SPANDIDOS PUBLICATIONS

Influence of pentoxifylline on natural cytotoxicity and expression of granzymes and PI-9, a specific granzyme B inhibitor

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Abstract. Pentoxifylline (PTX) is an unspecific inhibitor of phosphodiesterase activity that increases intracellular concentration of cyclic nucleotides, mainly cAMP. Since PTX improves microcirculatory blood flow, it is commonly and often chronically used in peripheral vascular diseases. On the other hand PTX also displays a variety of immunomodulatory activities. PTX inhibits natural cytotoxicity and it has previously been suggested that it could partially act also through its influence on perforin/granzyme-dependent pathways. However, the underlying mechanisms are obscure and it remains unknown whether PTX inhibits natural cytotoxicity influencing only leukocytes or also acting on target cells. In this study, we show that PTX inhibits expression of granzyme A in human leukocytes probably due to suppression of phosphodiesterase activity. Contrary, PTX does not affect expression of granzyme B and H. On the other hand we hypothesized that PTX could inhibit natural cytotoxicity not only affecting leukocytes but also due to generation of resistance to leukocyte-mediated cytotoxicity in target cells e.g. through overexpression of PI-9, a specific granzyme B inhibitor. We found that at the mRNA level, PTX stimulates expression of PI-9 in K562 cells. However, we did not observe such an influence at the protein level, in either K562 cells or in human leukocytes. It may suggest that other PTX-triggered molecular events may interfere with PI-9 overexpression in these cells at the further, post-transcriptional levels. According to these results, PTX did not affect resistance of target cells to natural cytotoxicity. Altogether, PTX inhibits natural cytotoxicity affecting mainly effector but not target cells and in case of the effector cells, besides previously reported mechanisms, it can also inhibit granzyme A expression.

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Introduction

Pentoxifylline (PTX) is a methylxanthine derivative, which inhibits phosphodiesterase activity and increases intracellular concentration of cyclic nucleotides, mainly cAMP (1,2). PTX was shown to improve microcirculatory blood flow and tissue perfusion (3-5) through reducing fibrinogen plasma concentration (6) and blood viscosity (3), inhibiting platelet aggregation (7) and enhancing erythrocyte flexibility (8). Therefore, PTX is commonly and often chronically used in patients with peripheral vascular diseases (7,9), usually being well tolerated (9,10).

On the other hand, it has been shown that PTX may promote tumor development in several animal models (11-13). The reasons of this action have not been unequivocally established, although it has been suggested that it might be in part due to immunomodulatory properties of pentoxifylline. It has been shown that PTX disturbs leukocyte polarization and migration (14) and reduces leukocyte infiltration of tumors in murine model (15). Moreover, PTX decreases secretion of TNF (16), IL-12 (17) and IFN-γ (18) and inhibits natural cytotoxicity in humans both *in vitro* and *in vivo* (19,20). However, the mechanisms of inhibitory influence of pentoxifylline on natural cytotoxicity have not been elucidated in detail, especially in humans. It remains unknown whether besides its influence on cytokine- and perforin-dependent pathways (16,19) PTX may influence also granzyme-dependent cytotoxicity pathways. Hoskin and coworkers reported that PTX inhibits perforin and granzyme B expression in murine lymphocytes (21) but in humans the mechanisms of PTX action are to some extent different and PTX does not influence e.g. perforin expression (22), although it inhibits perforin-dependent natural cytotoxicity (19). Moreover, since influence of PTX on natural cytotoxicity was evaluated mainly in mixed cultures of effector and target cells (13,15,19), it is difficult to discriminate which cells are actually affected by PTX. As we have previously hypothesized (22), it cannot be

excluded that besides its previously established influence on leukocytes, PTX could inhibit natural cytotoxicity also through increasing resistance of target cells to lymphocyte-mediated killing. It has been shown that cancer cells overexpressing PI-9 (protease inhibitor 9), a specific inhibitor of granzyme B activity (23), are resistant to lymphocyte-mediated killing (24). Thus, the aim of the study was on the one hand to evaluate influence of PTX on granzymes expression in human leukocytes and on the other to establish influence of PTX on PI-9 expression and resistance of target cells to natural cytotoxicity.

Materials and methods

Chemicals. In all the experiments aqueous solution of pentoxifylline for intravenous injections (Pentohexal™, Hexal AG, Holzkirchen, Germany) was used. Isobutyl-methylxanthine (IBMX), phorbol 12-myristate 13-acetate (PMA), ionomycin (Iono) and dibutyryl cyclic AMP (dbcAMP) were purchased from Sigma (St. Louis, MO).

Cells. Human erythroleukemia-derived (K562) cell line (obtained from American Type Culture Collection) was maintained in suspension in RPMI culture medium (RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and antibiotic-antimycotic solution, all from Gibco, Paisley, Scotland) at 37˚C, 5% CO_2 in humidified air.

RNase protection assay (RPA). Freshly isolated human PBMC were obtained by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation of heparinized blood from healthy volunteers. Then PBMC (or K562 cells - for further semi-quantitative PCR analysis) were incubated for 6 h (or 12 h, data not shown) in RPMI-1640 culture medium supplemented with 10% FCS, 2 mM L-glutamine and antibiotic-antimycotic solution, according to the scheme: 1, control; 2, PTX (1 mM); 3, PTX (0.1 mM); 4, PMA (7 ng/ml) + Iono (350 ng/ml); 5, PMA (7 ng/ml) + Iono (350 ng/ml) + PTX (1 mM); 6, PMA (7 ng/ml) + Iono (350 ng/ ml) + PTX (0.1 mM); 7, dbcAMP (1 mM); 8, dbcAMP (0.1 mM); 9, IBMX (1 mM); 10, IBMX (0.1 mM).

Total RNA was isolated from PBMC (or also from K562 cells for PCR) using TRIzol preparation method (Gibco BRL, Grand Island, NY). RNA (2 μg) was then hybridized with riboprobe (25), generated using [α-^{32}P]-UTP (Amersham Pharmacia-Biotech, Buckinghamshire, UK), hAPO-4 template set and RPA Starter Kit, according to the protocol provided by manufacturer (PharMingen, San Diego, CA). After RNase digestion, the protected hybrids were separated on 4.5% polyacrylamide-denaturing gel and scanned by Molecular Imager FX (Bio-Rad, Hercules, CA). The length of obtained products was calculated by comparison to the standard curve, which was computed from the riboprobe fragments with known length, serving as a marker. The amounts of thus identified mRNA for perforin and L32 were analyzed using QuantityOne software (Bio-Rad). The experiment was performed twice and similar results were obtained. A similar trend was observed for 6- and 12-h incubations and only data from a representative experiment with 6-h incubation is further presented.

PCR. RNA from K562 cells isolated as briefly described above was used for standard semi-quantitative PCR analysis (26). PCR for human PI-9 was performed with 5'-TCT GCC CTG GCC ATG GTT CTC CTA-3'and 5'-CTG GCC TTT GCT CCT CCT GGT TTA-3' as primers at an annealing temperature of 58˚C with 30 cycles, yielding 475-bp product. For human ß-actin PCR was performed with 5'-TAC ATG GCT GGG GTG TTG AA-3' and 5'-AAG AGA GGC ATC CTC ACC CT-3' primers at an annealing temperature of 58˚C with 25 cycles, yielding 310-bp product. Each experiment was performed twice and similar results were obtained.

Western blot. The K562 cells or PBMC were cultured for 24 h (or 48 h, data not shown) with PTX, dbcAMP, IBMX or medium alone, according to the scheme: 1, control; 2, PTX (1 mM); 3, dbcAMP (1 mM); 4, IBMX (1 mM).

On the basis of the previous experiments concerning influence of PTX, dbcAMP and IBMX on PI-9 mRNA expression, the higher (1 mM) concentration of these chemicals was chosen for Western blot studies. After the incubation, cells were rinsed with cold PBS and incubated at 4˚C for 15 min with lysing buffer. Next the lysates (15 μl/sample) were separated by 4%/12% SDS/PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). Membrane was incubated with anti-PI-9 mouse antibodies (MONOSAN, cat. no. 2065) at 125 ng/ml before anti-mouse goat secondary antibodies (Dako, Glostrup, Denmark, cat. no. E0433) at 0.8 μg/ml were added (both incubations at room temperature for 3 h or 1 h, respectively). Then, membranes were incubated with streptavidin and biotinylated alkaline phosphatase (1:3,000) for 1 h at room temperature and afterwards NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) in DMF (dimethylformamide) were added. The reaction was stopped with distillated water. The internal control for protein loading was ß-actin expression measured by Western blot (primary antibody; Sigma-Aldrich, cat. no. A5441). Optical density was measured with GRAB-IT 2.0 Gel Documentation System (UVP, Cambridge, UK). Each experiment was performed twice and similar results were obtained. A similar trend was observed for 24- and 48-h incubations and only data from a representative experiment with 24-h incubation is further presented.

^{51}Cr release assay. K562 cells were incubated with PTX or dbcAMP at 1 mM or with medium alone for 24 h. Subsequently the cells ($2x10^5$ of cells per batch) were labeled for 90 min with 100 μCi of $Na_2{}^{51}CrO_4$ (POLATOM, Swierk, Poland) in 0.5 ml of RPMI culture medium (un)supplemented with PTX or dbcAMP at 1 mM, respectively. Afterwards the cells were washed three times by centrifugation in PBS, resuspended in RPMI culture medium and $2x10^4$ cells/well were seeded in triplicates in 96-well flat-bottom microtiter plates (Nalge Nunc Int., Denmark). Then, freshly isolated human PBMC were added to each sample in effector-target ratio (E:T) 100:1; 50:1 or 25:1, respectively (similar trend was observed at the each ratio and only representative graph for ratio 100:1 is further presented). Subsequently PTX or dbcAMP (both at the final concentration of 1 mM) were added. Each group included its own maximal and minimal cytotoxicity controls, which corresponded to cells lyzed by Triton X-100 (Sigma)

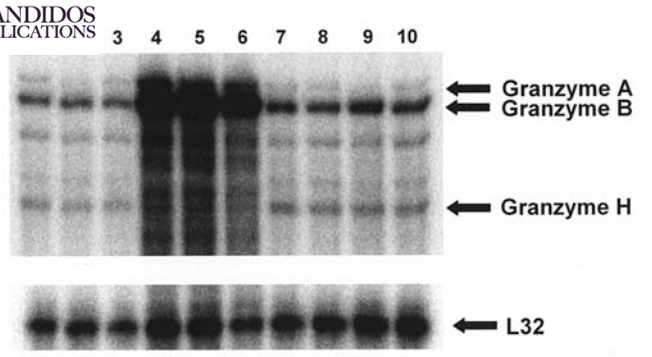

Figure 1. Expression of mRNA for granzymes A, B and H in human PBMC (6-h incubation), estimated using RNase protection assay. The following samples are shown: 1, control; 2, PTX (1 mM); 3, PTX (0.1 mM); 4, PMA (7 ng/ml) + Iono (350 ng/ml); 5, PMA (7 ng/ml) + Iono (350 ng/ml) + PTX (1 mM); 6, PMA (7 ng/ml) + Iono (350 ng/ml) + PTX (0.1 mM); 7, dbcAMP (1 mM); 8, dbcAMP (0.1 mM); 9, IBMX (1 mM); 10, IBMX (0.1 mM). The internal control for RNA loading was L32.

or RPMI medium-maintained culture, respectively. The samples were incubated for 4 h and the radioactivity of cell-free supernatants was measured by TriLux MicroBeta 1450 counter (Wallac, EG&G, Finland). The specific cytolysis was calculated as follows (27):

Cytotoxicity (%) = (test. cpm - min. cpm)/(max. cpm - min. cpm) x 100%

where test. cpm, radioactivity (counts per minute) of tested supernatants; min. cpm, radioactivity of supernatants of cells cultured in medium alone; max. cpm, radioactivity of supernatants obtained by incubation of cells with 2% Triton X-100.

Results

Using RNase protection assay we showed that after 6-h (or 12-h, data not shown) incubation, PTX and IBMX at the 1 mM concentration, but not dbcAMP, inhibit granzyme A expression in human PBMC, although, at the lower concentration they did not display such an influence (Figs. 1 and 2a). In contrast to granzyme A, PTX, IBMX and dbcAMP did not significantly affect expression of granzyme B (Figs. 1 and 2b) and H (Figs. 1 and 2c) in human PBMC at any concentration or incubation time.

To establish whether PTX could influence not only leukocytes but also target cells and affect their resistance to granzyme B, we investigated influence of PTX (as well as dbcAMP and IBMX) on expression of PI-9, a specific inhibitor of granzyme B activity, in human erythroleukemia cells - K562. Analysis using semi-quantitative PCR, revealed that PTX, dbcAMP and IBMX stimulated expression of mRNA for PI-9, and this influence was dose-dependent in all the tested substances (Fig. 3). Thus for further studies we chose the higher concentrations (1 mM) of PTX, dbcAMP and IBMX and we examined their influence on PI-9 expression in K562 cells at the protein level using Western blot technique. Unexpectedly, we did not observe a significant influence of PTX dbcAMP or IBMX on PI-9 expression in these cells (Fig. 4a). Since it protects against granzyme B hyper-

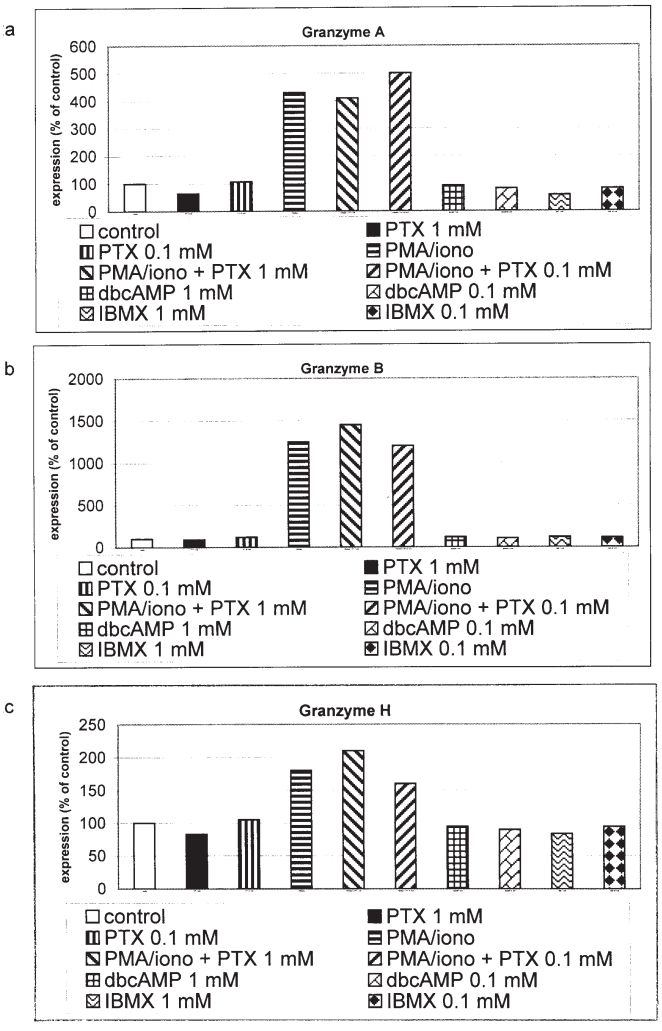

Figure 2. Expression of mRNA for granzymes A (a), B (b) and H (c) in human PBMC (6-h incubation), estimated using RNase protection assay, presented as the relative intensity of bands.

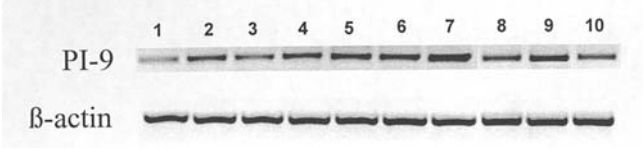

Figure 3. Expression of mRNA for PI-9 in K562 cells (6-h incubation), estimated using semi-quantitative PCR. The following samples are shown: 1, control; 2, PTX (1 mM); 3, PTX (0.1 mM); 4, PMA (7 ng/ml) + Iono (350 ng/ml); 5, PMA (7 ng/ml) + Iono (350 ng/ml) + PTX (1 mM); 6, PMA (7 ng/ml) + Iono (350 ng/ml) + PTX (0.1 mM); 7, dbcAMP (1 mM); 8, dbcAMP (0.1 mM); 9, IBMX (1 mM); 10, IBMX (0.1 mM). The internal control for RNA loading was ß-actin.

activity PI-9 is usually expressed in leukocytes (23), where the mechanisms regulating its expression might be different than in transformed cells. We studied its influence on PI-9 expression at the protein level also in human PBMC. Similarly to K562 cells, Western blot analysis revealed that the tested substances did not affect PI-9 expression in PBMC as well (Fig. 4b).

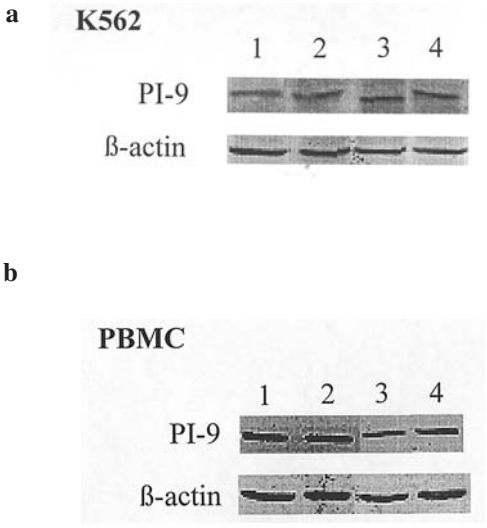

Figure 4. Expression of PI-9 at the protein level in K562 cells (a) and human PBMC (b) (24-h incubation), estimated using Western blot. The following samples are shown: 1, control; 2, PTX (1 mM); 3, dbcAMP (1 mM); 4, IBMX (1 mM). The internal control for protein loading was ß-actin.

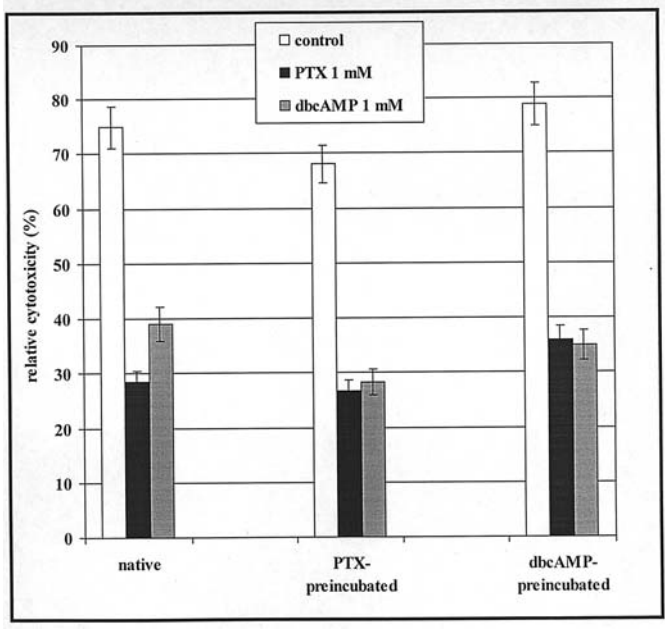

Figure 5. Influence of PTX and dbcAMP on natural cytotoxicity against K562 cells (4-h assay; effector:target ratio 100:1), which had previously been preincubated for 24 h with PTX (1 mM), dbcAMP (1 mM) or medium alone, measured by ^{51}Cr release assay. The graphs represent mean relative cytotoxicity (± SD) in appropriate groups.

To establish whether PTX or dbcAMP can increase resistance of target cells (K562) to natural cytotoxicity we performed ^{51}Cr release cytotoxicity assay. It has been found that 24-h preincubation with PTX (or dbcAMP) does not influence the resistance of K562 to natural cytotoxicity (Fig. 5). On the other hand both PTX and dbcAMP inhibited natural cytotoxicity when added to the mixed culture of effector and target cells, both in case of native and PTX- or dbcAMP-preincubated cells (Fig. 5).

Discussion

It has been widely reported that besides its beneficial influence on microcirculation, pentoxifylline displays immunomodulatory activities. Although it has previously been suggested that PTX may affect also perforin/granzyme-dependent cytotoxicity, the mechanisms of this action remain obscure.

In this study, we showed that pentoxifylline can influence granzyme expression in human leukocytes. However, whereas PTX inhibited granzyme A expression it did not reveal such an activity in case of granzyme H and more importantly granzyme B (Figs. 1 and 2), one of the most potent and best studied granzyme in humans (28). This effect seems to depend on phosphodiesterase inhibition but it may be independent from the changes in intracellular cAMP concentration. Another unspecific phosphodiesterase inhibitor - IBMX but not dbcAMP, a stable cAMP analog (Figs. 1 and 2) displayed an action similar to PTX. The differential influence of PTX on granzyme expression could result from the fact that genes encoding granzyme A and granzyme B/H are located on different chromosomes and probably their expression is regulated by different sets of factors (29,30). The functional significance of the described PTX-dependent inhibition of granzyme A expression in PBMC remains uncertain, especially in the context of the lack of influence on granzyme B/H expression. However, at least hypothetically, it could be partially involved in the PTX-dependent inhibition of natural cytotoxicity.

On the other hand, it seemed to be possible that PTX-dependent inhibition of natural cytotoxicity could be due to its influence on not only effector but also target cells. Since overexpression of PI-9, a specific granzyme B inhibitor, was shown to lead to increased resistance of cancer cells to leukocyte-mediated cytotoxicity (24), we studied influence of PTX on PI-9 expression in K562 cells. Analysis at the mRNA level confirmed that PTX is able to stimulate expression of PI-9 mRNA in these cells and this effect could be due to inhibition of phosphodiesterase activity and increase of intracellular cAMP concentration, similar results were obtained for IBMX and dbcAMP (Fig. 3). However, we have found that at the protein level PTX surprisingly did not influence PI-9 expression in K562 cells (Fig. 4). Similar results were obtained also in case of PBMC (Fig. 4), where PI-9 is natively expressed and serve probably as a protection against granzyme B hyperactivity. It may suggest that other, possibly PTX-initiated, molecular events may be responsible for the lack of PI-9 overexpression at the further, post-transcriptional levels. Moreover, it strongly argued against the possibility that PTX could lead to PI-9 overexpression in target cells and consequently to increase in their resistance to leukocyte-mediated cytotoxicity. In fact, we have finally confirmed and showed that inhibition of phosphodiesterase activity and/or increase of intracellular cAMP concentration does not influence the resistance of cancer cells to natural cytotoxicity (Fig. 5).

Altogether, it suggests that pentoxifylline inhibits natural cytotoxicity mainly through its influence on effector but not on the target cells, and in addition to the previously described mechanisms, inhibition of granzyme A expression might be possibly involved in this action as well.

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