Pentoxifylline inhibits perforin-dependent natural cytotoxicity in vitro

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Abstract. Pentoxifylline (PTX) is commonly used in peripheral blood vessel diseases, however it has also been found to decrease the level of proinflammatory cytokines such as IL-12, TNF-α and IFN-γ. Moreover, some authors reported that PTX suppresses spontaneous cytotoxicity of peripheral blood mononuclear cells (PBMC) in vitro. It could influence the mechanism of killing target cells by PBMC. For this reason we evaluated the influence of PTX on spontaneous cytotoxicity of PBMC against K562 and CaSki cell lines. Subsequently, we compared this effect to that evoked by dexamethasone, one of the most effective anti-inflammatory drugs. Our study revealed that PTX inhibits natural cytotoxicity preferentially through inhibition of perforin-mediated cell membrane damage, without a statistically significant influence on apoptosis induction. Furthermore, pentoxifylline inhibits natural cytotoxicity as effectively as dexamethasone. However, the result of PTX inhibitory influence is observed much earlier than that of dexamethasone. Currently PTX is commonly used in diseases that occur more frequently in elderly patients. We suggest that PTX, inhibiting perforin-dependent PBMC cytotoxic activity, could weaken anti-cancer action of immune system thus accelerating the progress of neoplasm formation in these patients.

Introduction

Pentoxifylline (PTX) is a methylxanthine derivative, which is commonly used in peripheral blood vessel diseases. It is a hemorheologic agent that increases erythrocyte flexibility, reduces blood viscosity and improves microcirculatory flow and tissue perfusion. It results in raised supply of oxygen to ischemic muscles of the limbs (1,2). Besides these well-known properties PTX has also been found to decrease secretion of inflammatory cytokines such as IL-12, TNF-α and IFN-γ (3-5), thus it could exert immunosuppressive activity (6,7). It has also been reported that PTX suppressed spontaneous cytotoxicity of peripheral blood mononuclear cells (PBMC) in vitro (8).

PBMC are able to kill target cells in two different ways. Firstly, by inducing apoptosis in target cells through TNF receptor family molecules and secondly, by perforin-dependent cell membrane damaging (9,10). However, it is unclear, which of these pathways could be affected by PTX. Therefore, we examined the influence of PTX on mechanisms of spontaneous cytotoxicity of PBMC and compared its effects to those evoked by dexamethasone (DEX), a well-known anti-inflammatory/immunosuppressive agent.

Materials and methods

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% CO₂ in humidified air.

Human skin cancer-derived adherent cell line (CaSki) was kindly provided by Dr P. Fuchs from Department of Virology, University of Koeln, Germany. The cells were maintained in Dulbecco's modified culture medium (DMEM supplemented with 10% FCS, 2 mM L-glutamine and antibiotic-antimycotic solution, all from Gibco). Cultures were tripinisized every 2-3 days, before they reached full confluence.
Chemicals. Pentoxifylline (Pentohexal™) was kindly provided by Hexal AG, Holzkirchen, Germany. Dexamethasone sodium phosphate (Dexaven™) was purchased from Jelfa, Jelenia Góra, Poland.

51Cr cytotoxicity assay. K562 or CaSki cells (2x10^5 cells per batch) were labeled for 90 min with 100 µCi of Na_2^51CrO_4 (Polatom, Swierk, Poland) in 0.5 ml of RPMI culture medium. 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 obtained by Ficoll-gradient centrifugation of heparinized blood from healthy volunteers were added to each sample in effector-target ratio (E:T) 100:1; 50:1 or 25:1, respectively. Subsequently PTX (at the final concentration of 0.1 mg/ml or 1 mg/ml) or DEX (at the final concentration of 0.01 mg/ml) were added. The used concentrations of both, PTX and DEX, corresponded to maximal concentrations achieved in vivo after intravenous administration in human. Each group included its own maximal and minimal cytotoxicity controls, which corresponded to cells lyzed by Triton X-100 (Sigma, St. Louis, MO) or RPMI medium-maintained culture, respectively. The samples were incubated for 4 h or 16 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: Cytotoxicity (%) = (test. cpm - spont. cpm)/(max. cpm - spont. cpm) x100% where: test. cpm - radioactivity (counts per minute) of tested supernatants, spont. cpm - radioactivity of supernatants of cells cultured in medium alone, max. cpm - radioactivity of supernatants obtained by incubation of cells with 0.5% Triton X-100.

Flow cytometry analysis. K562 or CaSki cells (2x10^4 per batch) were suspended in RPMI culture medium and mixed with 10^6 of PBMC in ratio (E:T) 50:1. In this proportion, as found in 51Cr release assay, the influence of PTX was the highest. Control samples of target cells without PBMC were also assayed. Next, PTX (the final concentration 0.1 mg/ml), DEX (the final concentration 0.01 mg/ml) or culture medium as control were added, respectively. The samples were incubated for 4 h. Then mixed cultures of cells were washed twice in cold PBS and incubated for 15 min with fluorescein-conjugated annexin V and propidium iodide (PI), according to the protocol provided by the manufacturer’s (R&D, Minneapolis, MN). Fluorescence of both target cells and PBMC was analyzed separately using FACS Calibur equipment with CellQuest software (Becton Dickinson, San Diego, CA).

Results

Our study revealed that PTX significantly decreased PBMC cytotoxic activity against K562 cell lines growing in suspension, as measured by 51Cr-release assay after 4 and 16 h...
We observed strong inhibitory activity of PTX already after 4 h and still remaining after 16 h. In case of adherent CaSki cells, due to their relatively low sensitivity to PBMC in general, the statistically significant inhibitory influence of PTX on PBMC cytotoxicity was observed only after 16 h (Fig. 1B). In both tested cell lines the observed inhibitory effect of PTX was dose dependent (Fig. 2). We also compared the effectiveness of inhibitory influence of PTX on PBMC to the dexamethasone, which has been used as a control immunosuppressive agent. The PTX inhibitory effect on PBMC cytotoxicity was visible already after 4 h, whereas DEX required much more time to show the effect of its influence. Moreover, the effectiveness of PBMC cytotoxicity inhibition by PTX after long incubation was comparable to that of DEX.

Flow cytometry analysis using Apoptosis Detection Kit revealed that both target cell lines were sensitive to PBMC in 4-h cytotoxicity assay. However, CaSki cell line was relatively more resistant to the cytotoxic influence of PBMC, as compared to K562 cell line. We observed that after 4-h incubation PTX significantly reduced the percentage of IP-stained target cells but not the percentage of annexin V-positive cells. In contrast, in 4-h assay DEX had no statistically significant effect on PBMC cytotoxicity (Fig. 3). Neither PTX nor DEX influenced the viability of target or effector cells.

**Discussion**

Among PBMC the cytotoxic activity is revealed by Tc lymphocytes, large granular lymphocytes (LGL) and monocytes. The last two populations, as well as some of Tc lymphocytes, demonstrate natural killer (NK) activity (11). NK cells play an important role e.g., in preventing tumor growth, especially in the early stage of oncogenesis (12,13). It seems that lowering NK activity may favor development of some types of neoplasms, e.g., leukemias, skin tumors (14,15). NK may kill target cells by causing rapid perforin-dependent lysis, or by inducing apoptosis (16). Necrotic cells with damaged cell membrane are stained with propidium iodide (17). Moreover, the result of this killing mechanism is visible in 51 Cr release assay very soon. On the other hand, apoptosis induction requires over a dozen hours to show the effect in 51 Cr release assay. However, the cells undergoing apoptosis may be detected much earlier since they express phosphatidylyserine on the outer leaflet of cell membrane and bind much more annexin V, than normal cells (17). Thus, the early stages of apoptosis are easily detectable using annexin V- and PI-binding assay, which also allows discrimination between apoptotic and necrotic cells.

Our study revealed that PTX inhibits natural cytotoxicity against non-adherent K562 cells, as well as against adherent CaSki cell line. PTX affects spontaneous cytotoxicity of PBMC preferentially through inhibition of perforin-mediated cell membrane damage, without a statistically significant influence on apoptosis induction. Although molecular mechanism of PTX action remains unclear, there are some reports suggesting its inhibitory activity on phosphodiesterase, thus increasing cellular level of cyclic nucleotides: cAMP and cGMP (18). Also, PTX seems to influence phospholipase C (19) or even NF-kB pathway (20). Moreover, Currie and coworkers demonstrated that PTX inhibits degranulation of neutrofiles, probably by influence on cytoskeletal architecture (21).

Numerous authors report that PTX inhibits expression of various cytokines, including proinflammatory and proapoptotic TNF-α (22) and LT-α (23). However, we did not find any statistically significant influence of PTX on PBMC-mediated apoptosis in target cells, as measured by annexin V-binding assay. Although this observation remains in contrast to reports of other groups, we did not observe any significant PTX influence on RNase protection assay-analyzed expression of CD95 (Fas) and its ligand CD95L (FasL) nor TRAIL and its receptors DR4 and DR5 in PBMC. Moreover, we did not observe any differences in expression of apoptosis pathway-engaged molecules such as FLICE (caspase-8) and FADD (data not shown).

In our study we compared the impact of immunosuppressive action of PTX and dexamethasone. Dexamethasone is well known as one of the most effective anti-inflammatory drugs exerting, like some other steroids, immunosuppressive activity...
(24). Immunosuppression weakens anti-tumor potential of the organism and favors tumor formation (25, 26). However, because of its mechanism of action (27), DEX requires more time than PTX to reveal its influence on cytotoxic activity. Surprisingly, the observed effect of PTX influence on PBMC after 16 h was comparable with DEX.

Recently it has been shown that PTX inhibits natural cytotoxicity in vivo in humans (28). Thus, it is possible that PTX could inhibit anti-tumor reaction of immune system, maybe even as potently as steroids. Currently PTX is commonly used in chronic peripheral blood vessels diseases occurring more frequently in elderly patients (29). The same group faces a higher risk of developing cancer than the rest of the population (30). On the basis of obtained results we suggest that PTX could allow the progress of neoplasm formation in such patients. However, such a hypothesis requires further prospective as well as retrospective study.

It is noteworthy that currently the presence of a tumor does not form a contra-indication for the use of PTX. It seems that in view of our data the risk of using this drug should be evaluated and contra-indications reconsidered, especially in patients treated for oncological reasons.

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References


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