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Antiproliferative Effect of Pentoxifylline on Psoriatic and Normal Epidermis

In vitro and In vivo Studies

A. GILHAR[1], N. GROSSMAN[2], S. KAHANOVICZ[3], H. REUVENI[2], S. COHEN[4] and A. EITAN[4]

[1]Skin Research Laboratory, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, [2]Investigative Dermatology Laboratory, Soroka Medical Center of Kupat Holim and Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, [3]Plastic Surgery Unit, Meir Hospital, Kfar Saba and [4]Teva Pharmaceuticals Industries Ltd., Netanya, Israel

Psoriasis is characterized by abnormal cell proliferation, inflammation and increased biosynthesis of various cytokines. The inhibitory effect of pentoxifylline on some cell functions has been reported widely. This property of pentoxifylline prompted an investigation of its possible role in controlling psoriasis. In the in vitro study normal human keratinocytes proliferation was determined and formation of cornified envelopes was assayed following treatment with pentoxifylline. The in vivo experiment consisted of nude mice grafted with psoriatic or normal skin treated with tetradecanyl phorbol 13 acetate. At the end of the treatment period, the grafts were excised and assessed for acanthosis and labelling index. The in vitro study showed that continuous exposure of normal human keratinocyte cultures to pentoxifylline resulted in a significant dose-dependent inhibition of proliferation, and in induction of cornified envelope formation. The in vivo experiments showed a significant reduction of epidermal thickness and of labelling index in psoriatic and tetradecanyl phorbol 13 acetate-treated normal skin, as compared to the initial values. *Key words: nude mice; autoradiography; tetradecanyl phorbol 13 acetate.*

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A. Gilhar, Skin Research Laboratory, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, POB 9649, Haifa 31096, Israel.

The xanthine derivative pentoxifylline (PTX) is a widely used vasoactive drug with clinical efficiency in various microcirculatory disorders (1). In recent years a potential use of pentoxifylline as an antiinflammatory drug has gained increasing interest. It has been found that a high concentration of PTX suppressed leukocyte function in vitro (2); it selectively inhibited cytokine release by peripheral mononuclear cells, and inhibited serum and IL-1B-proliferation of normal human dermal fibroblasts (3). In situ it activated fibroblasts from keloidal and scleroderma tous skin (4). Elevation of cAMP levels of the cells, following inhibition of phosphodiesterase (5), seemed to be an important factor in the mechanism of action of PTX.

These properties of PTX prompted an investigation of its possible role in controlling psoriasis. Psoriasis is a hyperproliferative disease characterized by abnormal cell proliferation and differentiation as well as by inflammation (6). Within psoriatic lesions this is increased biosynthesis of various cytokines, including tumour necrosis factor alpha (TNF-α). Nickoloff (7) has hypothesized that TNF-α plays a crucial role in the psoriatic pathology. PTX can antagonize TNF-α production and activity (8).

The question raised in the present study was whether PTX has a beneficial effect on psoriatic skin. This question was addressed in two experimental systems: in vitro, using proliferative normal human keratinocytes cultured over a feeder layer of normal human fibroblasts, and in vivo, employing congenitally athymic nude mice grafted with human psoriatic skin, or with long-term tetradecanyl phorbol 13 acetate (TPA)-treated normal human skin, which showed a similar epidermal proliferation as seen in psoriatic skin (9). The antiproliferative effect of PTX has been demonstrated in all experimental systems, suggesting a potentially beneficial effect against psoriasis.

SUBJECTS AND METHODS

In vitro study

Materials. Tissue culture media, sera, antibiotics and trypsin were from Biological Industries (Beth Haemek, Israel); growth factors and chemicals were purchased from Sigma (St. Louis, MI). Tissue culture plasticware was from Costar (Cambridge, MA).

Cells, growth media and growth conditions. Normal human epidermal keratinocyte cultures were initiated from young foreskins (1 to 4 years old) according to Rheinwald & Green (10). Normal human fibroblast cultures (NHF) were initiated from dermal explants. Normal human keratinocyte (NHK) cultures were propagated on metabolically active NHF, pretreated with mitomycin C (4 μg/ml, 2 h in the dark, 37°C) (MC-NHF). NHK cultures at second to fourth passage were used in all experiments. Growth medium for keratinocytes was a slight modification of Green's growth medium (11) and comprised of DMEM: F12 (3:1), 8% FCS, glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (50 μg/ml); adenine (0.18 mM), hydrocortisone (0.1 μg/ml), transferrin (5 μg/ml) and insulin (5 μg/ml). Cells were refed with fresh medium every other day. Fibroblasts were maintained in DMEM-based medium containing 10% FCS, glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml) and were refed twice weekly. Cultures were incubated at 37°C, 8% CO_2, 95% humidity.

The in vitro experimental system. The in vitro experimental system comprised normal human keratinocytes growing on a feeder layer of normal human metabolically active fibroblasts (MC-NHF) (12). MC-NHF were seeded in 96-well plates at 8×10/well. Two hours later, proliferative keratinocytes were seeded at 1.5×10/well, in 200 μl of growth medium. Cells were incubated at 37°C, 8% CO_2. The first treatment with drugs (time zero) was started on the third day after cultures were seeded. Each treatment was performed in triplicate. Cells were refed every other day. Cell proliferation was determined after treatment for 48, 72, 96 and 160 h. Formation of cornified envelopes was assayed after 160 h of treatment.

Cell proliferation assay. Cells were counted manually in a counting chamber (Fuchs-Rosenthal, Germany) under a phase contrast microscope, after a selective removal of feeder cells with EDTA (0.02%) in calcium and magnesium-free PBS (13). Keratinocytes were suspended in 100 or 200 μl of trypsin (0.25% plus EDTA 0.05%). At least three fields were counted from each well. In preliminary experiments, viability was assessed by trypan blue exclusion. The number of dead

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cells per well in cultures treated with 0.1–2.5 mM PTX for 72 h never exceeded the number of dead cells in untreated cultures (1067 ± 753/well vs. 1041 ± 427/well, respectively; mean $\pm$ SD of three experiments). This stable rate of viability (representing $0.78 \pm 0.29\%$ of the initially seeded cells) indicated that PTX was not cytotoxic to the cells, and that total counts may be used to quantitate the effect of PTX on the cells.

Cornified envelopes assay. Keratinocytes grown following treatment for 160 h were collected from the wells, in 200 µl of trypsin, after the feeder cells had been selectively removed. Total cell number was determined using up to 25 µl. Cornified envelopes were prepared from the rest of the cells as follows: 160 µl aliquots were pelleted in a minifuge (3 min, 15,000*g*). Pellets were resuspended in 40 µl of SDS (1%) and β-mercaptoethanol (0.2%), and after 5 min boiling envelopes were counted (14). For each well, the percentage of cells forming envelopes was calculated.

In vivo study

Subjects. Seven patients (5 men, 2 women, aged 34–67 years) with generalized psoriasis and 6 normal, healthy human subjects (4 men, 2 women, aged 37–57 years) who underwent cosmetic procedures served as skin graft donors. The extent of skin lesions was noted in each case. All psoriatic lesions had been relatively stable for 4 weeks and had not received systemic or topical treatment for at least 4 weeks prior to skin observation. Informed consent was obtained from each patient before split thickness skin grafts were taken from the patients. Areas of skin bearing established plaque-type psoriatic lesions were selected on the legs or forearms. Split thickness skin samples of 0.4 mm thickness, 1.5×1.5 cm in diameter, were obtained under local anaesthesia with 1% lidocaine, using a dermatome (Brown Electro-Dermatone model 666, Zimmer USA, Warsaw, IN).

Mice. Congenitally athymic Balb/C nude mice, 2–3 months of age, were obtained from the pathogen-free animal facility at the Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa. The mice were grafted with one of two types of skin: normal human skin or skin from psoriatic subjects, involved with disease.

Grafting procedures. The split thickness skin graft was transplanted onto the subcutaneous tissue over the lateral thoracic cage of the mouse, as previously described. Graft sites were covered with petroleum-impregnated gauze and standard adhesive bandage and surgically stapled to the ventral surface of the animals.

Treatment

Psoriatic skin grafts. Split thickness skin grafts of the psoriatic patients were grafted onto the nude mice. Three mice received skin of the same patient. Three groups of 7 mice each were included in this study. Following a 7-day healing period (i.e. on day 8), each group of psoriatic skin-grafted mice was treated with the following agents: Group A: vehicle (hydrophilic ointment; 0.1 g); Group B: 2% PTX (2% PTX was chosen on the basis of preliminary experiments); Group C: betamethasone 17 valerate 0.01% (Betnovate cream, Glaxo) 0.1 g. All agents were administered by painting the grafted area once every 12 h for the next 7 days. On day 15, the day following the end of the 7-day treatment period, the animal was sacrificed and the entire graft was excised for histological and autoradiographic evaluation.

Normal skin grafts. Following complete healing (i.e. 1 week after normal human skin engraftment), TPA (2%) (Sigma, St. Louis, MO) was applied on the grafted area in 0.05 ml. The TPA was solubilized in a vehicle composed of equal parts of DMSO and acetone (D/A) (20). Because of the potential problem of transfer of agents among animals by direct contact, the treated areas were occluded with bandaids. After drying, tested compounds were applied on the gauze pad of bandaid and applied on the graft site. The TPA application on human skin grafted onto nude mice, combined with the application of the tested compounds, was performed for 6 alternate days. The mice were divided into 4 groups, each one containing 6 mice. One group received TPA alone, and the other 3 groups received TPA and 2 h later the grafts were painted with the tested compound. Each group received a different compound, as follows: Group A: TPA alone, Group B: TPA plus vehicle, Group C: TPA plus 2% PTX, Group D: TPA plus betnovate. On day 13 following first TPA application, the entire graft was excised for histological and autoradiographic evaluation.

Histological and autoradiographic analysis

Psoriatic grafts. Two-mm sections were obtained from each psoriatic graft prior to skin engraftment in order to assay the rate of proliferation by autoradiography ([^{3}H]-thymidine incorporation) and to determine the epidermal thickness of each graft (16). At the end of the treatment period (day 13) the entire graft was excised and was used to assess changes in the rate of proliferation by autoradiography and sectioned for microscopic histological assessments.

Assessment of acanthosis and labelling index were determined and calculated by two independent observers who were unaware of the various treatment groups. Quantitation of the epidermal mass (acanthosis) was performed with an ocular micrometer by measuring 20 points in each specimen. Histological assessment was by routine light microscopy sections that were obtained from each graft before and after transplantation. Autoradiographic sections were obtained separately in order to avoid difficulties in histological assessment because of a possible background deposition. Incorporation of [^{3}H]-thymidine and determination of labelling index was performed as previously described (16). Briefly, before and after grafting, pieces of tissue (1–2 mm) were incubated for 4 h in a shaking incubator at 37°C with 4 µl [^{3}H]-thymidine in 2 ml RPMI, specific activity 20 Ci/mmol at a concentration of 25 mCi/ml (New England Nuclear, Boston, MA). Autoradiographs were prepared using the dipping technique (16), and the epidermal labelling indices given in the results indicate the percentage of labelled cells counted in 1,000 basal cells.

Statistical assessment

The data were analysed by multiple analysis of variance, i.e. one-way or two-way analysis of variance, with appropriate post test as needed.

RESULTS

In vitro study

Effect of PTX on proliferation of normal keratinocyte cultures. Proliferation of NHK cultures was inhibited by PTX with a dose-dependent kinetics versus the untreated control cultures. The percentage of inhibition due to PTX was calculated for each experimental condition, and a summary of four such experiments is depicted in Fig. 1A. Growth inhibition by 0.1 mM PTX was less than 10% after 48 h of treatment and became significant (ANOVA, $p < 0.001$) only after 72 h of treatment. An earlier and greater effect was observed at higher PTX concentrations. Thus, significant inhibition of $36.9 \pm 5.9\%$ and $47.6 \pm 4.8\%$ (ANOVA $p < 0.01$) was observed after 48 h of treatment with 0.5 mM and 1 mM PTX, respectively, and increased further with time (Fig. 1A). However, the final growth inhibition after 160 h of treatment with PTX (in 3 experiments) was dose-dependent as well: $38.5 \pm 1.0\%$, $54.1 \pm 8.8\%$, and $76.6 \pm 9.8\%$ at 0.1 mM, 0.5 mM and 1 mM, respectively. The calculated IC_{50}, after 72-h treatment, was 0.55 mM. Proliferation of NHK growing without a feeder layer of MC-NHF was inhibited by PTX in identical dose-response curve and kinetics with time (not shown). The calculated IC_{50}, after 72-h treatment, was 0.8 mM. The anti-proliferative effect of PTX was not due to lethal toxicity to the initially seeded NHK, as the number of dead cells, visualized by trypan blue dye exclusion, in cultures treated with 0.1–2.5 mM PTX for 72 h never exceeded the number of dead

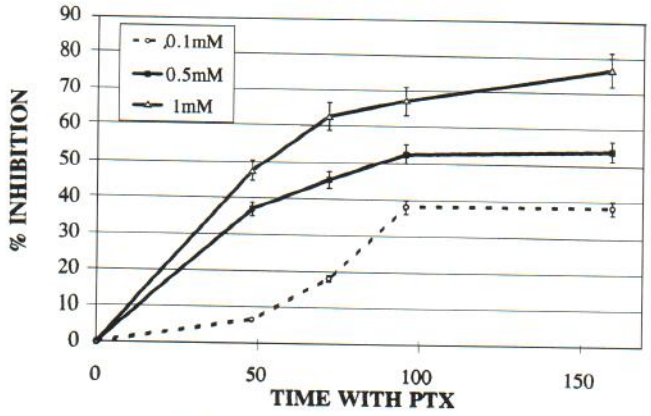

Fig. 1. Effect of PTX on the proliferation of cultured normal human keratinocytes (NHK). NHK were treated with varying concentrations of PTX, beginning 48 h after seeding and every 48 h thereafter. At various time points following the first exposure, keratinocytes were removed from the wells using trypsin, after selective removal of the fibroblast feeder layer. Cells were quantitated microscopically and per cent inhibition was calculated vs. untreated control. Assays were performed on triplicate cultures counted three times. The results of four experiments are presented as means ±SD. Kinetics of inhibition by 0.1 (○), 0.5 (■), and 1.0 mM (△) PTX.

cells in untreated cultures. In addition, there was no reduction in the number of cells in the PTX-treated cultures below the initial number at seeding, indicating that PTX had no lytic effect on the cells. Neither was there a shut-off of the metabolic activity of the treated cells, since the colorimetric response of PTX-treated cells in MTT assay was inhibited to a lesser extent than their proliferation. (For example – the proliferation of cells treated with 1 mM PTX for 72 h was inhibited by 59.17±6.16%, while their MTT response by only 32.2±93% vs. untreated cells).

Effect of PTX on differentiation of normal keratinocyte cultures. To examine whether PTX induced differentiation in NHK, cornified envelopes were purified from NHK treated for 160 h with 0.5 mM or 1 mM PTX. The method used to purify cornified envelopes was based on their resistance to heating in a solution of SDS/β-mercaptoethanol (14). The percentage of cornified envelopes appearing in these cultures was taken as the degree of differentiation. The relative induction of differentiation was calculated for each experiment, as the ratio of percent cornified envelopes in untreated wells. The results of three experiments are summarized in Table I. As shown, the degree of cornified envelopes formation was very low in the untreated control, although the cells had been growing in 1.8 mM Ca^{++}. It is also clear that PTX induced cornified envelope formation significantly, in a dose-dependent fashion, in treated keratinocytes: 0.5 mM PTX induced a 2.5-fold increase in cornified envelopes (ANOVA, $p<0.01$) and 1 mM PTX induced an 8.4-fold increase in cornified envelopes (ANOVA, $p<0.001$).

In vivo study

Psoriatic grafts – epidermal thickness. The initial mean epidermal thickness increased with the vehicle from 108±31 μm to a mean of 146±24 μm, an increase of 30.4% (Tukey-Kramer multiple comparison test, $p<0.05$). Four out of 7 grafts showed a marked increase of epidermal thickness.

Table 1. *Induction of cornified envelope (CE) formation by pentoxifylline*

CE were purified from keratinocytes treated for 160 h with varying PTX concentrations, according to Sun & Green (18). The per cent of CE formed was calculated relative to total NHK in each well, and the relative induction of CE formation by PTX was calculated versus untreated controls. Results are a summary of three experiments (mean±SD).

PTX concentration (mM)	% CE formed	Relative induction of CE formation	p*
0	0.11±0.03	1	
0.5	0.28±0.01	2.56±0.87	<0.01
1.0	0.90±0.08	8.37±1.36	<0.001

*p with respect to 0 mM PTX.

However, all grafts treated with either 2% PTX (group B), or betnovate (group C) showed a marked reduction in epidermal thickness. The mean epidermal thickness of group B treated with 2% PTX was 75±13 μm, and the mean epidermal thickness of group C treated with betnovate was 80±12 μm, a mean reduction of 30.5% and 25.9%, respectively, compared to grafts treated with vehicle (group A). In group B, 5 out of 7 grafts showed reduced epidermal thickness as compared to the initial values, whereas in group C a reduction was noted in 6 out of 7 grafts. Grafts treated with PTX showed a significant reduction of the epidermal thickness at the end of the topical application, as compared to the initial values (Tukey-Kramer multiple comparison test, $p<0.05$). A non-significant reduction of epidermal thickness was observed in grafts treated with BV as compared to the initial value (ANOVA with Tukey-Kramer multiple comparison test).

Psoriatic grafts – autoradiography. The labelling index of grafts treated with 2% PTX (group B) or betnovate (group C) was markedly reduced, as compared to the initial values (multivariate analysis of variance (MANOVA), $p<0.01$ and $p<0.0053$, respectively). The mean labelling index of groups B and C was similar (labelling index = 3.8±0.7 and 2.9±0.7, respectively). The labelling index evaluation did not show a reduction of mean value of group A (vehicle) as compared to the initial values (10±6.2 versus 12.9±5.7). Thus, the vehicle failed to induce an inhibitory effect on epidermal proliferation, contrary to a marked effect observed with PTX or betnovate. MANOVA was performed on the data in order to use both epidermal thickness and labelling index parameters in detecting differences between the three treatment groups. The results demonstrated a significant correlation between epidermal thickness and labelling index ($r=0.65675$, $p<0.0012$, $n=21$). A significant difference was found between the three treatment groups: vehicle versus before, $p<0.0254$, vehicle versus PTX, $p<0.0130$, and versus betnovate, $p<0.0161$.

Normal skin grafts treated with TPA. The results of this study indicated that group B (TPA+vehicle) did not inhibit the effect of TPA on epidermal proliferation. The mean labelling index of group B was similar to that of group A (mice treated with TPA alone). Treatments with 2% PTX (group C) and betnovate (group D) caused a striking reduction of labelling index, and a significant difference was found between the mean labelling index of these groups and the vehicle group (MANOVA, $p<0.001$ and $p<0.001$, respectively). Betnovate inhibited further and more significantly the proliferative effect

of TPA, in comparison to PTX, and a significant difference was found between the mean labelling index of both groups (labelling index group C treated with 2% PTX, 4.2 ± 0.8, versus group D treated with betnovate, 2.6 ± 0.54, MANOVA, $p < 0.05$).

DISCUSSION

The present study shows an in vitro and in vivo inhibitory effect of PTX on proliferation of normal and psoriatic keratinocytes. PTX is a methylxanthine having a phosphodiesterase inhibitory potential (1). The compound is well known for its inhibitory properties on various functions of neutrophils, platelets, lymphocytes and polymorphonuclear cells, probably via the preservation of intracellular cAMP (2–5, 17). In addition, it was recently found that PTX, in a concentration range similar to that used in our study, blocked TNF-α RNA levels and interleukin-1 (IL-1) accumulation in murine and human leukocytes (8, 18). PTX was shown to inhibit TNF-α-induced intracellular adhesion molecule-1 (ICAM-1) expression on keratinocytes and Langerhans' cells (19). Recently, Schwarz et al. (20) demonstrated a suppressive effect of PTX on the elicitation phase of contact hypersensitivity. Taken together, these data demonstrate an anti-inflammatory effect of PTX. Furthermore, an in vitro exposure to PTX resulted in a prominent reduction in fibroblast proliferation without lethal toxicity (4).

The results presented in this study demonstrate that in vitro exposure of normal human keratinocytes to PTX resulted in a dose-dependent inhibition of cell proliferation, without lethal toxicity, during the first 48 to 96 h of treatment (Fig. 1A), and in increased differentiation, measured by percentage of cornified envelopes formed after 160 h of treatment. The calculated IC_{50} for NHK grown on a feeder layer of fibroblasts (MC-NHF) was 0.55 mM after 72 h of treatment and that for NHK grown without a feeder layer was 0.8 mM (not shown). The latter result indicated that PTX affected keratinocyte proliferation directly, and that these cells were slightly more sensitive to PTX than NHF, for which an IC_{50} of 2 mM, following 96 h of treatment, was reported (4). The difference in IC_{50} at 72 h between the two in vitro systems of NHK might reflect the difference in growth rate of the cultures in those experiments (a mean generation time of 31.9 ± 3.6 h for NHK grown on a feeder layer and 47.2 ± 5.5 h for NHK grown without feeders). However, the possibility that the higher sensitivity of NHK to PTX, when grown over metabolically active NHF, was in part due to an added effect of modulation of fibroblasts-derived factors by PTX, cannot be excluded. The present in vivo experiments strongly confirm the antiproliferative effect of PTX. Topical application of the drug significantly reduced the proliferative rate of psoriatic and normal TPA-treated skin grafted onto nude mice. Engraftment of psoriatic skin onto nude mice leads to regression of most of these histological features, including reappearance of the granular layer of the epidermis. However, the aberrant epidermal hyperplasia characteristic of psoriasis remains and is detectable as increased labelling index and epidermal thickness (21). In agreement with other studies, we found that on day 15 post engraftment the involved psoriatic skin maintained many of its psoriasiform features such as papillomatosis and acanthosis (21). The labelling index for involved epidermis was significantly higher than for normal skin (data not shown). This study showed a significant difference between the labelling index of the vehicle-treated grafts as compared to the PTX ($p < 0.01$) and to the betnovate-treated grafts. A marked reduction of labelling index was noted in the groups treated with PTX and betnovate as compared to the initial values. Similarly, reduced epidermal thickness was observed in both the PTX- and betnovate-treated groups. Many of the biochemical and histological features of psoriasis resemble those of TPA-treated skin (9). Prolonged in vivo TPA treatment resulted in a marked stimulation of keratinocyte proliferation. PTX and betnovate inhibited significantly the TPA-induced proliferative effect in a manner similar to that observed in the psoriatic grafts. Glucocorticosteroids are known as potent inhibitors of the proliferation induced by TPA (22). Indeed, a more significant suppressive effect was noted in the betnovate-treated group than the PTX-treated group. The anti-proliferative effects of PTX observed in these in vivo and in vitro studies suggest that the drug might be of potential use as an antipsoriatic agent. Furthermore, the inhibitory effect of PTX on TNF-α and of IL-1 release, two mediators shown to be produced by keratinocytes (23), may also contribute to the efficacy of PTX in their control (24, 25). The beneficial effect of PTX on psoriatic lesions observed in the current study warrants clinical studies on its potential usefulness in the treatment of psoriatic patients.

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