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INFLUENCE OF CORTICOSTEROIDS ON CHEMOTACTIC RESPONSE AND COLLAGEN METABOLISM OF HUMAN SKIN FIBROBLASTS*

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Abstract—Following chronic administration of corticosteroids *in vivo*, a number of complications occur, which mainly involve the metabolism of connective tissue cells. Therefore, several attempts have been made to develop corticosteroids, which show less pronounced side effects. Fibroblasts were kept in monolayer cultures and were exposed to corticosteroids demonstrating similar anti-inflammatory activity (prednicarbate, desoximetasone). Chemotaxis of fibroblasts was studied over 4 hr, protein and collagen synthesis were estimated using proteinchemical methods and also by dot blot hybridization. Corticosteroids used in a high dosage (10 μM) affected all biosynthetic capacities of the investigated fibroblasts. Protein synthesis and production of collagen types I and III were reduced and a similar decrease of mRNA levels for collagen type I could be found indicating an influence on the pretranslational control. In the same concentrations desoximetasone was much more active than prednicarbate. Fibroblast migration was dosage dependently inhibited from 10^{-9} M to 10^{-5} M for desoximetasone, while incubation with prednicarbate did not cause a reduction of the chemotactic response at concentrations lower than 10^{-7} M.

These data suggest that modifications of corticosteroids might result in a dissociation of some of their biological activities and can specifically influence their effects on biosynthetic capacities of fibroblasts.

Topical corticosteroids are commonly used in the treatment of a variety of dermatologic diseases [1]. Although their benefit is unquestionable, it soon became clear that the long-term use of potent corticosteroids induces profound side effects mainly by influencing the dermal connective tissue. Thinning, atrophy, induction of striae, or impaired wound healing has been reported [2].

Therefore the effects of corticosteroids on cultured dermal fibroblasts have already been thoroughly investigated [3–5]. It is well established that corticosteroids inhibit cell proliferation and mitosis [6], decrease synthesis of glycosaminoglycans [7] and have an inhibitory effect on general protein and collagen synthesis [8]. This has been investigated in detail on a molecular level and was found to be accompanied by a reduction of mRNA levels due to reduced transcription [9–11] and increased halflife time of type I procollagen mRNA [12]. These effects on cellular functions have been thought to sufficiently explain side effects on skin and connective tissue observed after long treatment with topical corticosteroids.

Fibroblasts also show a chemotactic response to a variety of mediators, which play an important role in wound healing and the formation of repair tissue [13]. Inhibition of the directional movement of fibroblasts into the injured tissue by corticosteroids would certainly severely affect tissue repair in wounds. There are various test systems available, which enable us to study the chemotactic response of fibroblasts *in vitro* and it was the aim of the present study to investigate whether corticosteroids can interfere with the movement of fibroblasts. In addition, during the last years several new corticosteroids have been introduced into clinical trials and have also been investigated concerning their inhibitory activity on collagen and protein synthesis in fibroblasts. Although several of these substances could be shown to be very powerful agents [14], high anti-inflammatory activity was usually associated with increased reduction of fibroblast metabolism [4]. Nevertheless, recently a component has been developed which structurally differs from the fluorinated corticosteroids but also shows a high anti-inflammatory activity [15].

Therefore, in this report we compare the activity of various corticosteroids including newly developed derivatives on chemotactic activity and collagen synthesis of human skin fibroblasts.

MATERIALS AND METHODS

Prednicarbate, hydrocortisone, desoximetasone were obtained from Dr Höhler (Cassella-Riedel). Betamethasone-17-valerate and betamethasone-17-propionate were gifts from Glaxo.

Cell culture. Fibroblast cultures were established from biopsies obtained from human skin and were used in 4th–8th passages. The cells were kept in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin (400 U/ml) and streptomycin (50 μg/ml), glutamine (400 μg/ml) and ascorbate (50 μg/ml) and subcultivated using

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Trypsin (0.05%) and EDTA (0.02%) in PBS.

Corticosteroids were dissolved in 40% ethanol and then added to the cell culture medium. Equivalent amounts of ethanol were used for controls. Cultures were examined by phase contrast microscopy. After 1, 3 and 5 days cell numbers were determined in triplicate cultures.

Determination of synthesis of collagenous and non-collagenous proteins. Radioactive labelling of cells was performed on 3-day-old cultures with L-(2,3)-^{3}[H]proline (10 μCi/ml) in Dulbecco's modified Eagle's medium (DMEM) for 24 hr. Corticosteroids were added as indicated previously for 3 days to the culture medium. After a preincubation of 24 hr in DMEM with 10% fetal calf serum and corticosteroids but without streptomycin, incubation was carried out in DMEM containing penicillin (400 U/ml), sodium ascorbate (50 μg/ml) and ^{3}H-proline (10 μC/ml) in the presence of corticosteroids.

Combined medium and cell layer were dialysed against 1 M $CaCl_2$, 0.05 M Tris, pH 7.4 for 12 hr and then extensively dialysed against several changes of 0.5% acetic acid. The samples were subsequently lyophilised, hydrolysed and analysed on an automatic amino acid analyser. The amount of radioactively labelled hydroxyproline and proline was used to calculate the synthesis of collagenous and noncollagenous proteins as described previously [16].

Characterization of newly synthesized collagens. Newly synthesized collagens were treated with pepsin (0.1 mg/ml, 6 hr, 17°) [17] and collagens I and III were precipitated by 2.7 M NaCl. Chromatography on Agarose A 5 m under denaturing conditions separated type III collagen (γ-components) from type I collagen (α-components). When the γ-components were collected and rechromatographed following reduction with mercaptoethanol, type III collagen migrated as α-components [18]. The ratio of type I and type III collagen was calculated from the radioactivity eluting from the Agarose columns. Isolated α-chains were also hydrolysed and subsequently analysed on an automated amino acid analyser to determine the ratio of labelled hydroxyproline and proline [16].

α1 (I) procollagen mRNA levels. Total RNAs were isolated from fibroblasts according to Chirgwin *et al.* [19]. For dot blot hybridization two-fold serial dilutions of RNA were applied to nitrocellulose filters using a dot blot template. Filters were baked, prehybridized and hybridized to ^{32}P-labelled radioactive cDNA probes specific for the α1 chain of type I collagen and tubulin [20]. The cDNAs were kindly supplied by Dr F. Ramirez (Hf 677) [21] and Dr D. W. Cleveland [22]. To remove unspecific hybridization filters were washed twice in 2 × SSC,* 0.1% SDS at room temperature, and then twice in 0.1 × SSC, 0.1% SDS at 50°. After exposure of the filters to Kodak X-Omat intensity of hybridization was quantitated by densitometry (Hirschmann Elscript 400).

Chemotactic activity of fibroblasts. Human skin fibroblasts were either incubated for three days in medium containing increasing concentrations of various corticosteroids or the substances were added prior to the chemotaxis assay. The chemotactic response was then studied in a blind well Boyden chamber with and without corticosteroids. Polycarbonate filters (diameter 13 mm, pore size 8 μm, Nuclepore, Pleasonton) were coated with gelatin (5 mg/l, Sigma) as described by Postlethwaite [23]. The lower compartment was filled with the chemoattractant (0.2 ml) and the gelatin-coated polycarbonate filter was placed above.

Fibroblasts were harvested by short trypsinization (0.25% Trypsin, 0.1% EDTA), suspended in DMEM containing 10% FCS for 5 min to inactivate the enzyme, centrifuged and resuspended in DMEM without FCS at cell density of 4 × 10^5/ml. Aliquots of these cell suspensions (0.8 ml) were added to the upper compartment of the chamber. Conditioned medium from embryonic human fibroblast cultures [24] was used as chemoattractant. Control cells and corticosteroid-treated cells were incubated for 4 hr at 37°, 95% air/5% CO_2. The filters were then removed, fixed in ethanol and stained with Hematoxylin-Eosin. Cells migrated to the lower side of the filters were counted in a 200-fold magnification field of a Leitz microscope. Each sample was assayed in triplicate and cells were counted in five unit fields.

RESULTS

Cell growth and morphology

Corticosteroids added to fibroblast cultures showed a strong dosage dependent influence on the proliferation of cells. Desoximetasone (10^{-5} M) and hydrocortisone (10^{-5} M) reduced the rate of proliferation to about 50% of the controls, while prednicarbate was less active (Fig. 1). No alteration in cell morphology (checked by phase contrast microscopy, Fig. 2) or viability was noted when cortico-

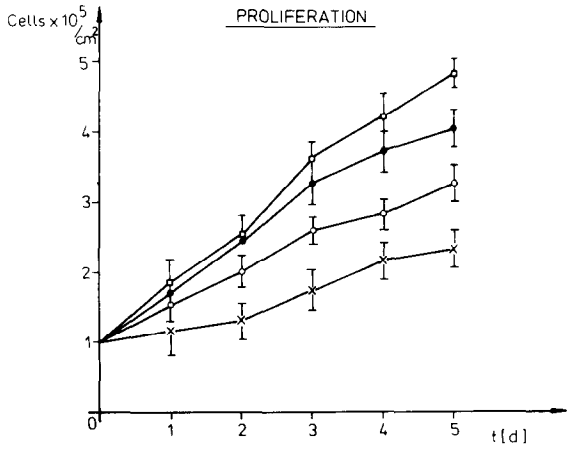

Fig. 1. Inhibition of proliferation of fibroblast cultures by various corticosteroids (10^{-5} M). The cells were seeded at low density and triplicate cultures were counted daily: □, controls; ●, prednicarbate; ○, hydrocortisone, ×, desoximetasone.

* Abbreviations used: EDTA, ethylenediaminetetraacetate; PBS, phosphate buffered saline, 1 × SSC, 0.15 M NaCl, 0.015 M sodium citrate; SDS, sodium dodecylsulfate.

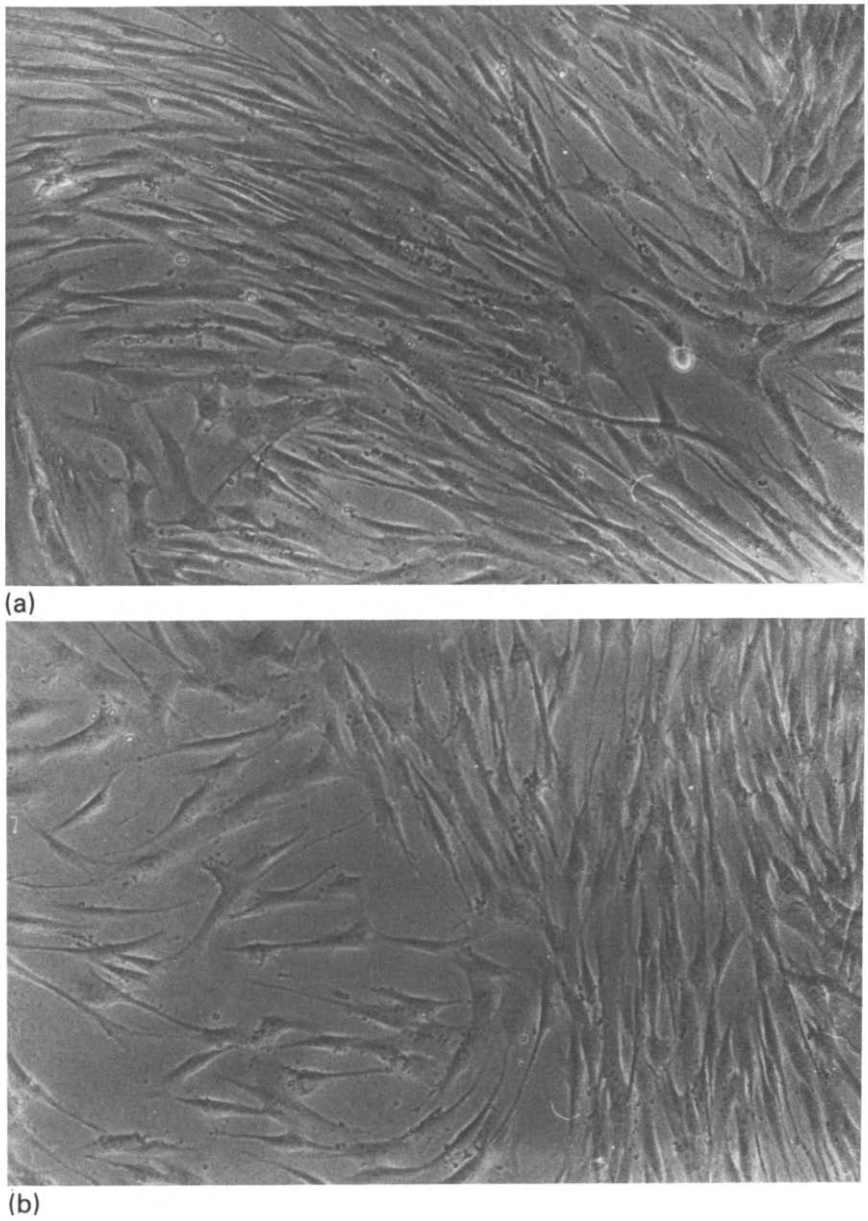

Fig. 2. Phase contrast microphotography of fibroblasts after 3 days in culture (a). Parallel cultures have been treated with desoximetasone (10 μM) (b). Similar pictures were obtained with other corticosteroids (10^{-5} M).

steroids were used in concentrations from 1 nM to 10 μM.

Ethanol used as solvent for the corticosteroids was added to the medium of fibroblasts in various concentrations (1–10 μl/ml) and did not reveal any effect on cell growth, collagen synthesis or chemotactic response (not shown).

Synthesis of collagen and non-collagenous proteins

To investigate the influence of corticosteroids on protein and collagen synthesis, fibroblasts were incubated for 3 days with or without corticosteroids (1 μM to 10 nM) and subsequently labelled with L-(2,3)^3[H]proline for 24 hr. As summarized in Table 1 synthesis of non-collagenous proteins was decreased with increasing concentrations of corticosteroids. However, major differences were noted when various corticosteroids were compared. Desoximetasone and hydrocortisone still inhibited synthesis of non-collagenous proteins at low levels, while prednicarbate affected protein synthesis only at high concentrations (10 μM) (Table 1).

When protein-bound hydroxyproline was used as a measure of collagen production strong inhibition was already found for desoximetasone at a concentration of 10^{-9} M, while prednicarbate did not inhibit collagen synthesis significantly at concentrations less than 1 μM (Fig. 3).

In addition, collagen α-chains were isolated after pepsin treatment by chromatography on Agarose

Table 1. Synthesis of non-collagenous proteins measured as protein-bound proline and calculated as described in Material and Methods

Substances	Concentration [nM]	cpm × 10^{-2}	%
Prednicarbate	10	55.47 ± 17.2	97
	100	47.94 ± 16.47	84
	1000	33.48 ± 1.85	58
Hydrocortisone	10	44.22 ± 7.78	77
	100	42.30 ± 3.82	74
	1000	22.33 ± 1.75	39
Desoximetasone	10	37.48 ± 1.95	66
	100	36.25 ± 3.18	65
	1000	23.68 ± 5.61	41
Controls	—	57.27 ± 4.19	100

N = 3.
The cells were kept for 3 days in the presence of various corticosteroids.

A5, hydrolysed and analysed on an amino acid analyser. No influence of corticosteroids on the hydroxylation of proline was found (not shown).

Determination of mRNA levels

To determine mRNA levels of α1 (I) procollagen, fibroblasts were grown for three days in the presence of different corticosteroids (10^{-5} M). RNA was then isolated and mRNA levels specific for α1 (I) procollagen were estimated by dot blot hybridization. Tubulin mRNA was used as control. As shown in Fig. 4, pretreatment with prednicarbate and desoximetasone resulted in reduced levels of collagen mRNA. However, comparison of both agents revealed the strongest inhibitory activity for desoximetasone (90%), whereas the reduction obtained after incubation with prednicarbate was less pronounced (65%).

Interestingly, tubulin mRNA levels were also reduced, but to a lesser extent than pro α1(I)-mRNA (Table 2).

Synthesis of collagen types I and III

Pepsin-treated collagens from confluent cultures were precipitated and type I and type III collagens were separated by Agarose chromatography under denaturing conditions. The ratio of both collagen types was similar in controls and cultures treated with different corticosteroids (Table 3).

Chemotactic activity

Whereas corticosteroids as chemoattractants did not reveal any activity for fibroblasts (not shown), preincubation of cells with various corticosteroids resulted in a marked decrease of chemotactic response of fibroblasts to conditioned medium. This inhibition was dosage dependent from 10^{-9} M to 10^{-5} M for desoximetasone (Fig. 5). Significantly reduced chemotactic response was not seen at concentrations less than 10^{-7} M when prednicarbate was used for preincubation.

Various corticosteroids were then tested in a concentration of 10^{-6} M (Table 4). Desoximetasone and clobetasol-17-propionate revealed the strongest inhibition (50–70%) compared to controls, while chemotactic response of fibroblasts was less affected by prednicarbate and hydrocortisone (20–40% inhibition).

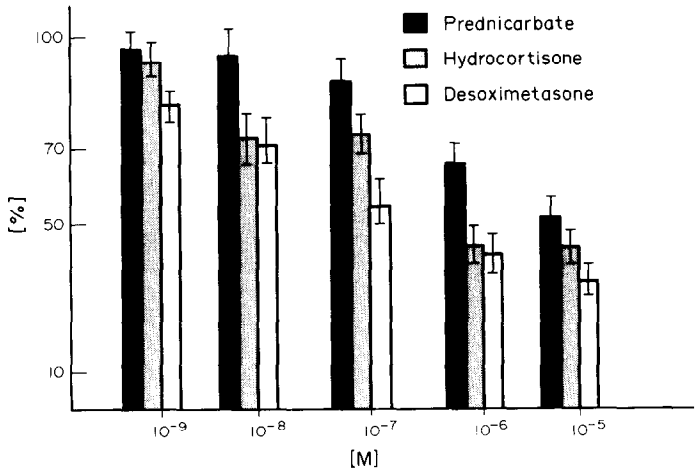

Fig. 3. Influences of corticosteroids on synthesis of collagen measured as protein-bound hydroxyproline. The values are expressed as a percentage of untreated cultures.

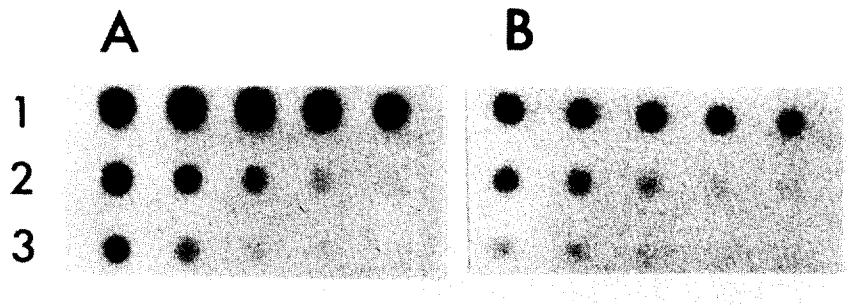

Fig. 4. Quantification of mRNA levels for pro α1 (I) collagen and tubulin in human fibroblasts treated with corticosteroids. Total cytoplasmatic RNA prepared from untreated (1) or treated fibroblasts (2 = prednicarbate 10^{-5} M, 3 = desoximetasone 10^{-5} M) was dotted on nitrocellulose in serial dilutions (3, 1.5, 0.75, 0.375, 0.187 μg). The filters were hybridized with nick-translated ^{32}P-labelled cDNA probes specific for pro α1 (I) collagen (A) and β-tubulin (B).

DISCUSSION

Corticosteroids are known to reveal a strong anti-inflammatory activity, but also to inhibit the metabolism of many cell types [5]. Recently substances have been developed, which are thought to show minor side effects [14]. We therefore intended to use different *in vitro* systems to characterize the response of fibroblasts to these agents. All corticosteroids were used in concentrations not affecting the viability of the cells as detected by the Trypan blue test. This was further supported by the unaltered morphology. As reported previously [6], the logarithmic growth of fibroblasts was significantly inhibited when high concentrations (10^{-5}) of corticosteroids were added to the cultures. Also consistent with previous observations [25] synthesis of non-collagenous proteins was inhibited significantly by most of the corticosteroids used. All corticosteroids resulted in a reduction of collagen synthesis, which was demonstrated by reduced amounts of radioactively labelled non-dialysable hydroxyproline and by comparing isolated collagen α-chains following molecular sieve chromatography on Agarose A5. In addition, this was supported by measuring specific mRNA levels for α1 (I) procollagen and tubulin. Differences in the relative inhibition of collagenous versus non-collagenous proteins, which have been previously reported [8, 10, 26] were also confirmed in our experiments.

The stable ratio of collagen types I and III indicates that synthesis of both collagens is similarly controlled by corticosteroids. A coordinate regulation of type I and III collagen has been reported by others [27] and was also found using other substances, e.g. reti-

Table 2. mRNA levels specific for pro α1 (I) collagen and β-tubulin of fibroblasts after treatment with corticosteroids in percent of controls

	Controls	Prednicarbate [10^{-5} M]	Desoximetasone [10^{-5} M]
β-tubulin	100	55	21
pro α1 (I) collagen	100	35	10

Total RNA was extracted and dotted onto nitrocellulose. After hydrization the relative amounts of mRNA were quantitated by densitometry. mRNA levels of untreated cells were taken as control (100%). The values represent the mean of three determinations.

Table 3. Influence of various corticosteroids on the ratio of types I and III collagen

Substances	Concentration [nM]	Type I collagen [%]	Type III collagen [%]
Control	—	85	15
Prednicarbate	1	86	14
	10,000	85	15
Deoximetasone	1	88	12
	10,000	90	10

$N_{\bar{x}} = 3$.

The collagens synthesized from confluent cultures were precipitated by 2.7 M NaCl, treated with pepsin and chromatographed on Agarose A5 m under denaturing conditions.

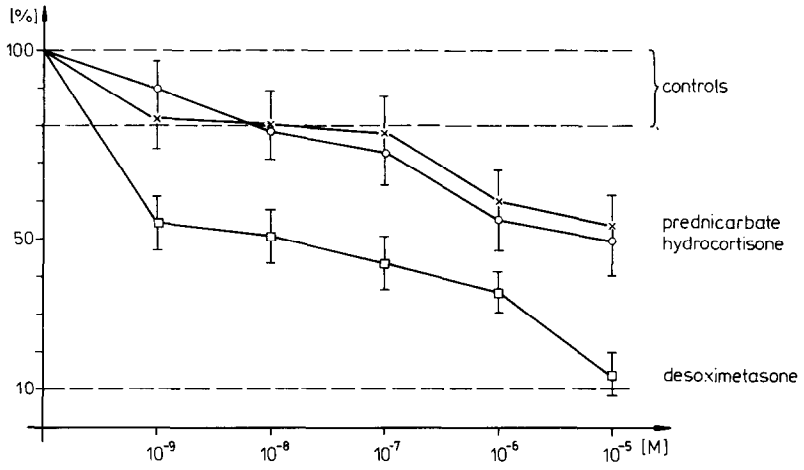

Fig. 5. Influence of corticosteroids on chemotactic response of fibroblasts. Cells were exposed for 3 days to various corticosteroids in concentrations from 10^{-9} to 10^{-5}. The chemotactic activity is expressed as a percentage of untreated cultures.

Table 4. Influence of various corticosteroids (10^{-6} M) on chemotactic response of fibroblasts

Substances	Migrated cells/unit field	[%]
Controls	62 ± 6.3	100
Prednicarbate	49 ± 5.4	79
Hydrocortisone	34 ± 6.1	55
Desoximetasone	30 ± 6.2	48
Clobetasol-17-butyrate	31 ± 5.5	50
Clobetasol-17-propionate	21 ± 4.9	34
RM = random migration	3 ± 0.7	5

N = 12.

noids [28]. Since activity of collagenase does not play a major role in the culture conditions used [29], reduced production of collagenous proteins is probably due to a decreased synthesis rather than an increased degradation. This is further corroborated by data indicating a reduced activity of collagenase in the presence of glucocorticosteroids [30]. As demonstrated by decreased levels of collagen mRNA collagen synthesis is regulated by corticosteroids on a pretranslational level. This could be due to decreased transcription [31] or to enhanced turnover of mRNA [12].

The chemotactic response of fibroblasts to various chemoattractants representing another function of these cells is thought to control the formation of repair tissue during wound healing [32] and is probably also important for the continuous remodelling of connective tissue. Similar to other substances affecting wound healing *in vivo* [33], corticosteroids were shown to reduce chemotaxis of fibroblasts without influencing random migration. This indicates a specific effect and not a secondary event due to reduced protein synthesis.

Reduced chemotaxis of fibroblasts was found to be affected even at low concentrations of potent corticosteroids and might therefore play an important role to explain delayed wound healing which is often observed during systemic or local treatment with corticosteroids [34]. Inhibition of the migration of fibroblasts could also play a role together with reduced collagen and protein synthesis in the development of atrophy following long term application of topical corticosteroids. Most of the effects of corticosteroids on the metabolism of fibroblasts were dose dependent. However, when different derivatives were compared, fluorinated compounds were found to be most active in affecting fibroblast metabolism. Prednicarbate, in contrast, turned out to have less activity in the systems tested, although it reveals a high anti-inflammatory activity similar to those known for fluorinated derivatives of corticosteroids [15].

Similar results were found for the chemotactic response as well as for the inhibition of collagen and protein synthesis. Therefore, it remains to be seen whether prednicarbate is internalized to a lesser extent than the other derivatives under *in vitro* conditions or whether the data indicate a dissociation of the inflammatory activity and inhibition of the biosynthetic capacities of fibroblasts.

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