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Polymorphonuclear Leukocytes Modulate Tissue Factor Production by Mononuclear Cells: Role of Reactive Oxygen Species

Yves Cadroy,1* Dominique Dupouy,* Bernard Boneu,* and Henri Plaisancié†

To determine whether polymorphonuclear leukocytes (PMN) modulate the production of tissue factor (TF) by monocytes, PBMC were incubated with increasing concentrations of PMN, PMN did not express any procoagulant activity. After 20-h cocultures, PMN enhanced or inhibited the TF production of PBMC, and this effect depended on the PMN/PBMC ratio. When the ratio increased from 1/1000 to 1/5, without or with LPS, the TF activity of PBMC increased to peak at 2.5-fold the baseline value (p < 0.01). The TF Ag and TF mRNA also increased. This potentiating effect was mediated by reactive oxygen species (ROS) released by PMN during the coculture; it did not require direct cell contact between PMN and PBMC, it was enhanced when PMN were stimulated by fMLP (a chemotactic peptide), and it was inhibited by two antioxidants, N-acetyl cysteine and pyrrolidine dithiocarbamate. In contrast, when the PMN/PBMC ratio was further increased from 1/2 to 2/1, the PBMC TF activity, Ag, and mRNA decreased and were inhibited compared with those of PBMC cultured alone (p < 0.01). This inhibitory effect required direct cell contact between PMN and PBMC, and it was not due to a PMN-mediated cytotoxicity. To confirm the role of ROS, H2O2 enhanced then inhibited the TF activity of PBMC in a dose-dependent manner, similarly to PMN. Thus, PMN may play an important role in the pathogenesis of thrombosis and atherosclerosis by exerting concentration-dependent regulatory effects on the TF production by PBMC via the release of ROS. The Journal of Immunology, 2000, 164: 3822–3828.

Thrombosis and atherosclerosis are multicellular processes in which the role of leukocytes is becoming increasingly recognized. Monocytes/macrophages express procoagulant activities (PCA), which are mediated to a large extent by cell surface-associated tissue factor (TF) (1–4). TF is the cellular receptor and cofactor for plasma factor VII(a), which initiates the coagulation protease cascade leading ultimately to the generation of thrombin and fibrin and is the main initiator of thrombogenesis in vivo (5). Quiescent monocytes synthesize and express TF on their membranes in response to a wide variety of stimulating agents, including LPS, cytokines, arachidonic acid, and immune complexes (6, 7).

Polymorphonuclear leukocytes (PMN) are another type of leukocyte whose role in several pathological processes related to thrombosis and atherosclerosis appears important (8). They act notably by releasing potent mediators which may interact with other blood cells. These mediators include proteolytic enzymes such as cathepsin G, and reactive oxygen species (ROS). Cathepsin G is a platelet-activating agent (9–11). ROS are derived from molecular oxygen by sequential monovalent reductions, yielding the superoxide radical (O2·−), hydrogen peroxide (H2O2), and the hydroxyl radical (·OH). A number of recent studies have shown that their targets include endothelial cells, vascular smooth muscle cells, and macrophages and that increased or uncontrolled ROS production is involved in the formation of thrombosis and atherosclerosis (12). For example, they act on endothelial cells and alter their production of prostacyclin and nitric oxide, two molecules with important endogenous antiplatelet and vasodilator properties (8). They induce the expression of TF (13) and increase the adheriveness of endothelial cells for PMN (14).

In the present study, we describe a new mechanism by which PMN may play an important role in the pathogenesis of thrombosis and atherosclerosis. We show that they modulate the TF production of PBMC, and that, depending on the experimental setting, this modulation is positive or negative. It appears to be mediated by the PMN production of ROS, underlining the role of these mediators in the development of these pathological processes.

Materials and Methods

Materials

PBS (pH 7.4) was obtained from Seromed, Biochrom (Berlin, Germany). Ficoll-Hypaque PLUS was purchased from Pharmacia Biotech (Uppsala, Sweden). M199 was purchased from ATGC Biotechnologie (Noisy-le-Grand, France). All other reagents were obtained from Sigma (Saint Quentin Fallavier, France).

tfMLP was dissolved in 100% DMSO. N-acetyl cysteine (NAC) was dissolved at 200 mmol/L in deionized water and neutralized by titration with NaOH. Xanthine (X) was dissolved in NaOH. All other reagents were dissolved in deionized water. In experiments involving pharmacological reagents, control experiments were always performed with the corresponding solvents.

Cell isolation and cell cultures

PMN and PBMC were isolated from healthy volunteers (12 females and six males, aged 20–50 years) that had not taken aspirin or other nonsteroidal anti-inflammatory drugs in the 7 days preceding the donation. Whole blood was obtained with a 19-gauge needle, anticoagulated with trisodium citrate (0.129 M; Becton Dickinson, Meylan, France), and centrifuged at 280 × g for 15 min at 4°C. Platelet-rich plasma was removed. The sedimented cells were diluted to twice the original blood volume with PBS and layered onto
Ficoll-Hypaque PLUS. After centrifugation at 400 × g for 35 min at 20°C, PMN and PBMC appeared in two separate bands. The lower band containing PMN and erythrocytes was resuspended in a lysis buffer containing 155 mmol/L NH4Cl, 2.96 mmol/L KHCO3, and 3.72 mmol/L disodium EDTA for 10 min. The cell suspension was then centrifuged, and PMN were finally resuspended at 50 × 10^6 cells/ml in the culture medium composed of M199, 2 mmol/L glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The upper band contained PBMC. As described earlier (7), PBMC were washed in 5 mmol/L EDTA-PBS four to six times to remove remaining platelets. The resulting mononuclear fraction contained less than two platelets per leukocyte. Nonspecific α-naphthyl-acetate esterase staining indicated that the mononuclear fraction contained 28.3 ± 6.4% (n = 4) of monocytes. PBMC were resuspended at 25 × 10^6 PBMC/ml in the culture medium and were plated (10 μL) in sterile 96-well polystyrene tissue-culture plates (Nunc, Roskilde, Denmark) containing 100 μL of culture medium. In some experiments, PBMC were preincubated for 15 min with different pharmacological agents before being plated. These agents included cycloheximide, actinomycin D, N, and pyrrolidine dithiocarbamate (PDTC). Then, 10 μL of culture medium containing no PMN or various concentrations of PMN were added. When indicated, PMN were incubated with polymyxin B for 15 min before being added to PBMC. PMN and mononuclear cell mixtures always originated from the same donor.

LPS, obtained from Escherichia coli 0111:B4, was incubated with PMN/PBMC cocultures at various concentrations (10 ng/ml–10 μg/ml). In selected experiments, PBMC and PMN were cultured separately using an inner-well system (Nunc Tissue Culture Inserts, 0.2 cm anopore membrane; Nunc), in which PBMC were plated in the tissue-culture plate and were then frozen and thawed three times. They were stored at −80°C, melted, and were then refrozen three times. They were stored at −80°C until assayed.

### Measurement of Lactic Acid Dehydrogenase

Cell viability, assessed by the measurement of lactate dehydrogenase (LDH) release in the supernatant of cultured cells (LDH Optimized, Sigma) and by trypan blue exclusion was >90%. All reagents used for cell isolation and culture were prepared with endotoxin-free water. The levels of endotoxin contamination in the different reagents incubated with PBMC, as assessed by a chromogenic Limulus assay (Chromogenix, Mölndal, Sweden), were very low (<0.001 ng/ml, final concentration). This level of endotoxin did not enhance the PCA of PBMC (data not shown).

### Measurement of PCA

PCA was measured on intact cells using a one-step plasma recalcification time assay. After incubation, plates were centrifuged for 10 min at 400 × g. The supernatant was collected, and cells or supernatant (80 μL) were incubated for 2 min at 37°C with citrated normal human platelet-poor plasma (100 μL). In selected experiments, the PCA was measured using a factor Xa-based assay (Stago, Asnières, France). Then, 100 μL of a 10 mmol/L CaCl2 was added to initiate the reaction. The change in optical density at 405 nm was quantitated using a microplate reader. Coagulation times were converted into arbitrary units (AU) using reference curves determined with a standard human brain TF preparation containing 100 AU/ml (Thromborel Behring, Marburg, Germany); the logarithm of the PCA was related to the logarithm of the coagulation time. The PCA was expressed in AU/106 PBMC. The PCA of PBMC cells was characterized by incubating the cells with a mixture of two mouse anti-human TF mAbs (10 μg/ml; American Diagnostica, Greenwich, CT) for 30 min at 37°C.

### Measurement of TF Ag

TF Ag was measured on cell lysates by commercially available immunoenzymoassay (Immundin Tissue Factor, American Diagnostica). Cell lysates were prepared by lysing PBMC in PBS containing 0.1% Triton X-100, 1 mmol/L EDTA, 16 mmol/L octyl phosphatidyl Ditolbecco’s glyco-pyranoside, 10 μmol/L pepstatin A, 10 μmol/L leupeptin, 0.1 mmol/L PMSF, and 100 Kallirein International Units/ml aprotinin. The cell lysates were then frozen and thawed three times. They were stored at −80°C until assayed.

### TF RT-PCR

Semiquantitative RT-PCR was used to calculate relative changes in TF mRNA levels. To correct variations in amplification efficiency in each reaction, both TF and HLA-DR class II MHC Ag (DR) mRNAs were detected. We chose DR as a standard because, like TF, DR is a receptor with an important role in immune recognition. We chose TF as a marker of thrombin generation because, like DR, TF is a membrane-bound receptor expressed in AU/106 PBMC. The PCA of PBMC cells was characterized by incubating the cells with a mixture of two mouse anti-human TF mAbs (10 μg/ml; American Diagnostica, Greenwich, CT) for 30 min at 37°C with citrated normal human platelet-poor plasma (100 μL). In selected experiments, the PCA was measured using a factor Xa-based assay (Stago, Asnières, France). Then, 100 μL of a 10 mmol/L CaCl2 was added to initiate the reaction. The change in optical density at 405 nm was quantitated using a microplate reader. Coagulation times were converted into arbitrary units (AU) using reference curves determined with a standard human brain TF preparation containing 100 AU/ml (Thromborel Behring, Marburg, Germany); the logarithm of the PCA was related to the logarithm of the coagulation time. The PCA was expressed in AU/106 PBMC. The PCA of PBMC cells was characterized by incubating the cells with a mixture of two mouse anti-human TF mAbs (10 μg/ml; American Diagnostica, Greenwich, CT) for 30 min at 37°C.

### Statistical analysis

All results are expressed as mean ± 1 SEM. In comparisons of two groups, probability values were calculated by Student’s t test. In experiments involving comparisons of multiple groups, the probability that differences existed between the means of the groups was determined by ANOVA, and then by a Neuman-Keuls test when the p value was ≤0.05. Differences were considered to be statistically significant when p was ≤0.05.

### Results

#### Effect of PMN on the PCA of PBMC

After 20 h of culture, isolated PMN did not express any significant TF mRNA, whereas isolated resting PMN expressed a low but detectable level of PCA and was probably related to the presence of cell-derived microparticles. Incubation of PBMC with PMN/PBMC cocultures at various concentrations of PMN (0–5 × 10^6 cells/*ml* were incubated in 96-well microtiter plates with cytochrome-c (75 μmol/L) and n-glucose (7.5 mmol/L) in M199. In selected experiments, the cells were also incubated with LPS (100 ng/ml) and/or fMLP (10 μmol/L). Immediately after addition of PMN, or fMLP when this agonist was used, the absorbance of the reaction wells was measured at 550 nm in a microplate spectrophotometer. The readings were recorded at 50-min intervals. Each experiment was performed in triplicate as a control reaction, which contained 300 U/ml superoxide dismutase. Production of superoxide anion was determined by use of the molar extinction coefficient of cytochrome-c (6.3 with a light path of 2 mm).

### Measurement of superoxide anion generation

Superoxide anion generation by PMN was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome-c. Increasing numbers of PMN (0–5 × 10^6 cells/*ml* were incubated in 96-well microtiter plates with cytochrome-c (75 μmol/L) and n-glucose (7.5 mmol/L) in M199. In selected experiments, the cells were also incubated with LPS (100 ng/ml) and/or fMLP (10 μmol/L). Immediately after addition of PMN, or fMLP when this agonist was used, the absorbance of the reaction wells was measured at 550 nm in a microplate spectrophotometer. The readings were recorded at 50-min intervals. Each experiment was performed in triplicate as a control reaction, which contained 300 U/ml superoxide dismutase. Production of superoxide anion was determined by use of the molar extinction coefficient of cytochrome-c (6.3 with a light path of 2 mm).

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### Results

#### Effect of PMN on the PCA of PBMC

When the PMN/PBMC ratio was further increased to 2/1, the cell PCA significantly increased to peak at 2.5-fold the baseline value (p < 0.01). This effect was not due to a contamination of PMN by endotoxin because the effect was comparable when PMN were incubated with PBMC in the presence of polymyxin B (10 μg/ml).

When the PMN/PBMC ratio was further increased to 2/1, the cell PCA significantly increased and, at the highest investigated PMN/PBMC ratio, it was significantly inhibited compared with that of isolated PMN (p < 0.01). This effect was not due to a PMN-mediated cytotoxicity of PBMC, as shown by the measurement of LDH, the cytoplasmic enzyme released by damaged cells, in the cell culture supernatant. During the 20-h culture, 2.5 × 10^6 PBMC alone released 0.98 ± 0.32 mU/ml of LDH (n = 5). Because the total amount of LDH present in 2.5 × 10^6 PBMC is 75 mU, we could determine that there was ~1% cell death. Regarding isolated PMN
(5 × 10⁶ cells/ml), which released 7.16 ± 2.57 mU/ml during the 20-h culture and contain 180 mU/5 × 10⁶ cells of LDH, the rate of cell death was higher (4%). When PBMC and PMN were cocultured for 20 h, the measured amount of released LDH (7.64 ± 1.61 mU/ml) was comparable to that expected if the amount of LDH released by isolated cells was summed (8.14 mU/ml).

In the presence of LPS (10 ng/ml–10⁶ g/ml), the PCA of PMN was still negligible, whereas that of PBMC increased in a dose-dependent manner to level off at 10 times the baseline values at 1 and 10 μg/ml of LPS (Fig. 2). The enhancing effect induced by low concentrations of LPS (10 and 100 ng/ml) was altered when PBMC were cocultured with PMN; it was further potentiated when the PMN/PBMC ratio was low (1/5), and it was inhibited when this ratio was high (2/1). Both the potentiating and inhibitory effects induced by PMN disappeared at higher concentrations of LPS (1–10 μg/ml).

**FIGURE 1.** Effect of increasing concentrations of PMN on the PCA of PMN/PBMC cocultures. PMN, PBMC, or PMN/PBMC cocultures were cultured at 37°C, and the PCA expressed on the cell surface was measured after 20 h. Statistical comparisons of the PCA of PBMC incubated with increasing concentrations of PMN vs that obtained without PMN are represented by the following: ***, p < 0.01 for potentiating effect; 00, p < 0.01 for inhibitory effect; n = 13.

After 5 h of culture, the PCA of isolated PBMC was very low, even in the presence of LPS (10 ng/ml; data not shown). Similarly, the potentiating and inhibitory effects of PMN were barely detectable. Therefore, except when indicated, all subsequent experiments were performed with 20-h cultures.

Finally, in all these experiments, the PCA present both at the cell surface and in the cell supernatant was identified as TF, as shown by the fact that >95% was abolished by a cocktail of neutralizing anti-TF mAbs (PCA < 0.1 AU/10⁶ cells; n = 1). In addition, there was no PCA when a factor VII-deficient plasma was used (PCA < 0.1 AU/10⁶ cells; n = 1).

**FIGURE 2.** Effect of increasing concentrations of PMN on the PCA of PMN/PBMC cocultures in the presence of increasing concentrations of LPS. PMN, PBMC, or PMN/PBMC cocultures were cultured at 37°C and the PCA expressed on the cell surface was measured after 20 h. Statistical comparisons of the PCA of PBMC incubated with increasing concentrations of PMN vs that obtained without PMN are represented by the following: *, p < 0.05; **, p < 0.01; n = 5 or 6.

**FIGURE 3.** Effect of increasing concentrations of PMN on the TF mRNA levels of PMN/PBMC cocultures. PMN, PBMC, or PMN/PBMC cocultures were cultured at 37°C, and after 2, 5, and 20 h, total RNA was extracted and used for RT-PCR studies with cDNA probes for TF and DR (n = 3, 7, and 7, respectively). The relative levels of TF mRNA were quantified by densitometric scanning and normalized according to the level of DR mRNA (which was not affected by PMN). PMN did not express significant levels of TF mRNA or DR mRNA. One representative experiment is shown. Statistical comparisons of the level of TF mRNA of PMN/PBMC cocultures incubated with increasing concentration of PMN vs that obtained without PMN are represented by the following: ***, p < 0.01.

The induction mechanism elicited by PMN required de novo protein synthesis. When PBMC were pretreated with actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml) for 15 min and then cocultured with PMN for 20 h, the potentiating effect induced by PMN was completely abolished; at the 1/5 PMN/PBMC ratio, the PCA of PBMC was 117 and 82% of that of PBMC cultured alone with actinomycin D and cycloheximide, respectively (n = 3).

Comparably to the PCA, when cells were treated with increasing concentrations of PMN, TF Ag levels increased to peak at 1.3-fold with a PMN/PBMC ratio of 1/5 from 166 ± 38 to 217 ± 31 pg/10⁶ cells (n = 8). With a PMN/PBMC ratio of 2/1, TF Ag levels were inhibited compared with those of PBMC cultured alone (105 ± 20 pg/10⁶ cells; p < 0.01).

TF mRNA transcripts were not detected in PMN cultured alone for 4 or 20 h but were detectable in PBMC (Fig. 3). At a PMN/PBMC ratio of 1/5, there was an increase of TF mRNA levels that was detectable at 2 h, maximal at 5 h, and stable during at least 20 h (p < 0.01). At a ratio of 2/1, the TF mRNA levels were slightly inhibited compared with those of PBMC cultured alone.

**Role of PBMC-PMN contact**

To ascertain whether direct cell-cell contact and/or secretory products were involved, PBMC and PMN were incubated separately using an inner-well system for 20 h. At the end of the incubation, the inserts containing different concentrations of PMN were removed and the PBMC-associated PCA was measured. PMN potently stimulated the PCA of PBMC, and the effect was even more...
Effect of PMN on the PCA of PBMC

PBMC were incubated with increasing concentrations of PMN together (No Insert) or separately using an inner-well system (Insert) for 20 h at 37°C. The PCA expressed on the cell surface was measured after 20 h. Statistical comparisons of the PCA of PMN incubated with increasing concentrations of PMN vs that obtained without PMN are represented by the following: *, p < 0.05; **, p < 0.01; n = 8, respectively.

Time course of PMN stimulation by PMN

We next determined the time required by PMN to maximally stimulate PBMC. PMN were deposited in inserts and cocultured with PBMC for 5, 15, 30, and 60 min and 20 h, after which the inserts containing PMN were removed. The PCA of PBMC was measured at 20 h. In one set of experiments, PBMC were cocultured with PMN incubated with fMLP (1 μmol/L) for 15 min before being removed. Results are expressed as a percentage of the PCA of PMBMC cultured alone for 20 h. Statistical comparisons of the PCA of PMBMC incubated with increasing concentrations of PMN vs that obtained without PMN are represented by asterisks (**, p < 0.01; n = 4).

Involvement of ROS in PMN stimulation of the PCA of PBMC

The secretory products of PMN include ROS. When PMN were incubated in polystyrene culture plates in the absence of any exogenous activator, there was a time-dependent and concentration-related increase of superoxide generation (Fig. 5), which confirms that adherence of PMN to polystyrene tissue-culture plates itself triggers the respiratory burst, even in the absence of any exogenous agonist (18). The production of superoxide was comparable when PMN was cultured with and without LPS (8.4 ± 2.2 and 11.9 ± 0.8 nmol/ml after 15 min, respectively; p > 0.05; n = 3). However, stimulation of PMN with fMLP elicited an acceleration of the rate of superoxide generation (55.5 ± 2.3 nmol/ml after 15 min; p < 0.01 vs nonstimulated PMN; n = 3).

The role of ROS in the PMN stimulation of the PCA of PBMC was assessed using the following two antioxidants: NAC, a sulfhydryl group donor that acts by increasing levels of the endogenous antioxidant-reduced glutathione peroxidase system and can directly scavenge free radicals; and PDTC, which scavenges free radicals and chelates transition metal ions. For example, the total amount of superoxide anion produced by PMN (5.0 × 10^6 PMN/ml) during 80 min decreased from 30 ± 5 nmol/L to < 1 nmol/L in the presence of NAC (2 mmol/L; n = 3), as already shown in a previous study (19). Incubation of PBMC in the presence of increasing doses of NAC (200 μmol/L and 2 mmol/L) or PDTC insignificantly enhanced the PCA of PBMC (p < 0.01), which indicates that the PMN stimulation accelerated the release of a soluble mediator(s) that subsequently acted on PBMC.

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Table 1. Time course of the effect of PMN on the procoagulant activity of PBMC

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Note: PMN were cultured with increasing concentrations of PMN for various time periods. PMN were deposited in cell culture inserts for 5, 15, 30, and 60 min and 20 h, after which the inserts containing PMN were removed. The PCA of PBMC was measured at 20 h. In one set of experiments, PBMC were cocultured with PMN stimulated with fMLP (1 μmol/L) for 15 min before being removed. Results are expressed as a percentage of the PCA of PBMC cultured alone for 20 h. Statistical comparisons of the PCA of PBMC incubated with increasing concentrations of PMN vs that obtained without PMN are represented by asterisks (**, p < 0.01; n = 4).

FIGURE 5. Effect of time on the superoxide anion production (O_2^−) by increasing concentrations of PMN. Resting PMN were plated in tissue-culture plate, and O_2^− generation was measured for 80 min as the superoxide dismutase-inhibitable reduction of ferricytochrome-c (n = 3).

FIGURE 4. Effect of PMN/PBMC contact on the modulatory effect of PMN on the PCA of PBMC. PBMC were incubated with increasing concentrations of PMN together (No Insert) or separately using an inner-well system (Insert) for 20 h at 37°C. The PCA expressed on the cell surface was measured after 20 h. Statistical comparisons of the PCA of PMBMC incubated with increasing concentrations of PMN vs that obtained without PMN are represented by the following: *, p < 0.05; **, p < 0.01; n = 8, respectively.

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X/XO or H₂O₂ directly stimulated PBMC. The enzyme system PCA of PBMC, we tested whether incubation of PBMC with scripts measured in PBMC cultured alone.

74 PMN/PBMC cell ratio was 1/5, they were 110 p was increased 5-fold from 4.92 had no effect on their PCA, whereas with both XO and X, the PCA expression on the cell surface was measured after 20 h. Statistical comparisons of the PCA of PBMC incubated with increasing concentrations of PMN vs that obtained without PMN are represented by the following: *, p < 0.05; **, p < 0.01; n = 4.

(10 μmol/L) resulted in an inhibition of the PMN stimulation of their PCA without (Fig. 6) or with LPS (data not shown). Finally, the PMN-mediated stimulation of TF mRNA levels was also abolished by NAC (2 mmol/L) and PDTC (50 μmol/L); when the PMN/PBMC cell ratio was 1/5, they were 110 ± 21% (n = 7) and 74 ± 32% (n = 7), respectively, of the level of TF mRNA transcripts measured in PBMC cultured alone.

To confirm that ROS were responsible for the stimulation of PCA of PBMC, we tested whether incubation of PBMC with X/XO or H₂O₂ directly stimulated PBMC. The enzyme system X/XO was chosen because it produces both O₂⁻ and H₂O₂ (20). Incubation of PBMC with either XO (1 mU/ml) or X (0.4 mmol/L) had no effect on their PCA, whereas with both XO and X, the PCA was increased 5-fold from 4.92 ± 1.28 to 23.0 ± 7.8 AU/10⁶ cells (p < 0.05; n = 5). Likewise, H₂O₂ alone or with LPS increased the PCA of PBMC in a dose-dependent manner (Fig. 7). Higher doses of XO (10 mU/ml) or H₂O₂ (Fig. 7) resulted in an inhibition of the PCA.

**Discussion**

This study demonstrates for the first time that PMN exert concentration-dependent regulatory effects on the expression of the procoagulant protein TF by resting or LPS-stimulated PBMC. First, cocultures of PBMC with low concentrations of PMN resulted in a marked increase in TF mRNA, TF protein, and TF activity of PBMC. Second, when PBMC were cultured with higher concentrations of PMN, the TF expression of PBMC was no longer stimulated but was even inhibited compared with that of PBMC cultured alone.

Different experimental findings indicate that the stimulating effect was mediated through molecules released by PMN; it did not require direct cell contact between PMN and PBMC (Fig. 4), it was reproduced with the supernatant of cultured PMN, and it was enhanced when the PMN release was stimulated by fMLP, a chemotactic peptide (Table I). Among the different potent mediators released by PMN, we focused our attention on ROS because they have been shown to induce the expression of TF by endothelial cells (13). Thus, the stimulating effect induced by PMN was completely prevented when PBMC were preincubated with two structurally unrelated antioxidants, NAC and PDTC (Fig. 6). In addition, the TF expression could be induced by exposing PBMC to H₂O₂ (Fig. 7) or to O₂ intermediates generated by the X/XO system. ROS have recently gained attention as important second messengers during cell activation. They induce the initiation of gene transcription and the translocation of transcriptional activating factors into the nucleus (21). The human TF gene contains consensus sequences for the binding of several transcription factors including NF-κB (22). This transcription factor participates in the induction of TF gene transcription (23, 24). Therefore, because ROS have been implicated as one of the intracellular second messenger molecules that induce NF-κB activation (25, 26), one can speculate that PMN stimulated or potentiated the TF expression of resting or LPS-stimulated PBMC by the release of ROS, which activated the TF gene of monocytes through transactivating NF-κB. However, it is also possible that PMN ROS acted on other cells or components present in PBMC, which could lead to TF expression on monocytes.

Mechanisms involved in the inhibitory effect induced by high concentrations of PMN appear to be more complex. In contrast to the previous phenomenon, this inhibitory effect was not reproduced with the cell supernatant, and it required direct cell contact between PMN and PBMC. However, despite these findings, we hypothesize that the inhibitory effect also was mediated through the release of ROS. It previously has been shown that cell exposure to ROS results in depressed protein synthesis (27). In addition, the LPS-stimulated expression of TF by PBMC was also inhibited when PBMC were exposed to increasing doses of H₂O₂ (Fig. 7). In this regard, one should note that it is difficult to compare the effect of ROS released by PMN that acted upon monocytes in a slow and continuous manner with that induced by purified H₂O₂ that was directly added to PBMC. The inhibitory effect was not due to a PMN-mediated cytolysis as shown by the measurement of LDH in the cell culture supernatant. Furthermore, monocytes cocultured with PMN for 20 h exhibited normal morphology (data not shown). In this regard, it is important to note that fresh monocytes have a high level of hydrogen peroxide-degrading enzymes (28) and, therefore, that in our experimental conditions their death by

**FIGURE 6.** Effect of NAC or PDTC on the PCA of resting PBMC (2.5 × 10⁶ cells/ml) cocultured without PMN (PBMC) or with increasing concentrations of PMN (0.05 × 10⁴ cells/ml, PMN/PBMC ratio of 1/50; 0.5 × 10⁴ cells/ml, PMN/PBMC ratio of 1/5; and 5 × 10⁴ cells/ml, PMN/PBMC ratio of 2/1). PBMC or PMN/PBMC cocultures were cultured at 37°C, and the PCA expressed on the cell surface was measured after 20 h. Statistical comparisons of the PCA of PBMC incubated with increasing concentrations of PMN vs that obtained without PMN are represented by the following: *, p < 0.05; **, p < 0.01; n = 4.

**FIGURE 7.** Effect of H₂O₂ on the PCA of resting or LPS-stimulated (100 ng/ml) PBMC. PBMC (2.5 × 10⁶/ml) were cultured at 37°C, and the PCA expressed on the cell surface was measured after 20 h. Statistical comparisons of the PCA of PBMC incubated with increasing concentrations of H₂O₂ vs that obtained without H₂O₂ are represented by the following: *, p < 0.05; **, p < 0.01; n = 9.
an apoptotic pathway is unlikely because it requires higher concentrations of \( \text{H}_2\text{O}_2 \) than those used in the present study (i.e., 5–8 mM; Ref. 29).

Cell contact between PMN and PBMC was required for the inhibitory effect. It is possible that specific ROS of particularly short half-life and different from those having a potentiating effect were involved in the inhibitory effect; the inhibitory effect was not reproduced when PMN and PBMC were separated by an insert or with the supernatant of PMN. In this regard, one may observe that the potentiating effect was less marked when PBMC and low concentrations of PMN were cultured together than when they were cultured separately (Fig. 4). Thus, we suggest that when PMN and PBMC were in contact, the inhibitory effect was present, but that it was overcome by a more potent stimulating effect. However, it is also possible that an effect not related to ROS was responsible for the inhibitory effect induced by PMN in contact with PBMC.

Overall, these results suggest that ROS elicit opposite reactions. This property previously has been shown in other experimental conditions in which opposite effects depended on the type of ROS and/or the dose of exposure. For example, ROS stimulated the proliferation of smooth muscle cell at low concentrations and induced cell death at higher concentrations (30). Likewise, short-term culture with oxidized low density lipoprotein (LDL) and/or mildly oxidized LDL activated transcription factor NF-κB, whereas with a longer incubation period and/or highly oxidized LDL, this transcription factor was inhibited (31–33).

Although our results suggest that PMN modulated the TF expression of PBMC through the production of ROS, the specific ROS involved in the different findings were not identified. The major contributor to ROS generation by PMN is the multicomponent membrane-bound enzyme NADPH oxidase. Oxygen is reduced to water through a four-step addition of electrons. This reduction generates superoxide anions (O\(_2^–\)), which are converted to \( \text{H}_2\text{O}_2 \) by superoxide dismutase. \( \text{H}_2\text{O}_2 \), although relatively inactive, can be reduced to the highly reactive hydroxyl radical (‘OH) by a metal ion through the Fenton reaction (6). These ROS have different properties. The hydroxyl radical is the most reactive species, but it cannot be involved in our findings because it does not pass the plasma membranes. The superoxide radical and \( \text{H}_2\text{O}_2 \) are not very reactive against major macromolecular components of the cell, but they can penetrate the membranes of surrounding cells either through anion channels for the former (34) or freely for the latter (8). In addition, \( \text{H}_2\text{O}_2 \) is relatively stable and is able to reach cell locations remote from the site of its formation. Early gene induction has been attributed to O\(_2^–\) (35), and inhibition of protein synthesis has been attributed to \( \text{H}_2\text{O}_2 \) (27), but these differential effects may depend on the type of the cell and the type of protein studied.

It has been shown that exposure to ROS results in depressed protein synthesis by affecting translation at the initiation step (27). In our study, stimulation and inhibition of TF expression appeared to be related to alterations in gene transcription because the level of TF gene transcripts evolved in a manner comparable to that of the procoagulant protein (Fig. 3). However, the present study does not establish whether the alteration of TF expression was related to alterations in gene transcription or in mRNA stability, events which are both affected by the redox state of the cell (36, 37).

Recently, it has been shown that PMN-derived microparticles act as potent proinflammatory agonists competent to initiate a broad pathway of signal transduction and gene expression in endothelial cells (38). These membrane microparticles were not involved in our results because depletion of the microparticle fraction from PMN suppressed endothelial cell activation. In our study, PMN still greatly enhanced the TF expression of PBMC when both cell populations were separated by a 0.2-μm filter (Fig. 4).

Finally, our study describes another situation in which the TF expression of monocytes is markedly influenced by surrounding cells through physical contact per se and/or biochemical interactions. Thus, platelets enhance LPS-induced TF activity in monocytes through the production of P-selectin, a cell adhesion molecule (39); platelet factor-4, a platelet α-granule (40); and 12-hydroxyeicosatetraenoic acid, a metabolic product of the platelet 12-lipoxygenase pathway (41). Likewise, adhesion of monocytes to activated endothelial cells induces TF generation on monocytes (42, 43). In another study, granulocytes amplified TF expression induced by LPS in a platelet-dependent manner (44). In this study, we show that granulocytes, in the absence of platelets, directly influence TF expression by PBMC.

In conclusion, this study shows that PMN may play an important role in the pathogenesis of thrombosis and atherosclerosis by exerting concentration-dependent regulatory effects on the TF production by PBMC. It describes another example where ROS exert different effects through activation or inhibition of cell functions, and it indicates a new mechanism by which they may represent a risk factor for cardiovascular events such as unstable angina and myocardial infarction, thereby confirming that clinical use of antioxidant in such pathology may be beneficial.

References


