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Treatment with recombinant tissue plasminogen activator (r-TPA) induces neutrophil degranulation in vitro via defined pathways

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ABSTRACT

Thrombolysis is recommended for reperfusion following acute ischemic stroke (AIS), but its effects on stroke-associated injury remain to be clarified. Here, we investigated the effects of recombinant tissue plasminogen activator (r-tPA) on neutrophil pathophysiology in vitro and in a case–control study with AIS patients submitted (n = 60) or not (n = 30) to thrombolysis. Patients underwent radiological and clinical examination as well as blood sampling at admission and after 1, 7 and 90 days. In vitro, 30-min incubation with 0.1–1 mg/ml r-tPA induced neutrophil degranulation in different substrate cultures. Pre-incubation with kinase inhibitors and Western blot documented that degranulation was associated with activation of PI3K/Akt and ERK1/2 pathways in Teflon dishes and PI3K/Akt in polystyrene. In thrombolysed patients, a peak of neutrophil degranulation products (matrix metalloproteinase [MMP]-9, MMP-8, neutrophil elastase and myeloperoxidase), was shown during the first hours from drug administration. This was accompanied by serum augmentation of protective tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. An increased rate of haemorrhagic transformations on day 1 after AIS was shown in thrombolysed patients as compared to non-thrombolysed controls. In conclusion, r-tPA treatment was associated with in vitro neutrophil degranulation, indicating these cells as potential determinants in early haemorrhagic complications after thrombolysis in AIS patients.

1. Introduction

The degranulation of neutrophil toxic products has been not only shown as a useful defense mechanism against bacterial infections, but also as a detrimental response in both acute and chronic diseases. In fact, the excessive and prolonged release of neutrophil proteases might per se injure tissues and worsen disease severity [1]. Different neutrophil products, especially matrix metalloproteinases ([MMPs],

Abbreviations: AIS, acute ischemic stroke; ASPECTS, Alberta Stroke Program Early CT Score; ECASS, European Cooperative Acute Stroke Study; ELISA, enzyme-linked immunosorbent assays; HDL, high density lipoprotein; HI, hemorrhagic infarction; hsCRP, high sensitivity C-reactive protein; HT, hemorrhagic transformation; MMP, matrix metalloproteinase; MPO, myeloperoxidase; mRS, modified ranking score; NE, neutrophil elastase; NCCT, non-contrast cranial computed tomography; NIHSS, National Institutes of Health Stroke Scale; PH, parenchymal hemorrhage; PMA, phorbol-12-myristate-13-acetate; r-tPA, recombinant tissue plasminogen activator; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; TOAST, Trial of ORG 10172 in Acute Stroke Treatment.

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such as MMP-9 and MMP-8), neutrophil elastase (NE), and myeloperoxidase (MPO), are stored in different neutrophil granules prone to be released on demand [2]. Once released and activated, such enzymes are able to digest collagen and other matrix proteins, thus promoting structural and functional impairment of inflamed tissues. The unbalance between neutrophil granule products and endogenous inhibitors (such as the tissue inhibitors of metalloproteinase [TIMPs]) may lead to irreversible injuries and increase disability, as largely reported for rheumatoid arthritis [3], atherosclerosis [4] and myocardial infarction [5]. Concerning acute ischemic stroke (AIS), the potential detrimental role of neutrophils is less proven. A large amount of non-specific neutrophil products were shown to be involved in neurovascular injury following experimental AIS [6–11]. For instance, serum levels of MMP-9 were suggested as predictors of post-stroke poor functional outcome in human beings [12–16]. However, the cellular source of this gelatinase in the early phases after AIS remains to be identified. On the other hand, incubation with recombinant tissue plasminogen activator ([r-tPA], the drug approved for the early re-establishment of cerebral reperfusion after ischemia) [17,18] was associated with in vitro neutrophil degranulation [19,20]. However, in vitro mechanistic insights on r-tPA-triggered intracellular pathways and the potential clinical relevance of r-tPA-induced neutrophil degranulation in AIS patients remain unexplored. Among different early complications associated with thrombolysis in which neutrophil degranulation might play a pathophysiological role, we focused on AIS hemorrhagic transformations [21,22], which range from of 1.7% to 13% of thrombolysed patients [23,24]. In addition, this translational study aims at investigating the potential degranulating activity of r-tPA and downstream activation of intracellular pathways in vitro on human neutrophils cultured on different substrate cultures. Finally, we performed a set of clinical observations in a case–control study with AIS patients in order to verify if thrombolysis was associated with an early serum peak of neutrophil products and accompanied by an increased rate of complications.

2. Methods

2.1. Human primary neutrophil isolation and culture

Neutrophils were obtained from 10 healthy volunteers after informed consent. The local ethical committee approved the investigation protocol that was conformed to the principles outlined in the Declaration of Helsinki. Human neutrophils were isolated from heparinized venous blood by dextran sedimentation followed by centrifugation on Ficoll-Hypaque (from Cedarlane Laboratories Ltd. [Ontario, Canada]) density gradient, as previously described [25]. The neutrophil isolation protocol was completed in 2 h from the blood sampling. Neutrophils resuspended in culture medium (serum-free RPMI 1640 medium containing 25 mmol/l Hepes) were >97% pure, as determined by morphologic analysis of Giemsa-stained cytopreparations (from Merck [Darmstadt, Germany]). Then, human neutrophils (5×10^5 cells per well) were cultured in the presence or absence of 10 ng/ml phorbol-12-myristate-13-acetate (PMA, positive control from Sigma-Aldrich, Buchs, Switzerland) [26], or different doses (up to 1 mg/ml) of r-tPA (Boehringer, Ingelheim, Germany) [19] for 30 min at 37 °C in a humidified atmosphere 5% CO_2 in Teflon dishes (to maintain cell suspension mimicking circulating conditions and to avoid any potential adhesion) or in adherence to polystyrene plates. In selective experiments, cells were 30 min pre-incubated in the presence or absence of different concentrations of intracellular kinase inhibitors LY294002 [PI3K inhibitor: 0.1, 1 and 10 μM from Sigma], U0126 [MEK inhibitor, 0.1, 1, 10 μM from Biomol Research Laboratories, Inc., Plymouth Meeting, PA], and SB203580 [p38 MAPK inhibitor, 0.1, 1, 1 μM from Biomol Research Laboratories, Inc.], and then, stimulated in the presence or absence of 0.1 mg/ml r-tPA.

2.2. Neutrophil product measurements in serum and neutrophil supernatants

Levels of MMP-9, MMP-8, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, MMP-9/TIMP-1 complex, MPO (all from R&D Systems, Minneapolis, Minnesota, USA) and NE (from eBioscience, Vienna, Austria) released in serum and cell supernatants were measured by colorimetric enzyme-linked immunosorbent assays (ELISA), following manufacturer's instructions. The limits of detection were 0.312 ng/ml for MMP-9, 0.156 ng/ml for MMP-8, 31.25 pg/ml for TIMP-1, 31.25 pg/ml for TIMP-2, 46.9 pg/ml MMP-9/TIMP-1 complex, 0.156 ng/ml for MPO, and 0.156 ng/ml for NE. Mean intra- and inter-assay coefficients of variation were below 6%.

2.3. Pro-MMP-9 zymographic assay

Pro-MMP-9 zymographic activity was assessed in human serum and cell supernatants. 9% SDS–polyacrylamide gels were copolymerized with gelatin (Sigma, St. Louis, MO). Equal amounts of serum (2 μl), cell supernatants (10 μl) and 1 ng of recombinant pro-MMP-9 standard (Calbiochem, Lucern, Switzerland) were loaded on gels in the absence of reducing agents. Then, gels were rinsed and stained with Coomassie Blue R-250. Zymographic results were expressed as pro-MMP-9 proteolytic activity and calculated on the basis of the following formula: Serum/supernatant pro-MMP-9 $= (I_{obs}/I_{std}) \times W_{std}$, where I_{obs} and I_{std} are intensities of lytic areas produced in gels by samples and by standard pro-MMP-9, and W_{std} is the weight (1 ng) of standard pro-MMP-9 loaded onto the gel. Zymographic data were expressed as ng/ml of serum/supernatants. Gelatinolytic bands were measured with a gel analysis system (GeneGenius, Syngene, Cambridge, UK).

2.4. Western blot analysis

Freshly isolated human neutrophils (1×10^7 cells/ml) from 3 healthy donors were lysed in 400 μl of Nonidet P40 buffer (20 mM Tris–HCl pH 7.5, 0.15 M NaCl, 10 mM NaF, 1% Nonidet P40, 10 μg/ml glycerol, 1 mM phenylmethanesulphonyl-fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM Na_3VO_4). In parallel experiments, neutrophils (from five different healthy donors) were incubated in Teflon dishes at 37 °C in a humidified atmosphere 5% CO_2 for different times (up to 30 min) in the presence or absence of culture medium, tumor necrosis factor (TNF)-α (positive control for kinase phosphorylation at 200 U/ml for 7 min, from R&D Systems) [27], or 0.1 mg/ml r-tPA (for 1, 5, 15 or 30 min). The incubations were stopped on ice and the cells were centrifuged at 4 °C. After removing supernatants, the pellets were lysed in 400 μl of Nonidet P40 buffer. Equal amounts of protein (50 μg for RANK and 20 μg for intracellular kinases) for each sample were boiled in loading buffer (62.5 mM Tris–HCl pH 6.8, 0.75% SDS, 3.75% 2-mercaptoethanol, 8.75% glycerol and 0.025% bromophenol blue) and resolved by 10% SDS–polyacrylamide electrophoresis. Then, proteins were transferred on nitrocellulose membrane at 4 °C for 45 min. After blocking 1 h in 5% non-fat dry milk and washing with Tris-buffered saline/Tween 20 (10 mM Tris-base pH 7.4, 154 mM NaCl and 0.05% Tween 20), membranes were incubated with appropriate dilution of anti-phospho-Akt (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-ERK1/2 (R&D Systems), anti-phospho p38 MAPK (R&D Systems) primary Abs, as well as corresponding secondary Abs. Blots were developed using the ECL system (Immobilion Western, Millipore, USA). Membranes were then stripped, reblocked and reprobed to detect total intracellular kinases (using anti-Akt [Santa Cruz Biotechnology], anti-ERK 1/2 [R&D Systems], or anti-p38 MAPK [Santa Cruz Biotechnology] Abs). Immunoblots were scanned and quantifications was carried out by Image Quant software version 3.3 (Molecular Dynamics, Sunnyvale, USA). Values of phospho-Akt, phospho-ERK 1/2, and phospho-p38 MAPK (obtained in three different experiments) were normalized to corresponding total amounts of Akt,

ERK 1/2 and p38 MAPK and expressed as percentages of control medium (defined as 100%).

2.5. Patients and clinical assessment

From April 2009 and December 2011, we conducted a prospective, case–control study enrolling consecutive patients admitted at Neurology Department of Ferrara University Hospital with diagnosis of first AIS treated according to the current recommended guidelines [28]. No blinding was done in this study. AIS was defined as an acute-onset focal neurological deficit combined with neuroimaging evidence of cerebral infarction [29]. Cases (n = 60) were defined as consecutive r-tPA-treated (intravenous or intra-arterial) patients admitted for AIS within 6 h from symptom onset. Patients admitted during the same period, but not submitted to thrombolysis were selected as controls (n = 30). Primary exclusion criteria were: primary hemorrhagic stroke, seizure, intracranial abscess or brain tumor, acute infection, recent (<30 days) myocardial infarction, malignancy and renal/hepatic failure. Instead, control patients were excluded from thrombolytic treatment for the following reasons: minor or rapidly improving stroke symptoms (n = 11); history of previous intracranial hemorrhage (n = 4); significant head trauma in previous 3 months (n = 4); elevated blood pressure (systolic > 185 mm Hg or diastolic > 110 mm Hg) (n = 3); active internal bleeding (n = 3); major surgery within previous 30 days (n = 3); admission after 4.5 h from onset of symptoms, in presence of a hypodensity > 1/3 cerebral hemisphere on non-contrast cranial computed tomography (NCCT) (n = 2). The study was approved by the Local Ethics Committee of Ferrara University Hospital and performed in accordance to the guidelines in the Declaration of Helsinki. The patients and their relatives gave informed consent prior to entering in the study. Patients were submitted to serial blood sample collection at baseline (time 0: within 7 h from symptom onset and 1 h from thrombolysis), 1, 7 and 90 days after stroke onset.

All patients were also categorized for stroke subtypes according to the TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria in: i) large-artery atherosclerosis, ii) cardioembolism, iii) small-vessel occlusion, iv) stroke of other determined etiology, or v) stroke of undetermined etiology [30]. Disease severity was assessed at onset and then 1, 7 and 90 days after stroke symptom occurrence by using the National Institutes of Health Stroke Scale (NIHSS) [31]. A NIHSS <5 defined a minor stroke [32] whereas NIHSS >15 identified severe/very severe stroke. Finally, clinical outcome at day 90 was measured by the modified Rankin scale (mRS) [33]. As previously described [34], mRS ≤2 and >2 were defined as good and poor outcomes, respectively.

2.6. Power study estimation

Although this is a "pilot" study, we calculated the study power according to previous studies [15,18]. MMP-9 was selected as reference parameter because it was the only neutrophil-related (though not exclusive) molecule so far investigated in thrombolysed AIS patients. Accordingly, our simple size allowed us to estimate large differences (effect size > 0.80) of baseline MMP-9 between treated and non-treated groups, with power of 95% and a two-sided alpha error of 5%.

2.7. Neuroimaging

NCCT was performed at onset and then 1, 7 and 90 days after stroke on a 64-slice Lightspeed VCT (GE Medical System, Milwaukee, WI; USA) from the skull base to the vertex by using an axial technique with the following imaging parameters: 120 kVp, 350 mA, 512 × 512 matrix, 25 cm-DFOV, 4 × 5-mm collimation, 1 s/rotation and table speed of 15 mm/rotation. All NCCT images were acquired along the orbito-meteal plane with 2.5-mm (8 images/rotation) and 5-mm (4-images/rotation) slice thickness reconstruction for posterior fossa and supra-tentorial region, respectively.

The extension of early ischemic changes (hypoattenuation, loss of the gray–white matter boundary and effacement of cortical sulci) was evaluated on NCCT at onset by Alberta Stroke Program Early CT Score (ASPECTS), a 10-point scale that rates the presence or absence of ischemia in 10 regions included in the middle cerebral artery territory assigning a score of 1 for normal and 0 for a region showing early ischemic signs [35]. ASPECTS was then dichotomized into >7 (minor stroke) and ≤7 (major stroke) [36]. As reported elsewhere, ischemic volume was calculated on NCCT on days 1, 7 and 90 after symptom onset with a multi-slice planimetric method by summation of the hypodense areas, manually traced on each slice in which they were detectable, multiplied by slice thickness [37]. The lesion volume obtained at 3 months was considered the final infarct size.

On NCCT, also the occurrence of hemorrhagic transformation (HT) was recognized on days 1 and 7 after stroke onset. Furthermore, four different categories of HT were identified according to the ECASS (European Cooperative Acute Stroke Study) II criteria [34]: i) hemorrhagic infarction type 1 (HI-1) defined as small petechiae along the margins of the infarct; ii) HI type 2 (HI-2) defined as more confluent petechiae within the infarcted area but without a space-occupying effect; iii) parenchymal hemorrhage type 1 (PH-1) defined as hematoma in ≤30% of the infarcted area but with some slight space-occupying effect; iv) PH type 2 (PH-2) defined as dense hematoma >30% of the infarcted area with substantial space-occupying effect or as any hemorrhagic lesion outside the infarcted area.

2.8. Blood collection and quantification

Blood samples were collected using a butterfly to reduce membrane shear stress to obtain serum. In cases, the first sample (time 0) was obtained within 1 h from the beginning of thrombolysis and within 7 h from symptom onset. In controls, the first sample (time 0) was obtained within 1 h from admission and within 9 h from symptom onset. Then, in both cases and controls, additional time points of blood collection were at 1, 7 and 90 days after stroke onset. Hematology parameters, blood chemistry including plasma glucose, triglycerides, total cholesterol, high-density lipoprotein, low-density lipoprotein cholesterol as well as high-sensitive C-reactive protein (hsCRP) were measured by routine autoanalyzer at different time points.

2.9. Statistical analysis

Patient characteristics were described at admission. Qualitative data were presented as absolute and relative frequencies and then compared with Pearson χ^2 test or Fisher's exact test when appropriate. Continuous variables were presented as median (interquartile range) and their comparison was performed by non-parametric Mann–Whitney *U* test (the normality assumption of the variables' distribution in both groups was violated). Intergroup comparison of marker serum levels at different time points was analyzed by Wilcoxon text. A 2-sided *p*-value <0.05 was considered statistically significant. Analyses were performed with IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.).

3. Results

3.1. Stimulation with the thrombolytic drug r-tPA promotes in vitro degranulation of human neutrophils both cultured in suspension and adherence patterns

To establish whether incubation with r-tPA induced neutrophil degranulation in different conditions, we tested two validated models of neutrophil culture using Teflon and polystyrene dishes

(mimicking circulating and adherent neutrophil patterns, respectively) [26].

In both cell culture models, incubation with r-tPA induced neutrophil degranulation of MMP-9 and increased pro-MMP-9 gelatinolytic activity in a dose-dependent manner (Fig. 1A–D). r-tPA concentration of 0.1–1 mg/ml was also shown to promote a significant release of MMP-8 and MPO (Fig. 1E and F), further suggesting a more specific role as a neutrophil degranulating factor for this drug. Concerning TIMPs, TIMP-2 raised in supernatants of both culture substrates in the presence of the positive control (PMA) but not r-tPA (Fig. 2). TIMP-1 was below the low range limit of detection (31.25 pg/ml), as previously reported [19].

3.2. Neutrophil degranulation induced by r-tPA is dependent on the activation of different intracellular pathways

In order to identify the potential signaling pathways regulating r-tPA-mediated effects, we focused on intracellular kinases recently

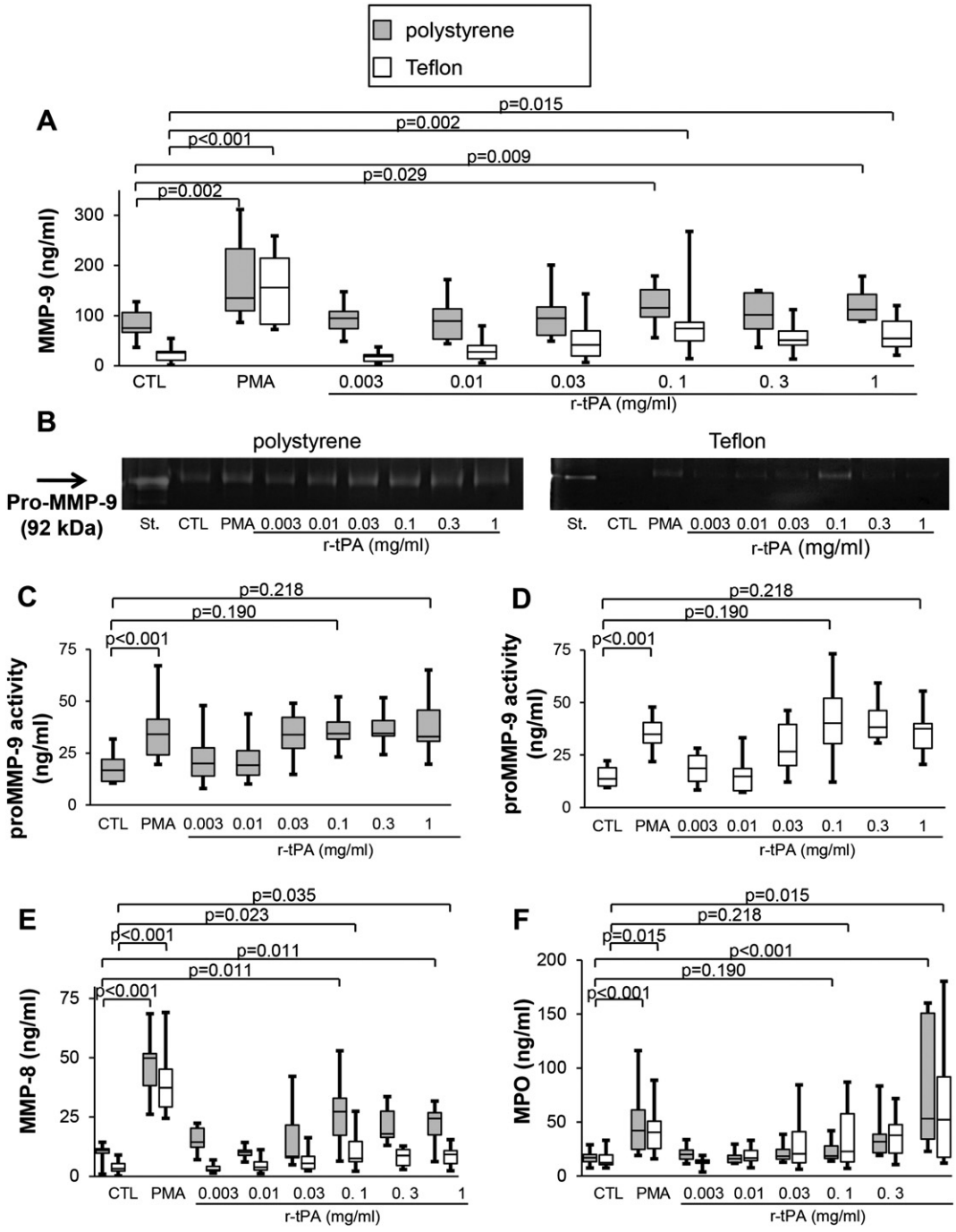

Fig. 1. r-tPA induces degranulation in human neutrophils cultured in polystyrene and Teflon dishes. A. MMP-9 release in supernatants of cells incubated in polystyrene (gray bars) or Teflon (white bars) dishes in the presence or absence of control medium (CTL), 10 ng/ml PMA (positive control), and different concentrations of r-tPA. B. Representative gels of MMP-9 zymography of 10 different experiments performed in polystyrene or Teflon dishes. White band (arrow) on the gel represents the pro-MMP-9 gelatinolytic activity of standard recombinant pro-MMP-9 (St.), and supernatants of cells treated with control medium (CTL) and with increasing concentrations of r-tPA. C–D. Results of densitometric quantifications of 10 different experiments performed in polystyrene (C) or Teflon (D) dishes. Data are expressed as median (interquartile range). E–F. Release of MMP-8 and MPO in supernatants of cells incubated in polystyrene (gray bars) or Teflon (white bars) dishes in the presence or absence of control medium (CTL), 10 ng/ml PMA (positive control), and different concentrations of r-tPA. Data are expressed as median (interquartile range), n = 10.

shown to participate to neutrophil degranulation [26]. Inhibition of PI3K by LY294002 significantly decreased r-tPA-induced degranulation of MMP-9 only in Teflon dishes (Fig. 3A and B), whereas a reduced MMP-8 release was exclusively observed in polystyrene substrate (Fig. 3C and D). Conversely, the release of MPO induced by r-tPA was independent of PI3K inhibition (Fig. 3E and F). Accordingly, Akt phosphorylation increased very early in the presence of 0.1 mg/ml r-tPA. This effect was observed within a few minutes (10–15 min), then prior to the time due for neutrophil degranulation in vitro (30 min) (Fig. 3G and H).

Pre-incubation with the MEK1/2 inhibitor U0126 dose-dependently abrogated cell degranulation of MMP-9, MMP-8 and MPO in Teflon, but not in polystyrene (Fig. 4A–F). Consistent with these results, an early increase of ERK1/2 phosphorylation was shown in both culture conditions within 15 min from r-tPA administration (Fig. 4G and H).

Finally, pre-incubation with the p38 MAPK inhibitor SB203580 was ineffective on r-tPA-induced degranulation except for a significant decrease of MPO degranulation on Teflon substrate (Fig. 5A–F). On the contrary, a significant enhancement of p38 MAPK phosphorylation was induced by r-tPA in both culture models (Fig. 7G and H).

These results suggest that r-tPA-induced neutrophil degranulation is partially dependent on activation of Akt in both culture substrates and exclusively mediated by ERK1/2 in Teflon. Conversely, despite r-tPA-induced activation of p38 MAPK, the inhibition of this pathway does not affect degranulation.

3.3. Clinical features of study population

At baseline, AIS patients treated with thrombolysis were similar for gender, age, comorbidities and medications as compared to non-thrombolysed controls. Among biochemical parameters, a weak reduction in total cholesterol, high density lipoprotein (HDL)-cholesterol and triglycerides was observed in thrombolysed patients as compared to non-thrombolysed controls (Table 1). According to TOAST classification, stroke etiology differed between groups with a higher prevalence of atherothrombotic stroke in the group treated with thrombolysis as compared to untreated controls (31.0% vs. 55.9%; $p = 0.028$) (Table 2).

At baseline, patients treated with thrombolysis were characterized by an increased disease severity according to NIHSS, while the radiological features of stroke according to ASPECTS were similar between the two groups (Table 2). Considering timing of hemorrhagic transformation, an increased number of hemorrhagic events were detected in patients with thrombolysis as compared to controls during the first 24 h, but not later till 7 days after stroke (Table 2). An increased number of type 1 parenchymal hemorrhages (by ECASS classification) was also observed in patients treated with thrombolysis as compared to controls within the first 7 days after stroke onset (Table 2). At 1, 7 and 90 days, no significant differences in stroke severity (assessed by NIHSS) and ischemic lesion volume (determined by CT) were shown between the study groups (Table 2).

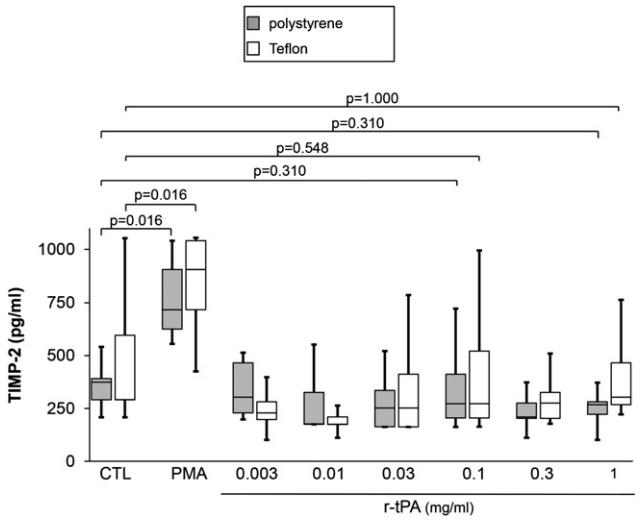

Fig. 2. r-tPA does not affect TIMP-2 levels in human neutrophil supernatants cultured in polystyrene and Teflon dishes. Release of TIMP-2 in supernatants of cells incubated in polystyrene (gray bars) or Teflon (white bars) dishes in the presence or absence of control medium (CTL), 10 ng/ml PMA (positive control), and different concentrations of r-tPA. Data are expressed as median (interquartile range), n = 5.

3.4. Thrombolysis is associated with an early increase in the serum levels of proteinases contained in neutrophil granules

Serum levels of MMP-9 (contained in neutrophil tertiary granules) and pro-MMP-9 gelatinolytic activity were significantly increased within 1 h after thrombolysis (time 0) as compared to patients without thrombolysis at the same time point (Fig. 6A and B). The baseline serum levels of MMP-9 and pro-MMP-9 activity were significantly higher when compared to serum samples from the same patients at 7 and 90 days after stroke onset (Fig. 6A and B). Similar to MMP-9, also MMP-8, NE and MPO (contained in neutrophil secondary [MMP-8] and primary granules [NE and MPO]) [5] were significantly increased at baseline in thrombolysed patients when compared to non-thrombolysed patients (Fig. 6C–E). The serum peak of neutrophil products was transient and significantly reduced at already 1-day follow-up (Fig. 6A–E). Interestingly, the sudden increase in neutrophil degranulation observed immediately after thrombolysis was not timely accompanied by a concomitant increase in the general inflammatory factor (i.e., hs-CRP) (Fig. 6F). A delayed increase on hsCRP (significantly rising at day 1 with a peak at day 7) was observed in the thrombolysis group as compared to baseline levels (Fig. 6F). No difference in hs-CRP serum levels between thrombolysis and non-thrombolysis groups was shown at any time points (Fig. 6F). These results were accompanied by a protective increase in TIMP-1 and TIMP-2 at baseline in thrombolysed patients as compared to untreated controls (Fig. 7A and B). Similar to proteinases, TIMP-2 but not TIMP-1 serum levels were significantly reduced at already 1-day follow-up (Fig. 7A and B). Partially confirming serum patterns of MMPs and TIMPs, the levels of the MMP-9/TIMP-1 complex had an initial weak increase in thrombolysed patients, without reaching the statistical significance ($p = 0.112$) (Fig. 7C). In thrombolysed patients, a significant reduction in serum levels of MMP-9/TIMP-1 complex was shown at 1-, 7- and 90-day follow-up when compared to baseline (Fig. 7C).

4. Discussion

This study shows that the thrombolytic drug r-tPA is an inducer of neutrophil degranulation. The release of neutrophil products was investigated in vitro, in two different models of culture, recently validated by our research group [26]. A significant increase of neutrophil degranulation was observed very early (within 30 min from r-tPA administration) in both Teflon dishes and polystyrene substrates, mimicking circulating and adherent patterns of neutrophil activation, respectively. Interestingly, r-tPA modified the physiological timing of neutrophil degranulation inducing a combined release of MMP-9, MMP-8 and MPO. In fact, together with tertiary granules (containing MMP-9) early released to promote extra-vascular migration, we also observed the degranulation of secondary (rich in collagenases, such as MMP-8) and primary granules (storing MPO and NE) [2].

Then, we investigated molecular mechanisms underlying neutrophil degranulation, especially focusing on intracellular pathways. As compared to the previous work by Cuadrado and co-workers [19], the activation of different intracellular pathways was investigated in cells incubated on adherence and suspension. At a concentration of 0.1 mg/ml, r-tPA significantly increased PI3K/Akt- and ERK1/2

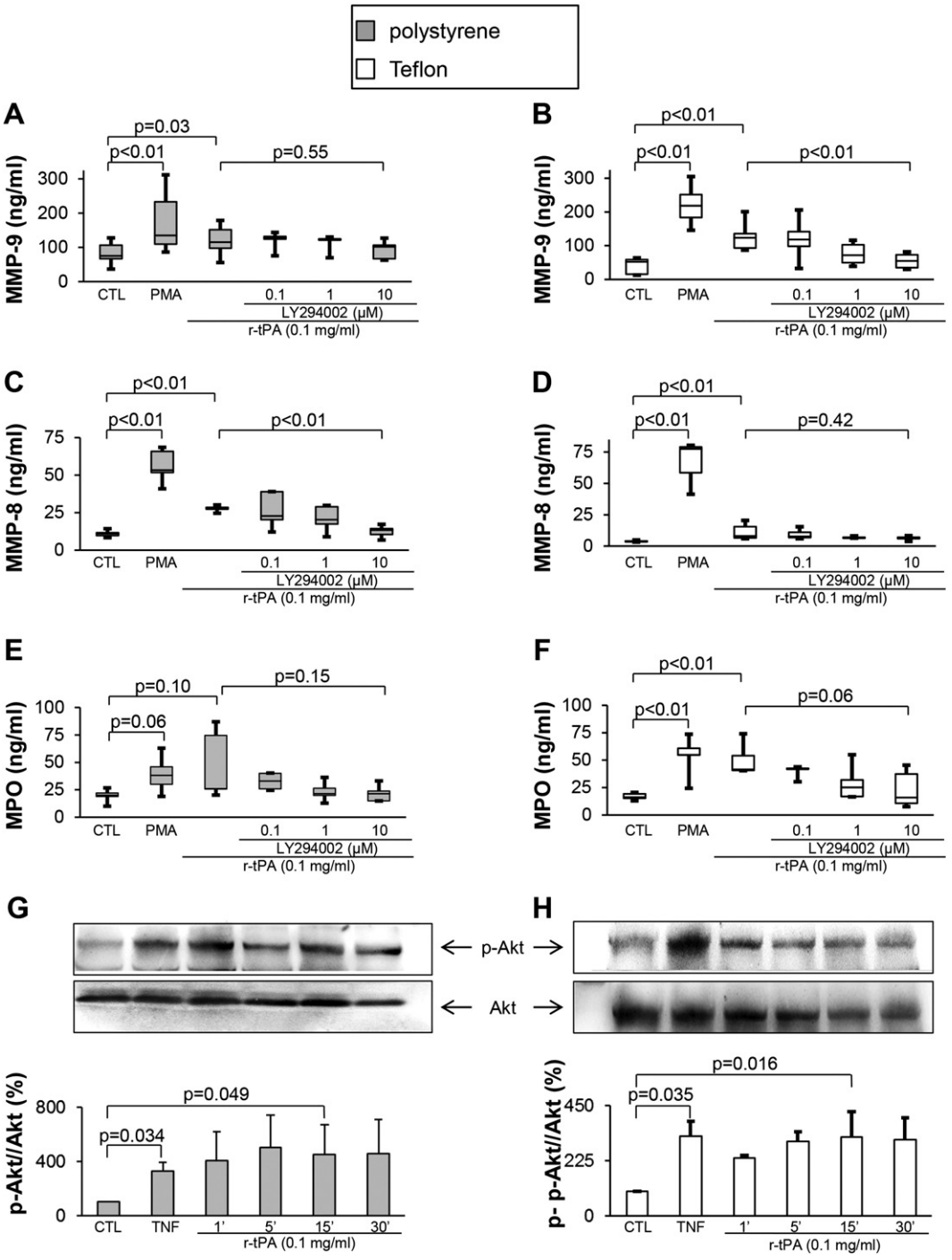

Fig. 3. r-tPA-induced release of neutrophil products is partially reduced by LY294002 (inhibitor of PI3K/Akt pathway). A–F. Neutrophils were 30-min pre-incubated in polystyrene or Teflon dishes in the presence or absence of different concentrations of LY294002 (PI3K inhibitor, 0.1, 1, 10 μM). Then, cells were stimulated with control medium (CTL), PMA (10 ng/ml, positive control) or 0.1 mg/ml r-tPA. Data are expressed as median (interquartile range), n = 5–10. Release of MMP-9 (A, B). Release of MMP-8 (C, D). Release of MPO (E, F). G–H. Representative western blot and densitometric analysis of Akt phosphorylation in the presence or absence of control medium (CTL), 200 U/ml TNF-alpha (for 7 min, positive control), or r-tPA (time course: 1 min, 5 min, 15 min, 30 min, all at 0.1 mg/ml). Values were normalized to total amounts of Akt and expressed as percentage of control medium (CTL), defined as 100%. Data are expressed as mean ± SEM (n = 3–4).

phosphorylation, independent of substrate culture. These effects, occurring within 15 min from r-tPA administration preceded degranulation and were consistent with previous studies supporting the crucial role of MAPK signaling in neutrophil activation [38]. On the contrary, p38 MAPK did not seem to be involved in r-tPA signaling transduction.

The use of PI3K/Akt inhibitor showed partial effects in inhibiting neutrophil degranulation in polystyrene and Teflon cultures, while the inhibition of ERK1/2 pathway abrogated r-tPA-induced neutrophil degranulation exclusively in Teflon cultures. Considering these partial beneficial results in vitro, we believe that more selective inhibitors of neutrophil degranulation or proteinase activity are needed to potentially improve early complications related to thrombolysis.

In the clinical cohort of AIS patients, a potential neutrophil degranulation was observed after thrombolysis. This was suggested by the early

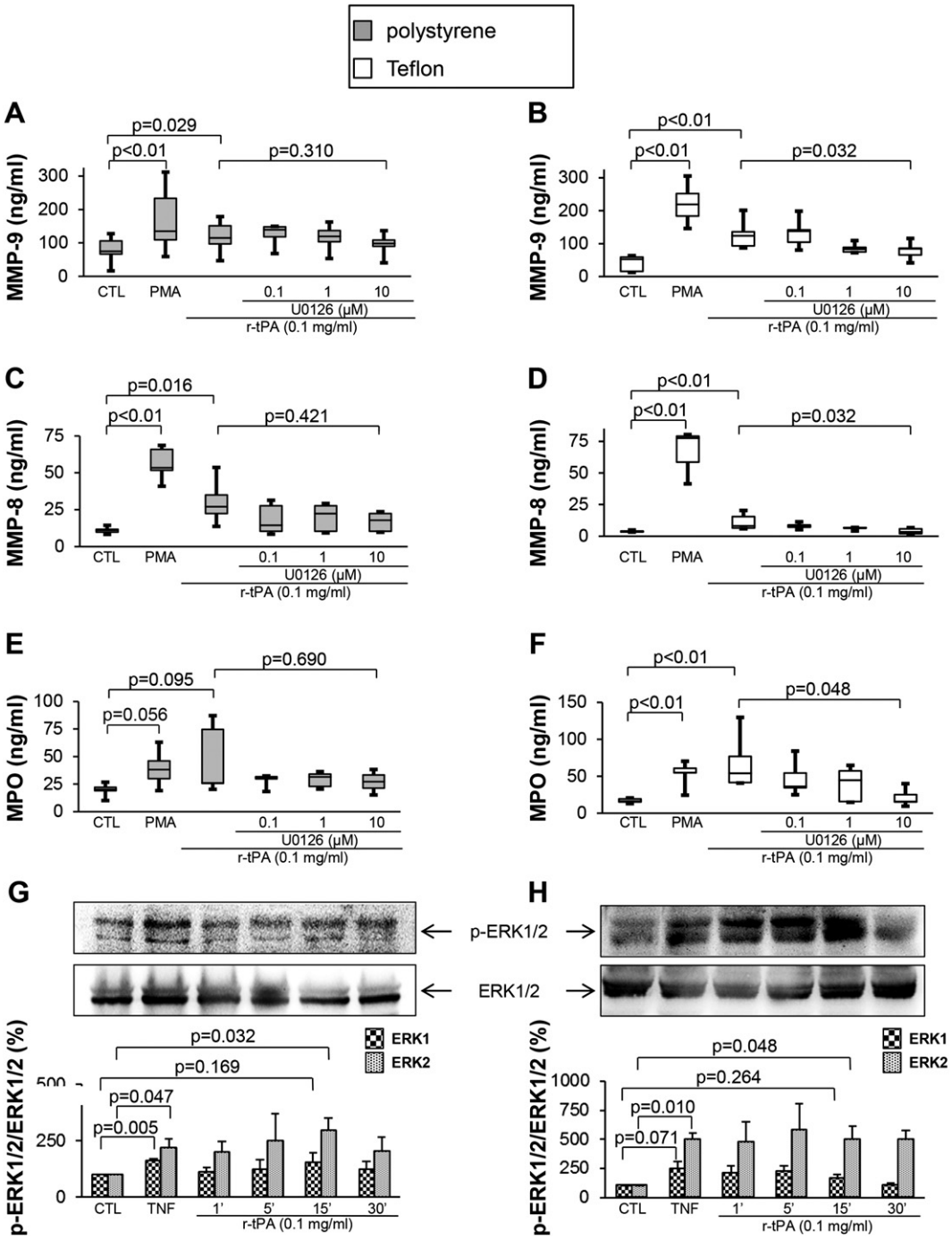

Fig. 4. r-tPA-induced release of neutrophil products in Teflon cultures is abrogated by UO126 (inhibitor of MEK1/2, a kinase directly activating ERK1/2). A–F. Neutrophils were 30-min pre-incubated in polystyrene or Teflon dishes in the presence or absence of different concentrations (0.1, 1, 10 μM) of UO126. Then, cells were stimulated with control medium (CTL) PMA (10 ng/ml, positive control) or 0.1 mg/ml r-tPA. Data are expressed as median (interquartile range), n = 5–10. Release of MMP-9 (A, B). Release of MMP-8 (C, D). Release of MPO (E, F). G–H. Representative Western blot of ERK1/2 phosphorylation in the presence or absence of control medium (CTL), 200 U/ml TNF-alpha (for 7 min, positive control), or r-tPA (time course: 1 min, 5 min, 15 min, 30 min, all at 0.1 mg/ml). Values were normalized to total amounts of Akt and expressed as percentage of control medium (CTL), defined as 100%. Data are expressed as mean ± SEM (n = 3–4).

serum peak of proteases (within 1 h from r-tPA in vivo administration) contained in neutrophil granules. This short-term effect was consistent with in vitro results (30 min of exposure). Although different neutrophil-specific enzymes were concomitantly increased at the same time point (time 0), not all proteases investigated were specific for neutrophil degranulation. For instance, the pathophysiology of MMP-9 is far from being known, also concerning the cellular sources. Microglial cells, vascular cells, astrocytes, neuronal cells and leukocytes may release MMP-9 and other MMPs potentially differing for timing, trigger and intracellular signaling [39]. Increased levels of MMP-9 were already associated with ischemic event and thrombolysis was shown to further enhance this effect [17,18]. Recent studies also emphasized the association between MMP-9 and adverse clinical outcome (increased infarct volume, hemorrhagic transformation and long-term disability) [13,15,40–42], thus raising the interest as a potential therapeutic target [43,44]. However, by combining the release of MMP-9

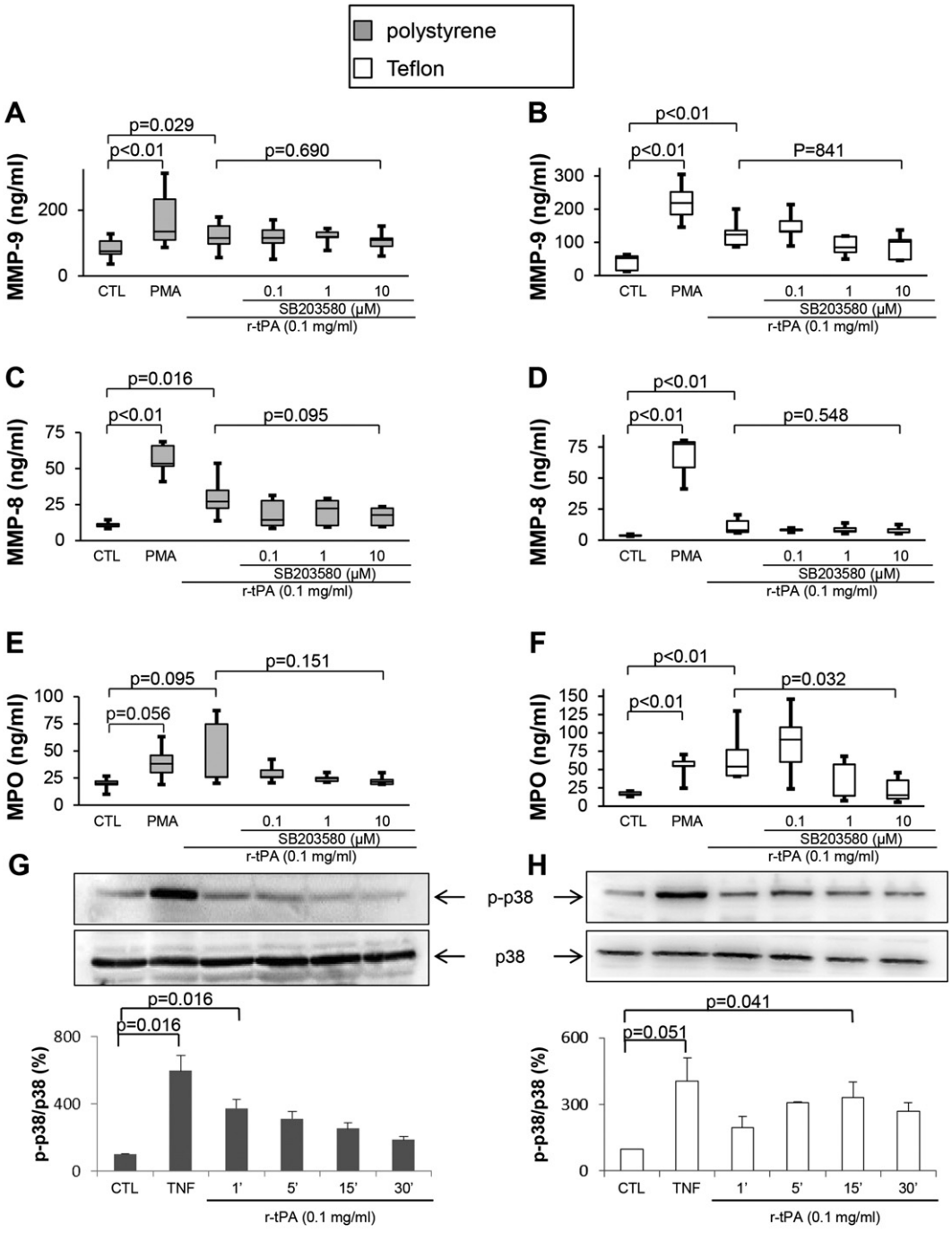

Fig. 5. r-tPA-induced release of MPO in Teflon is reduced by SB203580 (inhibitor of p38 MAPK pathway). A–F. Neutrophils were 30-min pre-incubated in polystyrene or Teflon dishes in the presence or absence of different concentrations (0.1, 1, 10 μM) of SB203580. Then, cells were stimulated with control medium (CTL) PMA (10 ng/ml, positive control) or 0.1 mg/ml r-tPA. Data are expressed as median (interquartile range), n = 5–10. Release of MMP-9 (A, B). Release of MMP-8 (C, D). Release of MPO (E, F). G–H. Representative Western blot of p38 MAPK phosphorylation in the presence or absence of control medium (CTL), 200 U/ml TNF-alpha (for 7 min, positive control), or r-tPA (time course: 1 min, 5 min, 15 min, 30 min, all at 0.1 mg/ml). Values were normalized to total amounts of Akt and expressed as percentage of control medium (CTL), defined as 100%. Data are expressed as mean ± SEM (n = 3–4).

with the specific neutrophil products MPO and NE, we speculated that the increase circulating levels of proteases might be originated from the activation of these cells in response to thrombolysis.

As additional finding, an increase of protective TIMPs was observed in our cohort concomitantly to the peaks on neutrophil degranulation, thus representing a potential beneficial response counteracting neutrophil proteases. Whether r-tPA has a direct role on this protective increase in TIMP-1 and TIMP-2 levels remains to be elucidated. Neutrophils were partially investigated as a potential source of TIMPs but differently from Cuadrado and colleagues [19], we failed in detecting any significant increase of TIMP-2 levels in supernatants of neutrophil treated with r-tPA. TIMP-2 was detected at very low concentration (in the range of pg/ml, 1000-fold less than other neutrophil enzymes), whereas TIMP-1 was confirmed at very low concentrations (below 31.25 pg/ml) in all neutrophil supernatants. Considering these findings and the higher concentration of TIMPs detected in serum of

Table 1
Clinical characteristics and medications of study population.

	Non-thrombolysis (n = 30)	Thrombolysis (n = 60)	*p*-Value
Demographic			
Age, yr. (IQR)	67 (56–76)	68 (55–75)	0.758
Males, no. (%)	14 (46.7)	37 (61.7)	0.186
Systolic blood pressure, mm Hg (IQR)	140 (130–180)	142 (130–150)	0.213
Diastolic blood pressure, mm Hg (IQR)	80 (80–100)	80 (70–90)	0.064
Hypertension, no. (%)	20 (66.7)	34 (56.7)	0.494
Current smoking, no. (%)	9 (31.0)	21 (35.0)	0.813
Previous smoking, no. (%)	5 (16.7)	6 (10.0)	0.496
Alcohol abuse, no. (%)	1 (3.3)	1 (1.7)	1.000
Type 2 diabetes, no. (%)	3 (10.0)	9 (15.0)	0.744
Dyslipidemia, no. (%)	9 (30.0)	12 (20.0)	0.303
Chronic CAD[a], no. (%)	2 (6.7)	6 (10.0)	1.000
Liver diseases, no. (%)	2 (6.7)	3 (5.0)	1.000
Medications			
RAAS[b] inhibitors, no. (%)	12 (40.0)	24 (40)	1.000
ACE-I[c], no. (%)	10 (33.3)	19 (31.7)	1.000
ARBs[d], no. (%)	2 (6.7)	5 (8.3)	1.000
β-Blockers, no. (%)	6 (20.0)	9 (15.0)	0.560
Calcium antagonists, no. (%)	3 (10.0)	3 (5.0)	0.396
Diuretics, no. (%)	6 (20.0)	22 (36.7)	0.148
Statins, no. (%)	4 (13.3)	8 (13.3)	1.000
Antiaggregants, no. (%)	6 (20.0)	10 (34.3)	0.224
Aspirin, no. (%)	5 (16.7)	17 (28.3)	0.301
Thienopyridine, no. (%)	1 (3.3)	3 (5.0)	1.000
Oral antidiabetics, no. (%)	0 (0.0)	4 (6.7)	0.297
Insulin, no. (%)	1 (3.3)	0 (0.0)	0.333
Biochemical			
Total WBC[e], no. × 10^9/l (IQR)	8.62 (5.98–10.41)	7.54 (6.55–8.61)	0.290
Neutrophils, no. × 10^9/l (IQR)	5.56 (3.64–7.40)	4.54 (3.70–5.91)	0.371
Lymphocytes, no. × 10^9/l (IQR)	1.99 (1.44–2.93)	1.97 (1.63–2.47)	0.751
Platelets, no. × 10^9/l (IQR)	220 (170–251)	213 (173–234)	0.513
Red blood cells, no. × 10^{12}/l (IQR)	4.64 (4.39–5.07)	4.68 (4.31–5.00)	0.656
Serum total-c[f], mg/dl (IQR)	215 (192–240)	193 (154–221)	0.008
Serum LDL-c[g], mg/dl (IQR)	45 (40–60)	133 (121–148)	0.290
Serum HDL-c[h], mg/dl (IQR)	133 (121–148)	111 (83–142)	0.019
Serum Triglycerides, mg/dl (IQR)	137 (97–181)	105 (82–147)	0.010
INR[i], no. (IQR)	1.09 (1.01–1.11)	1.10 (1.01–1.17)	0.575
Plasma fibrinogen, mg/dl (IQR)	264 (220–294)	280 (246–319)	0.132
Serum glycemia, mg/dl (IQR)	111 (97–136)	110 (97–146)	0.857

Data are expressed as median (interquartile range [IQR]) or number [no.] (percentages [%]).
[a] CAD: coronary artery disease
[b] RAAS: renin–angiotensin–aldosterone system.
[c] ACE-I: angiotensin converting enzyme inhibitor.
[d] ARBs: angiotensin receptor blockers.
[e] WBC: white blood cells.
[f] Total-c: total cholesterol.
[g] LDL: low density lipoprotein.
[h] HDL: high density lipoprotein.
[i] INR: International normalized ratio.

Table 2
Clinical and radiological characteristics of ischemic stroke in the study groups.

	Non-thrombolysis (n = 30)	Thrombolysis (n = 60)	*p*-Value
Clinical features at onset (time 0)			
Time window to CT[a], no. (%)			
<3 h	18 (60.0)	52 (86.7)	0.007
3–6 h	10 (33.3)	8 (13.3)	0.023
>6 h	2 (6.7)	0 (0.0)	0.109
TOAST[b] classification, no. (%)			
Atherothrombotic	9 (31.0)	33 (55.9)	0.028
Cardioembolic	9 (31.0)	18 (30.5)	0.959
Lacunar	11 (37.9)	8 (13.6)	0.009
Seizure at onset, no. (%)	0 (0.0)	0 (0.0)	1.000
Headache at onset, no. (%)	2 (6.7)	1 (1.7)	0.257
Previous cognitive decline, no. (%)	1 (3.3)	4 (6.7)	0.661
NIHSS[c] ≥ 5, no. (%)	19 (63.3)	55 (91.7)	0.002
ASPECTS[d] ≤ 7, no. (%)	7 (23.3)	7 (11.5)	0.216
1 day			
NIHSS ≥ 5, no. (%)	13 (43.3)	39 (65.0)	0.070
CT lesion volume at 24 h, mm^3 (IQR)	1.35 (0.60–17.97)	13.05 (1.24–46.95)	0.066
Hemorrhagic transformation, no. (%)	1 (3.3)	13 (21.7)	0.023
7 days			
NIHSS ≥ 5, no. (%)	8 (26.7)	25 (41.7)	0.246
CT lesion volume at 7 days, mm^3 (IQR)	2.15 (0.70–51.37)	14.30 (1.52–66.50)	0.104
Hemorrhagic transformation, no. (%)	6 (20.0)	22 (36.7)	0.148
ECASS[e], no. (%)			
None	24 (80.0)	38 (63.3)	0.118
Hemorrhagic infarction type 1	3 (10)	6 (10.0)	0.981
Hemorrhagic infarction type 2	3 (10)	1 (1.7)	0.067
Parenchymal hemorrhage type 1	0 (0.0)	10 (16.7)	0.018
Parenchymal hemorrhage type 2	0 (0.0)	5 (8.3)	0.106
90 days			
NIHSS ≥ 5, no. (%)	7 (23.3)	14 (23.3)	1.000
mRS[f] > 2, no. (%)	4 (13.3)	11 (18.3)	1.000
CT lesion volume at 3 months, mm^3 (IQR)	1.10 (0.40–23.00)	6.00 (0.80–59.20)	0.130
CT lesion volume variation (%)	21.5 (−0.01–33.3)	18.4 (−0.56–48.54)	0.827

Data are expressed as median (interquartile range [IQR]) or number [no.] (percentages [%]).
[a] CT: computed tomography.
[b] TOAST: Trial of ORG 10172 in Acute Stroke Treatment.
[c] NIHSS: National Institutes of Health Stroke Scale.
[d] ASPECTS: Alberta Stroke Program Early CT Score.
[e] ECASS: European Cooperative Acute Stroke Study.
[f] mRS: modified ranking scale.

AIS patients, we believe that the peak detected after thrombolysis might be independent of neutrophil degranulation. Despite this increase in serum TIMP levels in patients treated with thrombolysis, the amount of MMP-9/TIMP-1 complex was not significantly augmented. These results might suggest that the increase in TIMP-1 serum levels might be not enough to bind and abrogate MMP-9 activity. Therefore, the pathophysiology of TIMPs in AIS still remains largely unknown, even though long-time recognized in experimental studies [45,46].

The pathophysiological relevance of early r-tPA-induced neutrophil degranulation needs to be validated in clinical AIS cohorts, including the potential association with post-stroke hemorrhagic transformation. Our cohort included a quite high rate of hemorrhagic transformation following thrombolysis, as compared with other studies [47]. The inclusion of all radiological findings (and then both symptomatic and asymptomatic lesion) certainly contributed to increase this number. Various neutrophil products have been suggested as potential mediators of reperfusion injury [48,49], but also many other risk factors were shown to be potentially associated with hemorrhagic transformation [50]. Therefore, our clinical findings remained as mainly observational and require further validation. Finally, our study has some limitations. In vitro results did not completely clarify r-tPA-mediated molecular mechanisms on neutrophil degranulation. Therefore, this point requires further investigation to identify which transmembrane receptor binds r-tPA on neutrophil surface as well as additional downstream intracellular pathways. In addition, the small sample size of clinical study did not allow any sub-analysis within groups, especially in thrombolysed patients. Larger studies are needed to further clarify the pathophysiological relevance of r-tPA-mediated neutrophil activation on post-stroke hemorrhagic transformation in vivo in patients. Therefore, our clinical study has to be considered as a “pilot” clinical observation, potentially suggesting a detrimental neutrophil activation in response to r-tPA. On the other hand, the in vitro experiments conferred to our

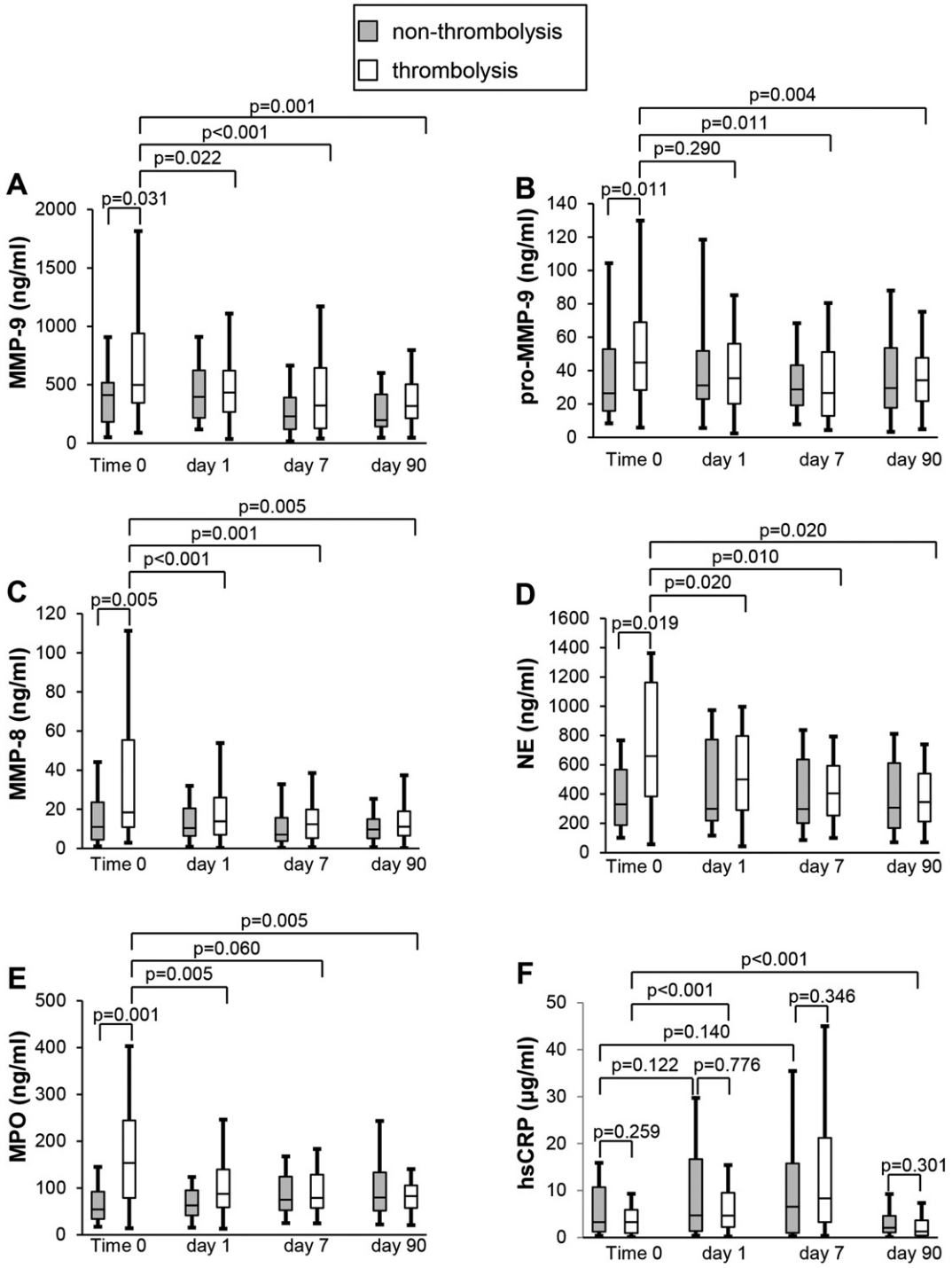

Fig. 6. Serum levels of neutrophil enzymes and pro-MMP-9 activity but not hsCRP are increased in patients immediately after thrombolysis (time 0). Serum MMP-9 (A), pro-MMP-9 activity (B), MMP-8 (C), NE (D), MPO (E) and hsCRP (F) at baseline (time 0), and 1, 7 and 90 days after the acute ischemic event in patients treated with (white bars) or without (gray bars) thrombolysis.

research a "translational" value, adding promising mechanistic insight on r-tPA-mediated effects on neutrophil pathophysiology.

In conclusion, this study shows that the administration of a thrombolytic drug r-tPA-induced early neutrophil degranulation both in vitro via defined intracellular pathways and potentially in vivo. In AIS patients treated with thrombolysis, neutrophil degranulation was mainly hypothesized on the basis of early MPO and NE serum peaks (proteases more specific of neutrophil granules). Considering serum MMPs, we should acknowledge that the serum profile of these proteases might be also influenced by a potential release from the damaged brain or blood–brain barrier into the blood stream. Neutrophil activation was early, but transient and accompanied by an increase in serum levels of protective TIMPs. Since no long-term effects were observed, these results do not invalidate the usefulness of thrombolytic approach. However, the combined inhibition of neutrophil activation together with the administration of r-tPA might be a promising strategy to further improve thrombolysis-mediated benefits after ischemic stroke.

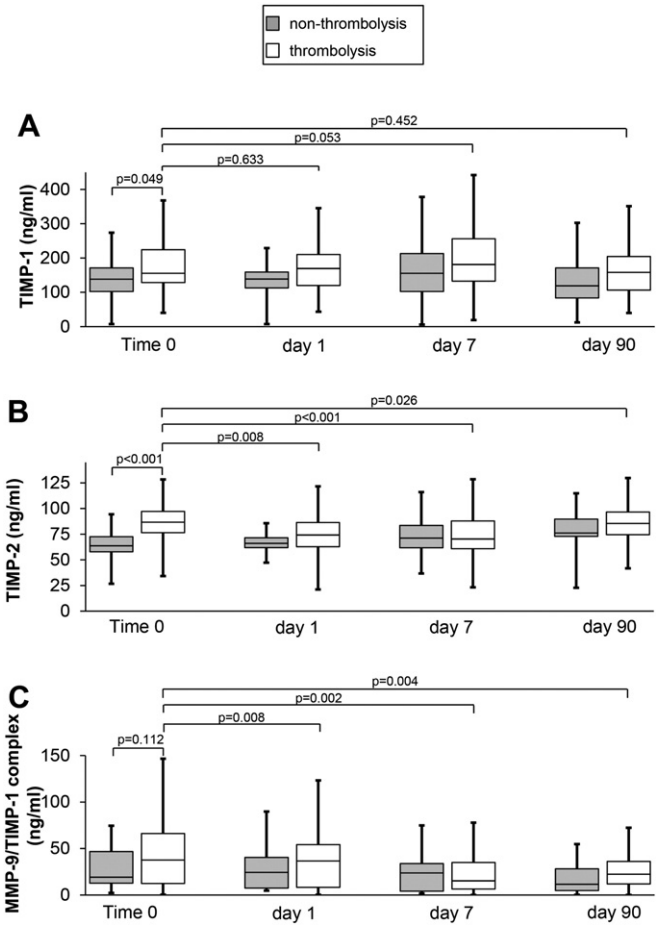

Fig. 7. Serum levels of protective TIMPs are increased in patients immediately after thrombolysis (time 0). Serum levels of TIMP-1 (A), TIMP-2 (B) and MMP-9/TIMP-1 complex (C) at baseline (time 0), and 1, 7 and 90 days after the acute ischemic event in patients treated with (white bars) or without (gray bars) thrombolysis.

Disclosure/conflict of interest

All authors declare that no conflict of interest exists.

Acknowledgments

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