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Pentoxifylline *in vivo* and *in vitro* down-regulates the expression of the intercellular adhesion molecule-1 in monocytes

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SUMMARY

Since pentoxifylline (PTX) was recently recognized as a substance with antiinflammatory capacities, we studied the *in vivo* and *in vitro* effect of PTX on the expression of the intercellular adhesion molecule-1 (ICAM-1) on human monocytes. For this purpose four healthy volunteers were treated with PTX (5×400 mg/day) for 2 days. Monocytes were isolated before and after PTX treatment and ICAM-1 expression was investigated. As shown by fluorescence-activated cell sorter (FACS) analysis, cultured monocytes isolated after oral application of PTX expressed significantly decreased amounts of ICAM-1 when compared with monocytes collected prior to oral PTX application. Northern blot analysis revealed reduced amounts of ICAM-1 mRNA in monocytes derived from volunteers after oral PTX treatment in comparison with monocytes isolated before oral PTX administration. Similarly, in monocytes treated with PTX (200 μg/ml) *in vitro* ICAM-1 was found decreased both at the protein and mRNA level in comparison with untreated cells. The inhibitory effect of PTX on ICAM-1 expression in monocytes could be reversed by the addition of exogenous tumour necrosis factor-α (TNF-α; 200 U/ml) suggesting that ICAM-1 down-regulation is mediated secondary to TNF-α suppression by PTX. The specific role of TNF-α in mediating ICAM-1 expression in cultured monocytes could be confirmed by the finding that a neutralizing anti-TNF-α antibody partially down-regulated ICAM-1 expression. The observed suppressive *in vivo* and *in vitro* effects of PTX on ICAM-1 expression in monocytes may contribute to the recently described antiinflammatory effects of PTX, e.g. in sepsis or allergic contact dermatitis.

INTRODUCTION

Monocytes and macrophages play a major role in the generation of immune and inflammatory responses. Interactions of the intercellular adhesion molecule-1 (ICAM-1), which is expressed on monocytes, with its major ligand lymphocyte function-associated antigen-1 (LFA-1) expressed on T lymphocytes, is critical in the adhesion process between T cells and monocytes, thereby triggering immunological reactions.[1,2] Monoclonal antibodies specific for ICAM-1 (CD54) and also LFA-1 (CD11/CD18) have been shown to inhibit antigen-specific responses. Moreover, transfection of the human ICAM-1 gene into mouse L cells markedly augmented their capacity to induce antigen-specific proliferation of human T cells.[3–5] Lymphocytes and ICAM-1-positive monocytes present in granulomatous lesions of sarcoidosis are associated closely within the granulomatous tissue indicating that an antigen-driven immune response initiating ICAM-1 induction appears to be responsible for this disorder.[6,7] A marked increase in the number of ICAM-1-positive monocytes is found in renal biopsies of primary glomerulonephritis.[8] Elevated numbers of monocytes are also present in synovial fluid of rheumatoid arthritis[9] and express high amounts of ICAM-1 after cell contact with synoviocytes.[10] Monocytes from human immunodeficiency virus type 1 (HIV-1)-infected patients express increased levels of ICAM-1, the expression appears to correlate with the clinical stage.[11]

Taken together, there is solid evidence that ICAM-1 plays an important role in inflammatory and immunologic reactions. Therefore, strategies to down-regulate or block ICAM-1 expression might have therapeutic implications. In this sense, blocking of ICAM-1 by specific antibodies proved beneficial,

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Abbreviations: cDNA, complementary deoxyribonucleic acid; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; LFA, lymphocyte function-associated antigen; mAb, monoclonal antibody; mRNA, messenger ribonucleic acid; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PTX, pentoxifylline; rh, recombinant human; TNF-α, tumour necrosis factor-α.

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e.g. in rats suffering from glomerulonephritis[12] or in patients suffering from rheumatoid arthritis.[13] However, due to the secondary antibody response the practical value of the clinical use of antibodies is limited. Therefore, identification of drugs down-regulating ICAM-1 expression might offer new therapeutic strategies.

Pentoxifylline (PTX) has been of value in the treatment of vascular disorders because of its capacity to change the formability of erythrocytes.[14] Recently, PTX has also been found to be able to inhibit the release of inflammatory cytokines including tumour necrosis factor-α (TNF-α).[15–17] Since TNF-α is involved in the mediation of a variety of inflammatory disorders, PTX can be considered a potential candidate for the treatment of inflammatory diseases. Accordingly, it has been shown that PTX can inhibit the elicitation of murine contact hypersensitivity,[18] a process in which TNF-α is critically involved. Subsequently, a pilot study revealed that PTX can also reduce contact dermatitis reactions in humans.[19] Recently we could demonstrate that PTX can inhibit the release of interleukin-1β (IL-1β), IL-6 and IL-8 by peripheral blood mononuclear cells both *in vitro* and *in vivo*.[17] TNF-α is a strong inducer of ICAM-1 expression and according to its suppressive effect on TNF-α production PTX has been shown to inhibit TNF-α-induced ICAM-1 expression in cultured keratinocytes.[20] Through this property, PTX may additionally influence immunologic and inflammatory reactions. Therefore, we addressed the question of whether PTX can affect ICAM-1 expression both *in vitro*, as well as under *in vivo* conditions.

MATERIALS AND METHODS

Healthy volunteers

After obtained consent, four healthy volunteers were treated orally with 5 × 400 mg PTX per day (Hoechst Corp., Vienna, Austria) for 2 days. For isolation of peripheral blood mononuclear cells (PBMC) blood was withdrawn by venepuncture with heparinized syringes before the start and after completion of PTX treatment.

Preparation of PBMC

PBMC were obtained from healthy human volunteers by density gradient sedimentation via Lymphoprep (Nycomed AS, Oslo, Norway) and resuspended at a density of 1×10^6 cells/ml, cultured in six-well plates in RPMI-1640 (Gibco Corp., Grand Island, NY), supplemented with 5% fetal calf serum (FCS; Gibco Corp.) for 24 hr at 37° in a humidified 5% CO_2 atmosphere.

For *in vitro* studies, 1×10^6 cells/ml were incubated with 200 μg/ml PTX for 24 hr on plastic Petri dishes. As control, PBMC were treated under identical conditions but in the absence of PTX. Where indicated, PBMC were treated with recombinant human (rh) TNF-α (200 U/ml) or TNF-α plus PTX, or with a monoclonal antibody (mAb) directed against TNF-α, respectively, at a dilution of 1:20. Cells were cultured for 24 hr as described above and prepared for fluorescence-activated cell sorter (FACS) analysis.

Antibodies and cytokines

Recombinant human TNF-α, and a monoclonal antibody directed against human TNF-α were obtained from Genzyme Corp. (Cambridge, MA); mAb directed against ICAM-1 was obtained from Immunotech (Marseille, France), a mAb directed against keyhole limpet haemocyanin (KLH) obtained from Becton Dickinson (San José, CA) was used as an isotype control, a fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse $F(ab')_2$ fragment was obtained from Grub, Kaumberg (Austria).

FACS analysis

After an incubation period of 24 hr in six-well culture dishes cells were detached by a cell scraper and incubated with anti-ICAM-1 mAb (1:40; Immunotech, Marseille, France), or anti-KLH mAb (Becton Dickinson) as an isotype control for 30 min at 4°, washed, and incubated with a 1:20 diluted FITC-conjugated goat-anti-mouse $F(ab')_2$ (Grub, Kaumberg, Austria) secondary antibody for 30 min at 4° in the dark. Expression of ICAM-1 was analysed using a FACS Scan (Becton and Dickinson, Mountain View, CA). Data are given either as histograms (ordinate indicating number of cells, abscissa fluorescence intensity). The expression was determined as the difference between cells stained with anti-ICAM-1 and those stained with the isotype-matched irrelevant control antibody. Monocytes were stained with an anti-CD14 mAb (Becton Dickinson), contaminating lymphocytes were gated.

Northern blot analysis

PBMC were isolated before and after oral PTX treatment and resuspended at a density of 1×10^8 cells/ml. For *in vitro* studies, 200 μg/ml PTX were added to PBMC, or, as control, PBMC were left untreated. PBMC were cultured for 4 hr, non-adherent lymphocytes were discharged and adherent monocytes gently detached by the use of a rubber policeman. For detection of ICAM-1 a 3-kilobase *Sal*/*Eco*RI (kindly provided by T. A. Springer, Boston) and for control purposes a 3·6-kb *Hind*III human β-actin cDNA (kindly provided by D. Gallwitz, Göttingen, Germany) were labelled with ^{32}P using a random primer labelling kit (Boehringer Mannheim, Mannheim, Germany) and used for hybridization. Cells were homogenized by the use of a homogenizer (Ultra Turrax T125, Aigner Corp., Vienna, Austria). Total cellular RNA was isolated by precipitation with 3 M lithium chloride–6 M urea overnight at 4° and subsequent extraction with phenol–chloroform–isoamyl alcohol and quantified by reading at 260 nm. Thirty micrograms of RNA was electrophoresed on 1% agarose/2·2 M formaldehyde gels followed by transfer to nitrocellulose. Equivalent loading and uniform RNA transfer were assured by ethidium bromide staining of the gels before and after Northern transfer. Northern blot analysis was performed as described previously.[21] Autoradiography was carried out at −70° using Kodak XAR 5 films (Kodak Corp., Rochester, NY).

RESULTS

PBMC obtained from untreated individuals showed significant ICAM-1 expression upon *in vitro* culture for 24 hr. In contrast, PBMC obtained from volunteers treated with PTX orally for 48 hr revealed significantly reduced ICAM-1 expression. (Fig. 1). The inhibitory effect of PTX on ICAM-1 expression could be confirmed at the mRNA level showing significantly decreased ICAM-1 mRNA transcripts in monocytes obtained from PTX-treated volunteers (Fig. 2).

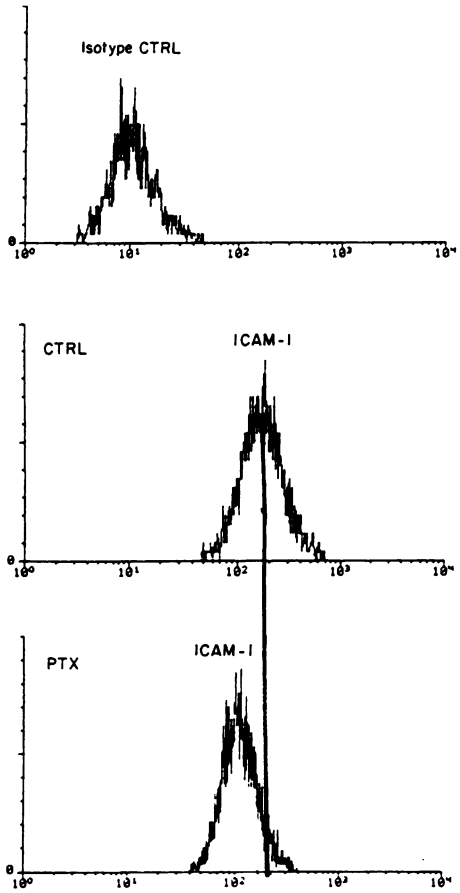

Figure 1. *In vivo* effect of PTX on the expression of ICAM-1 in monocytes. Volunteers were treated orally with PTX (5 × 400 mg/day) for 2 days. PBMC were obtained before and after PTX treatment and cultured for 24 hr. Adherent monocytes were evaluated for ICAM-1 expression by FACS analysis. An anti-KLH mAb was used as an isotope control. Contaminating lymphocytes were gated.

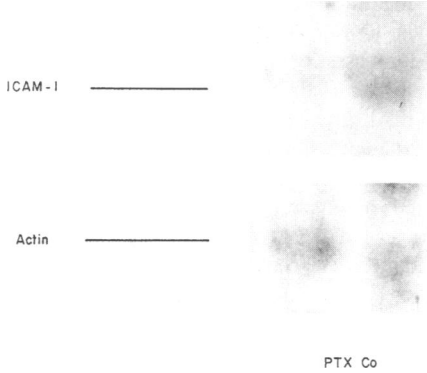

Figure 2. *In vivo* effect of PTX on ICAM-1 mRNA expression. PTX was applied orally 5 × 400 mg/day for 2 days. PBMC were isolated before (Co) and after completion of PTX treatment (PTX). After culturing for 4 hr total RNA was extracted from adherent monocytes and hybridized with a cDNA encoding for ICAM-1. Equal loading of RNA was determined by hybridization for β-actin.

Since PTX inhibits the release of TNF-α, which is known to induce ICAM-1, we addressed the question whether the reducing effect of PTX on ICAM-1 expression in monocytes might be mediated via inhibition of TNF-α release. As shown

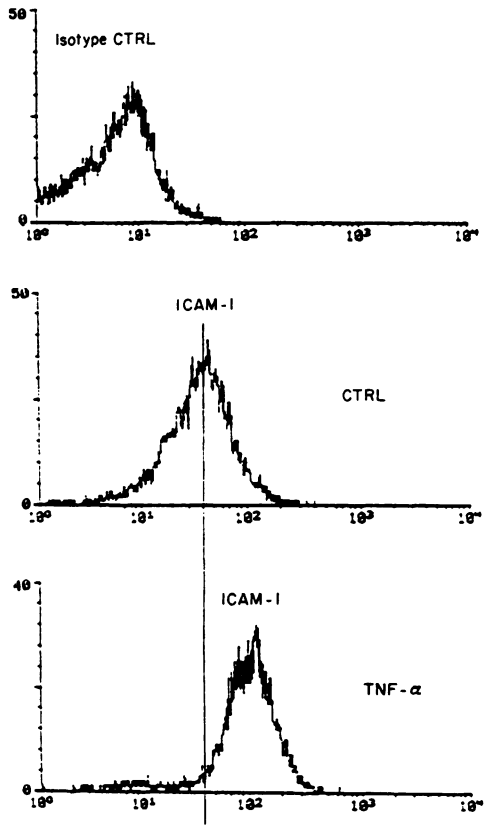

Figure 3. Effect of TNF-α on ICAM-1 expression in monocytes. PBMC were cultured for 24 hr in the presence of 200 U/ml rhTNF-α. As control, PBMC were left untreated. Adherent monocytes were evaluated for ICAM-1 expression by FACS analysis. An anti-KLH mAb was used as an isotype control. Contaminating lymphocytes were gated.

in Fig. 3, TNF-α up-regulates ICAM-1 expression in monocytes after a culture period of 24 hr. The *in vivo* effect of PTX on ICAM-1 expression in monocytes could be confirmed under *in vitro* conditions, where addition of 200 μg/ml PTX to cultured PBMC down-regulated the expression of ICAM-1 (Fig. 4). The reduction of ICAM-1 by PTX *in vitro* was also confirmed by Northern blot analysis (Fig. 5). As further demonstrated by FACS analysis, the down-regulatory effect on ICAM-1 expression by PTX could be almost completely reversed by addition of rhTNF-α, suggesting that PTX down-regulates ICAM-1 expression indirectly via inhibition of the release of TNF-α (Fig. 4). To further confirm that inhibition of autocrine TNF-α production by PTX might inhibit up-regulation of ICAM-1 expression, the effect of a neutralizing anti-TNF-α antibody on ICAM-1 expression in monocytes was investigated. FACS analysis revealed a partial down-regulation of ICAM-1 expression in the monocyte fraction treated with anti-TNF-α antibody (Fig. 6), suggesting that autocrine TNF-α production is involved in ICAM-1 induction in cultured monocytes.

DISCUSSION

Monocytes express significant levels of ICAM-1 after a culture period of 24 hr. In contrast, as recently reported, ICAM-1 is

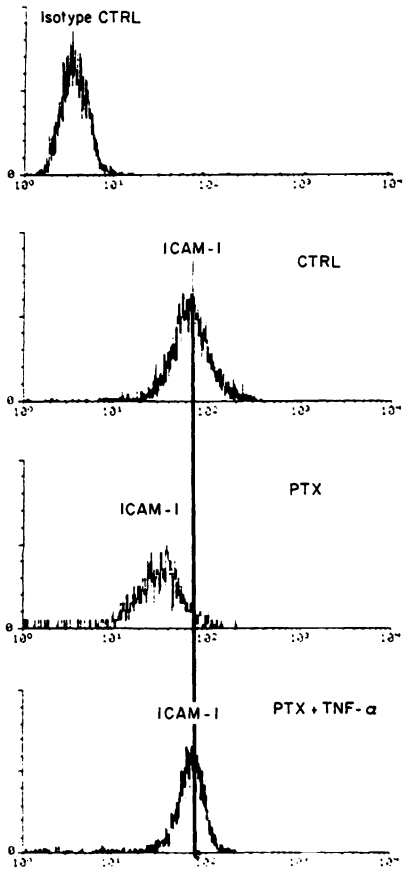

Figure 4. *In vitro* effect of PTX on the production of ICAM-1 in monocytes in the presence or absence of TNF-α. PBMC were cultured for 24 hr in the presence (PTX) or absence (CTRL) of PTX (200 μg/ml). Immediately after PTX treatment, rhTNFα was added (PTX+TNFα). Adherent monocytes were evaluated for ICAM-1 expression by FACS analysis. An anti-KLH mAb was used as an isotype control. Contaminating lymphocytes were gated.

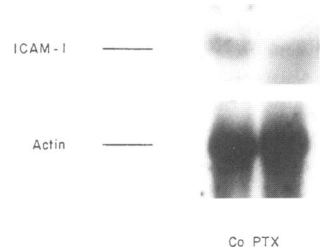

Figure 5. *In vitro* effect of PTX on ICAM-1 mRNA expression in monocytes. PBMC were cultured for 4 hr in the presence of PTX (200 μg/ml, PTX). As control PBMC were treated without PTX (Co). Total RNA was extracted from adherent monocytes and hybridized with a cDNA encoding for ICAM-1.

not detectable on freshly isolated monocytes.[22,23] Despite the absent ICAM-1 surface expression ICAM-1 mRNA transcripts are detectable in freshly isolated monocytes.[24] ICAM-1 expression induced by culture on plastic appears to be induced post-transcriptionally, since ICAM-1 mRNA levels remain unchanged during culture.[24] The suppressive *in vivo* and *in vitro* effects of PTX observed in this study suggest that PTX inhibits ICAM-1 at the transcriptional level. However, to date

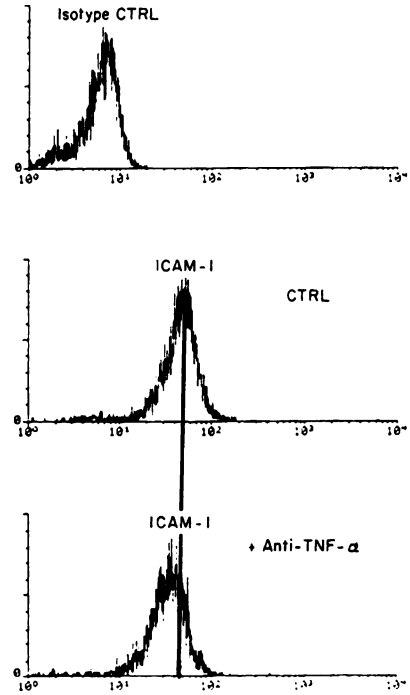

Figure 6. Effect of a neutralizing anti-TNF-α antibody on the expression of ICAM-1 in cultured monocytes. PBMC were cultured for 24 hr in the presence of anti-TNF-α (200 U/ml). As controls PBMC were cultured alone (CTRL). Adherent monocytes were evaluated for ICAM-1 expression by FACS analysis. An anti-KLH mAb was used as an isotype control. Contaminating lymphocytes were gated.

we cannot rule out that PTX affects ICAM-1 mRNA stability resulting in decreased amounts of ICAM-1-specific mRNA.

TNF-α is one of the mediators that induce ICAM-1 expression in a variety of cells including mono-myeloblastoid cell lines, keratinocytes, Langerhans' cells and endothelial cells.[20,25–27] In our system, PTX most likely inhibits ICAM-1 expression via TNF-α. This assumption is based on the facts that PTX suppresses TNF-α production; TNF-α reverses the inhibitory effect of PTX on ICAM-1 expression; and a neutralizing mAb directed against TNF-α could partially suppress ICAM-1 expression in untreated monocytes during culture. In accordance to the data obtained by Möst *et al.*,[24] a mAb directed against interferon-γ (IFN-γ), another well-known inducer of ICAM-1, had no effect on ICAM-1 expression (data not shown), confirming that IFN-γ does not appear to be an autocrine factor responsible for ICAM-1 induction in monocytes. However, the anti-TNF-α antibody was not as effective as PTX in inhibiting ICAM-1 expression in monocytes. This might be attributed to the fact that the TNF-α scavenging effect by the anti-TNF-α antibody was not sufficient to reveal a complete inhibition of the effect of TNF-α, while PTX completely blocks the effect of TNF-α by abrogating TNF-α mRNA production.[17]

Möst *et al.*[24] could show that addition of whole blood to cultured monocytes inhibits ICAM-1 expression, implying that a serum factor might be responsible for the inhibitory effect on ICAM-1 expression in circulating monocytes. Since PTX inhibits ICAM-1 expression in isolated monocytes under *in vitro* culture conditions as well, it is unlikely that the inhibitory

effect of PTX *in vivo* is because of induction of an ICAM-1 suppressor factor in the serum. Since monocytes were cultured in the presence of lymphocytes, one has to consider the possibility that the inhibitory effect of PTX on ICAM-1 expression is because of the effect of PTX on lymphocyte-derived mediators. Although lymphocytes do not seem to have an influence on the production of ICAM-1 in cultured monocytes,[24] it cannot be ruled out with absolute certainty that PTX might activate an ICAM-1 inhibitor derived from lymphocytes. Recently, IL-10 and IL-13 have been identified as lymphocyte-derived mediators, which are able to suppress cytokine and ICAM-1 production.[28,29]

The present study demonstrates that PTX both *in vitro* and *in vivo* down-regulates ICAM-1 expression. Since ICAM-1 is an adhesion molecule known to participate in a number of inflammatory reactions, this effect by PTX may contribute to its antiinflammatory capacity.

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