Interleukin-10 and pentoxifylline inhibit C-reactive protein-induced tissue factor gene expression in peripheral human blood monocytes

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Abstract Fibrin deposition is an integral feature of the inflammatory response. In response to C-reactive protein (CRP), an acute-phase reactant, blood monocytes synthesize and express tissue factor (TF), the main initiator of blood coagulation. We report the inhibitory effect of interleukin 10 (IL-10) and that of pentoxifylline, a methyl xanthine derivative, on monocyte expression of TF activity, TF protein and TF mRNA in response to CRP. These agents may be of use in diseases where a TF-induced prothrombotic state is detrimental.

Key words: Monocyte; Tissue factor; C-reactive protein; Interleukin-10; Pentoxifylline

1. Introduction

Activation of human blood monocytes by a variety of proinflammatory agents induces these cells to synthesize and express on their surface tissue factor (TF), a transmembrane glycoprotein which is the main initiator of blood coagulation [1]. The role of this aberrant TF expression in triggering excessive activation of coagulation and both extravascular and intravascular fibrin deposition is now well established in a number of immunological and non-immunological inflammatory diseases [2].

C-reactive protein (CRP), an acute-phase reactant, the concentration of which can increase from trace levels to several hundred $\mu g/ml$ within 24 h, has recently been shown to trigger TF synthesis by monocytes [3]. This effect is probably mediated through the binding of CRP to specific monocytic receptors [4] and involves TF mRNA production [3].

The aim of this work was to assess the effect of interleukin-10 (IL-10) and that of pentoxifylline (PTX), a methylxanthine derivative [1-(5-oxo-hexyl)-3,7-dimethylxanthine] (Torental, Hoechst), on CRP-induced TF activity, TF antigen and mRNA expression. These two compounds have been shown to downregulate LPS-induced TF synthesis [5,6] and have great potential therapeutic value.

2. Materials and methods

Mononuclear cell suspensions containing 20–30% monocytes and 70–80% lymphocytes were isolated by Ficoll-Hypaque gradient sedimentation as described in [7]. To obtain monocyte-enriched preparations (75–95%), monocytes were further purified by adherence to gelatin/fibronectin-coated Petri dishes [8]. Cell suspensions or adherent monocytes were incubated with or without IL-10 or PTX for 30 min at 37°C, before stimulation with 25 $\mu g/ml$ CRP (Sigma, St. Louis) for 1–8 h. Incubation was performed with 5% CO_2, in RPMI medium without foetal calf serum but otherwise supplemented as described in [6]. Because of slight contamination of CRP with LPS, the incubation medium was routinely supplemented with 5 $\mu g/ml$ polymyxin B (Sigma, St Louis). We checked that, under these conditions, the effect of LPS was completely neutralized (data not shown). Purified human IL-10 was generously provided by DNAX (Palo Alto, CA, USA). After incubation, the supernatant was discarded and cells were washed and stored for less than 1 week at −80°C until TF measurement of RNA extrac-

tion. Since monocytes are the only circulating cells to produce TF, mononuclear cell suspsensions were used to measure TF activity and TF antigen levels; adherent monocytes were used for RNA extraction; TF activity was also measured in the latter.

After thawing, cells were lysed and TF activity was assayed as described in [7,8]. Procoagulant activity (PCA) was expressed as TF mU/10^6 monocytes. To determine what proportion of PCA was TF-initiated, PCA was measured in some experiments after incubation of the cell lysate for 30 min with a neutralizing monoclonal antibody to human TF (MoAb4; Corvas, San Diego, CA).

Total RNA was extracted by the guanidinium-thiocyanate/cesium chloride method [8]. Aliquots (5 μg) were applied to slots on a microsample filtration manifold and transferred onto nitrocellulose membranes. Filters were hybridized for 24 h at 42°C, to the TF and G3PDH cDNA probes consecutively. The autoradiograms were scanned by means of laser densitometry, using the Sebia Preference R apparatus, to determine relative levels of mRNA in treated and untreated monocytes.

cDNA probes were labeled with $[\alpha^{32}P]dCTP$ (300 Ci/mmol) by random hexamer primer extension, using the Amersham Multiprime kit. The 641 bp TF cDNA probe, kindly provided by T.S. Edgington [9], was excised from plasmid pUC8 with *Eco*RI. The G3PDH probe was purchased from Clontech (Palo Alto, CA).

TF antigen was determined by an ELISA method using the Imubind TF kit (American Diagnostica Inc., Greenwich) according to manufacturer's instructions, with slight modifications. Cells were solubilized in PBS containing 1% Triton X-100 and directly transferred to the ELISA plate.

3. Results and discussion

As already shown [3], incubation of freshly isolated moncytes with increasing CRP concentrations (2.5–50 $\mu g/ml$) for 7 h led to a concentration-dependent increase in TF activity, from 130 to 1600 mU/10^6 monocytes (Fig. 1, inset). Procoagulant activity was identified as TF, as more than 97% was inhibited when cells were incubated with a neutralizing monoclonal antibody to TF.

We then investigated the effect of graded concentrations of IL-10 and PTX on CRP-induced TF activity. As shown in Fig. 1, both agents had a concentration-dependent inhibitory effect which reached 80% of the CRP-induced effect; IC_{50} values were about 5 U/ml IL-10 and 90 μM PTX. The effects of IL-10 and PTX on CRP-induced TF protein expression were then analyzed by means of ELISA: treatment with 25 $\mu g/ml$ of CRP produced the expected induction of TF expression from 22.5 (cells without CRP) to 368 pg/10^6 monocytes. With increasing concentrations of IL-10 or PTX, TF activity and

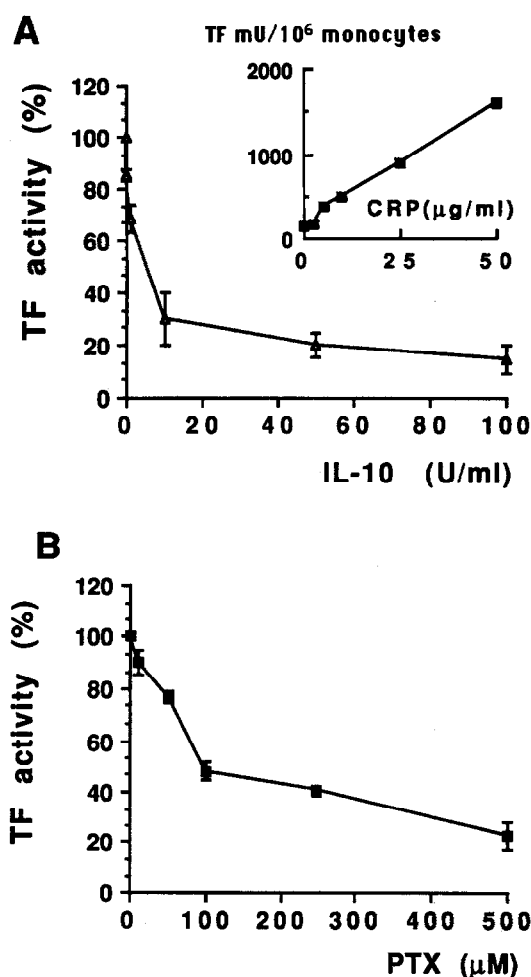*Corresponding author. Fax: (33) (1) 40 25 88 53.

Fig. 1. Effects of IL-10 and PTX on TF activity produced in response to CRP in human monocytes. The effect of increasing concentrations of CRP on TF activity of mononuclear cells is shown in the inset. Mononuclear cell suspensions were preincubated for 30 min with graded concentrations of IL-10 (A) or PTX (B) and subsequently stimulated with 25 μg/ml CRP. Results are means ± S.E.M. of four separate experiments.
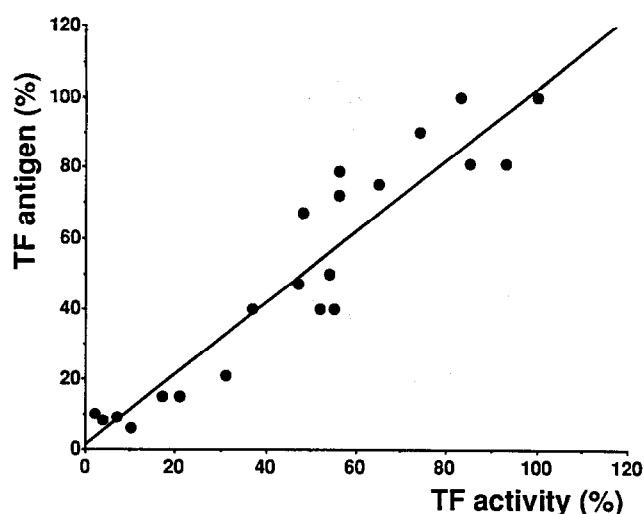

Fig. 2. Correlation between TF activity and TF antigen expression of CRP-exposed monocytes in the presence of increasing concentrations of IL-10 (1, 10, 50, 100 U/ml) or PTX (50, 100, 250, 500 μM) ($r = 0.95$, $P < 0.001$; $y = 1.01x + 1.57$). Results are the percentage of maximal TF expression induced by CRP (25 μg/ml) in the absence of inhibitor.

antigen levels measured in the same experiments ($n = 2$) correlated strongly ($r = 0.95$; $P < 0.01$) (Fig. 2). Moreover, the measured IC_{50} values were similar regardless of which TF assay was used.

As recently stated by McGee et al. [10], three theoretical mechanisms could be involved in modulating membrane TF activity, namely changes in lipid membrane cofactor, de novo synthesis of TF pathway inhibitor (TFPI), the main TF inhibitor, and changes in TF protein levels. Our results suggest that the inhibitory effect of PTX and IL-10 on CRP-induced TF activity is mainly, if not exclusively, secondary to a decrease in membrane TF protein expression.

The time–course of the induction of TF activity by CRP was then studied (Fig. 3). Monocytes were incubated with 25 μg/ml CRP and, after a lag time of 3 h, TF activity rapidly increased between the 4th and 8th hour; PTX and IL-10 inhibited this increase as early as the fourth hour, and continued to do so at 6 and 8 h.

We then studied the effect of IL-10 and PTX on CRP-induced TF mRNA levels. As shown in Fig. 4, 25 μg/ml of CRP

induced a tenfold increase in TF mRNA relative to untreated cells. The CRP-induced increase in TF mRNA was completely abolished when cells were preincubated with 50 U/ml IL-10 or 250 μM PTX, suggesting that the decrease in TF protein levels was secondary to a decrease in TF mRNA expression.

Monocytes and macrophages are present in inflammatory lesions. Expression of TF activity is believed to be an important factor in the thromboembolic manifestations often associated with inflammatory diseases such as lupus erythematosus [11], Crohn's disease [12], post-operative conditions [13] and infectious diseases [14]. The role of inflammation in unstable angina has been recently underlined by Liuzzo et al. [15] who cofirmed the elevation of CRP in these patients at the time of admission; they also showed the prognostic value of CRP: the 20 patients who had levels of CRP ≥ 0.3 mg/dl had more ischemic episodes in the hospital than those with levels < 0.3 mg/dl. Unstable angina has also been reported to be associated with an increase
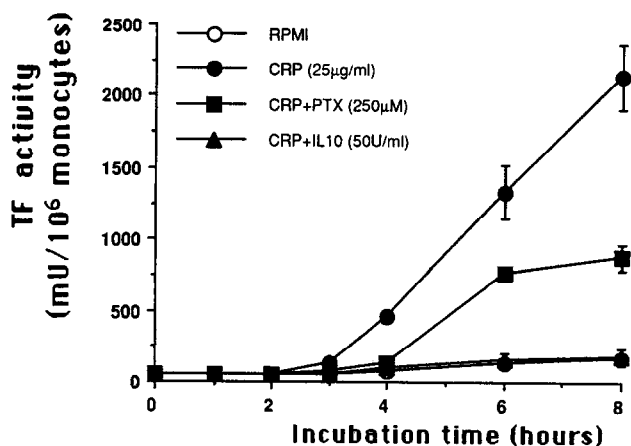

Fig. 3. Kinetics of the inhibition by IL-10 and PTX of CRP-induced expression of TF activity from 0 to 8 h. Mononuclear cells were incubated for 0, 1, 2, 3, 4, 6 and 8 h with RPMI medium alone, CRP, CRP + IL-10, or CRP + PTX.
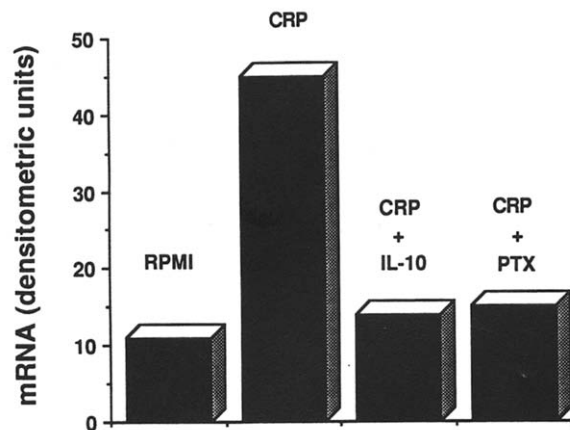
Fig. 4. Slot blot analysis of TF mRNA. 5 μg of total RNA was analyzed. RNA was extracted from untreated monocytes (RPMI; 1) and from cells exposed for 8 h to 25 μg/ml CRP (2), CRP + IL-10 at 50 U/ml (3) or CRP + PTX 250 μM (4). The membrane was hybridized to TF and G3PDH probes. Signal intensity was measured by means of laser densitometry and is expressed relative to the G3PDH control.

in monocyte TF activity [16,17]. As previously suggested [3,13], monocyte stimulation by CRP could be one of the mechanisms involved in TF expression. In view of the deleterious effect of TF expression, down-regulation of its expression by administration of IL-10 or PTX could be of great clinical value. Recently, both agents have been shown to down-regulate TF expression efficiently in animal models of endotoxemia. It was not determined whether they acted directly on LPS-induced TF production or indirectly by decreasing levels of tumor necrosis factor, another TF inducer. Our in vitro results suggest that PTX and IL-10 could also be useful in the prevention of thrombosis and extravascular fibrin deposition associated with inflammatory diseases.

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