
400	0.0 ± 0.0**	
Iodo-acetate (mM)		
0	100	
25	0.0 ± 0.0**	ND
Hirudin (U/ml)		
0	100	100
5	100.2 ± 0.7	116.1 ± 27.5
25	100.8 ± 0.6	258.8 ± 34.6*
100	101.7 ± 5.5	>1000**
PLA2 (U/ml)		
0	100	100
10	88.1 ± 8.0	>1000**
PLD (U/ml)		
0	100	100
10	91.8 ± 5.6	186.8 ± 73.1

EDTA, ethylene-diamine tetra acetic acid; PLD, phospholipase D; PLA_2 phospholipase A_2. The effect of various inhibitors was tested on the amplitude of ADP-like aggregation (height of the peak) and on the coagulation time after addition of 2×10^6 V8-line SMC to heparinized rat PRP. Results are expressed in % of amplitude of the control aggregation (height of the peak) or % of control coagulation time without inhibitor, values are mean ± S.D.
*$P < 0.05$ and **$P < 0.001$ vs. assays without inhibitor.

The amplitudes of aggregation obtained with supernatants of transformed SMC were significantly higher than those obtained with the corresponding cell suspensions (Fig. 5B). The difference between the two curves increased with the cell number, the dose-effect relation being linear for the supernatants but not for the cell suspensions. Addition of particulate suspensions of cells did itself cause a negative shift in the baseline because of a reduction in light transmission, thus, at least partly, masking a variation in light transmission due to platelet aggregation.

3.4. Characterisation with inhibitors (Table 1)

The nature of the PRP responses to addition of transformed SMC was further characterized by the use of inhibitors. Table 1 shows the results obtained with V8-line cells; similar results were observed with V6-line cells (not shown). EDTA reduced the amplitude of the ADP-like aggregation and delayed the coagulation induced by transformed SMC. Dependence on calcium ions was also indicated by the weak response in citrated plasma (not shown). Apyrase inhibited the ADP-like aggregation, as well as control ADP-induced aggregations, but did not significantly affect the coagulation time. Incubation of PRP with hirudin did not affect the platelet aggregation but lengthened the coagulation time up to a complete inhibition of coagulation at high dose (100 U/ml). Concerning the responses with phospholipases, their effect was tested only at the concentration previously used to discriminate different types of tumor cell-induced platelet aggregations [30]. For the dose tested, phospholipase A2 (10 U/ml) was able to completely inhibit the coagulation but did not significantly alter the ADP-like aggregation, whereas phospholipase D (10 U/ml) did not significantly alter the ADP-aggregation nor the co-

agulation. Preincubation of the SMC suspensions with iodoacetate completely inhibited the SMC-induced aggregation, suggesting a metabolic control of the ADP released by the transformed SMC rather than a non-specific leakage. This latter hypothesis can be ruled out based on the results of cell viability tests. In summary, the SMC-induced aggregation of platelets was inhibited by EDTA, apyrase and iodoacetate and thus was ascribable to ADP released by the transformed SMC. The coagulation was inhibited by EDTA, hirudin and phospholipase A2 (10 U/ml) indicating thrombin generation through activity of the SMC membrane tissue factor.

4. Discussion

This study was designed to examine the effect of transformed arterial SMC on the platelet function. The results indicate that transformed rat SMC induce rat platelet aggregation in a potent manner through a mechanism of ADP release by the SMC.

The previously described V6- and V8-SMC lines [24] and their normal parental counterparts, V6- and V8-parents, were tested for their effect on rat platelet aggregation. V6- and V8-SMC are transformed lines of rat arterial smooth muscle origin. They exhibit different morphologies, a cobblestone pattern for V8 and an elongated shape for V6; both are highly tumorigenic in nude mice. The rat PRP response to addition of a suspension of either transformed V6- or V8-SMC, was biphasic, with a first ADP-like platelet aggregation followed, after a lag period, by plasma coagulation. This type of PRP response was also obtained with transformed SMC established from tumors induced in nude mice after V6- or V8-line cells injections (not shown). By comparison, normal SMC, either V6- or V8-parent SMC or other control rat arterial SMC, induced coagulation only. These effects were specific since amplitude of the platelet aggregation and coagulation time depended on the number of SMC added to the aggregation system. The transformed SMC-induced platelet aggregation was reversible in our experimental conditions as was typical ADP-induced aggregation of platelets in heparinized PRP of rat [35]. ADP-induced aggregation of platelets presents species specificities such as reversibility for rat [36] unlike human or rabbit, but we chose to work with an homologous rat system. V6- and V8-lines did not significantly differ in inducing platelet aggregations. They only differ in their abilities to induce coagulation, the V6-line cells exhibiting a much weaker activity than the V8-line cells which behave similarly to normal SMC. Platelet aggregating activity is a property shared by numerous transformed cells of various tissues [25–28]. On the other hand, procoagulant activity is expressed by some tumor cells [27]. It is also an ability of stimulated vascular cells, endothelial or SMC, but not necessarily transformed [37,38], and is due to generation of thrombin through activation of the extrinsic coagulation pathway after induction of tissue factor expression [39].

The induction of ADP-like platelet aggregation, characteristic of the transformed V6- or V8-cell suspensions, was reproduced by supernatants of the cell suspensions and was abolished in PPP. The platelet aggregation was induced by a soluble factor. This aggregation was dose-dependently inhibited by apyrase and not affected by hirudin. This pattern is consistent with an ADP-induced aggregation [30]. The aggregation was abolished by incubating the transformed V6- and V8-SMC suspensions with iodoacetic acid and was due to ADP released by the transformed SMC themselves. The ADP release was the result of a metabolic control rather than of a non-specific leakage with cell lysis after cell harvesting. Viability of the cell suspensions was greater than 90% throughout the whole experiments, as evidenced by both trypan blue dye exclusion and LPH release measurements. Moreover, iodoacetic acid might be expected to increase a non-specific leakage [31]. Coagulation was observed with all SMC, even if some variations between the cell lines were observed in the coagulation time. Coagulation, characterized by its occurrence in PPP, was not induced by supernatants of SMC suspensions but only by suspensions of cells. It was inhibited by EPTA, hirudin and phospholipase A2. This pattern is consistent with a plasma clotting due to the generation of thrombin through activation of the coagulation cascade by the SMC membrane tissue factor thromboplastin [40]. Requirement of Ca^{2+}

ions for both SMC-induced platelet and plasma responses was demonstrated by their absence in citrated PRP or with EDTA in heparinized PRP, an observation classically reported for various tumor cell-induced platelet aggregations [36,41–43].

Several types of mechanisms have been described for tumor-cell induced platelet aggregation: 1 — generation of thrombin due to activation of clotting factors at various steps of the coagulation cascade: (a) activation of the extrinsic pathway by the action of tissue factor-like activity of the tumor cells, a mechanism described by the vast majority of publications [41,44–47]; two subclasses have been defined based on the presence or absence of sensitivity to phospholipase D [30]; (b) activation of the intrinsic pathway by a protease directly activating factor X into Xa [48,49], with the possible involvement of a release of cysteine-proteinase cathepsin B [50,51], or (c) activation of prothrombin into thrombin by a phospholipid providing a surface for prothrombinase complex formation [52]; 2 — ADP release [42,53–56], from the tumor cells [30,31].

Tissue factor, or thromboplastin, a transmembrane lipoprotein which activates the extrinsic pathway of blood coagulation [40], is the essential cofactor of factor VII. In the damaged vessel wall, tissue factor activity is the main mechanism responsible for the induction of the haemostatic response [57–60]. Its localization in the subendothelium [61] and in the matrix secreted by SMC [62] has been described and it is rapidly induced in arterial smooth muscle after balloon injury [63]. In vitro, intact vascular endothelium and SMC do not express tissue factor coagulant activity while other intact non-vascular cells do [37]. By contrast, perturbed, lysed or detached SMC express a high tissue factor activity compared to detached endothelial cells [38,64]. More recently, it has been shown that tissue factor, not expressed in quiescent SMC cultures, is rapidly induced by stimulation by growth factors or other agonists [39]. The present observations, using cell suspensions, showed that transformed otherwise normal SMC, expressed tissue factor procoagulant activities. In the present system, the SMC were stimulated by the detachment process [37,38]. Tissue factor has been implicated in cardiovascular disease. In human atherosclerotic plaques, in situ hybridization demonstrated high synthesis of tissue factor mRNA both in mesenchymal and macrophage cells, the protein being mostly present in the necrotic core and in foam cell regions of the plaque [65]. Thus, besides its main effect on SMC-induced platelet aggregation, the present in vitro work further underlines the possible importance of the SMC contribution to the procoagulant activity and fibrin formation in the atherosclerotic lesion through tissue factor induction.

Vascular endothelial or SMC cultures selectively release ATP, ADP, AMP and adenosine [66,67], the low basal release being activated by enzymes such as trypsin, thrombin or collagenase [68]. The relative extracellular amounts of ATP and ADP depend on the extracellular metabolism by membrane nucleotidases, ecto-ADPase and ATPase [69]. The suspensions of transformed arterial V6- and V8-SMC contained ADP in an amount compatible with the induction of measurable platelet aggregation. The increased release of ADP in transformed compared to normal SMC suspensions may be due to one or several of the following changes in the transformed cells: (a) an increased intracellular ADP content, (b) a higher efficacy of the nucleotide release, (c) a higher efficacy of the ecto-ATPase, (d) a lower activity of the ecto-ADPase.

This potent ability of transformed SMC to aggregate platelets through a release of ADP may be of pathophysiological relevance for atherosclerosis. Smooth muscle cell transformation is one mechanism that has been hypothesized [11] to account for the monotypism and even monoclonality of the atherosclerotic plaques [12]. It has already been shown in vitro that SMC transformation resulted in modifications of cell lipid metabolism consistent with some lipid features of atherosclerosis [23]. Interestingly, the present in vitro experiments showed that cell transformation resulted for arterial SMC in the expression of a platelet proaggregant activity. A parietal proaggregant activity is consistent with the thrombotic features of atherosclerosis. In addition, ADP has been shown to play an important role in in vivo platelet aggregation [70]. Thrombosis associated with

atherosclerotic lesions could be regulated in part by adenine nucleotide release and metabolism. Thus, the present results suggest a role for the SMC of the plaque as a source of ADP, in addition to the erythrocyte source which may occur during plaque fissuring [2]. Thus antithrombotic therapy may be useful against progression of coronary disease by interfering with the ADP pathway of platelet activation, in addition to the thrombin target. The high ADP release from transformed arterial SMC and its platelet proaggregant consequences could be of interest if a transformation of SMC occurs during atherogenesis.

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