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Tumor-promoting Phorbol Esters Cause a Stable Reduction of Dermal Collagen in Mouse Skin

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Abstract—*Chronic treatment with the tumor-promoting phorbol esters* 12-O-*tetradecanoylphorbol*-13-*acetate* (*TPA*) *and* 12-O-*retinoylphorbol*-13-*acetate* (*RPA*) *causes permanently increased levels of active collagenolytic enzymes in the dermis and leads to a stable reduction of dermal collagen content. Non-promoting skin mitogens like the* Ca-*ionophore A* 23187 *or the* 4-O-*methylether of TPA, while being active stimulators of collagenolytic enzymes, do not support chronic collagen degradation throughout the experimental period.*

On the other hand, TPA-induced collagen degradation is not necessarily influenced by inhibition of tumor promotion. Fluocinolone acetonid (*FA*), *an inhibitor preventing not only tumor development but also chronic inflammation and the establishment of a stationary hyperplasia, has been compared with retinoic acid* (*RA*) *which has no influence on either the inflammatory reaction or hyperplasia. While FA inhibited the dermal effects of TPA almost completely, RA at a dose that prevented tumor development by* 80% *had no effect whatsoever in this respect.*

Therefore, we conclude that both epidermal proliferation and inflammation are accompanied by collagenolytic reactions in the dermis. During chronic treatment sustained collagenolysis correlates with inflammation and/or the establishment of a stationary hyperplasia. Like these it can be regarded as a necessary but insufficient condition of tumor promotion (*second stage*).

INTRODUCTION

WE HAVE observed that tumor development during the treatment of the skin with the tumor promoter TPA is preceded by a decrease in dermal collagen content which is caused by an increase of collagenolytic activity [1]. To find out whether the observed collagen degradation was a promotion-specific effect we have examined the stimulation of collagen degradation in the dermis after topical application of skin mitogens which evoke different proliferative and inflammatory responses in the epidermis. 4-*O*-Methyl-TPA leads to an enhancement of proliferation without any increase in total cell number and without an inflammatory response. The phorbol esters TPA and RPA as well as A 23187 cause a different kind of proliferation which is accompanied by strong inflammation and hyperplasia. In the case of TPA and RPA, but not A 23187, chronic effects can be established by repeated applications and tumor development is supported [2, 3]. After one mitogen application the induction of collagenolytic enzymes in the dermis was correlated with the inflammatory effect of the agents [4]. However, tumor promotion is a long term process, of which chronic inflammation as well as stationary hyperplasia are regarded to be essential parts [2, 5]. Therefore, we used the same tumor-promoting and non-promoting skin mitogens to observe their effect on collagenolytic degradation during chronic application.

Furthermore, the influence of fluocinolon acetonid (FA) and of retinoic acid (RA) on TPA-induced collagen degradation was examined. Both substances are active inhibitors of tumor promotion, but while FA also is an anti-inflammatory drug and prevents formation of a stationary hyperplasia [5, 6]. RA has no effect in these respects [7].

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Abbreviations: DMBA: dimethylbenzanthracene; TPA: 12-*O*-tetradecanoylphorbol-13-acetate; RPA: 12-*O*-retinoylphorbol-13-acetate; FA: fluocinolone acetonid; RA: retinoic acid.

MATERIALS AND METHODS

1. *Chemicals*

RPA was kindly supplied by Professor Hecker (German Cancer Research Center, Heidelberg, F.R.G.). DMBA, A 23187, retinoic acid and fluocinolon acetonid were purchased from Sigma (St. Louis, MO, U.S.A.) and TPA and 4-*O*-methyl-TPA from Consolidated Midland Corporation (Brewster, NY, U.S.A.).

2. *Animals and treatment*

Female Swiss albino mice (11–12 weeks old) were used. They received 100 nmol DMBA dissolved in 0.1 ml acetone on their shaved back skin. After 1 week tumor promotion was performed by twice weekly applications of either 10 nmol TPA, 10 nmol RPA, 100 nmol A 23187 or 400 nmol 4-*O*-methyl-TPA dissolved in acetone or solvent only. 17 nmol RA were applied 1 h before TPA and 1 μg FA together with TPA to inhibit tumor formation.

3. *Sample preparation*

The animals were killed at various intervals in the course of the treatment 2 days after the respective applications and the dermis was isolated mechanically as described recently [1].

4. *Collagen content*

Individual dermis samples of five skins per group were dried to constant weight and extracted with 0.3 M trichloroacetic at 90° C twice for 30 min. Hydroxyproline in the extract was assayed according to Woessner [8].

5. *Collagenolytic activity*

Pooled dermis samples of five skins each were extracted with 5 M urea dissolved in 20 mM Tris–HCl pH 7.5 containing 5 mM $CaCl_2$. They were concentrated by precipitation with saturated ammonium sulfate and dialyzed. Aliquots were incubated with fibrillae of tritiated acid soluble rat type I collagen (0.41 mCi/mg, NEN, Boston, MA, U.S.A.) in 20 mM Tris–HCl pH 7.5 and 5 mM $CaCl_2$ at 30° C for 17 h. Intact fibrillae were removed by centrifugation and the solubilized radioactivity in the supernatant counted in a Packard Tri Carb 4530 scintillation counter. Digestion conditions were chosen so that up to 50% of the substrate were digested by the dermal extracts and less than 10% by trypsin. Activity was calculated as ng collagen digested per mg wet wt of extracted dermis and increases of activity as multiples of the corresponding control. All buffers contained 1 μg/ml aprotinin to inhibit non-specific proteases.

6. *Tumor development*

Twenty animals were kept alive to observe tumor development. We determined the time until the first tumors appeared and tumor rate and tumor yield at 15 weeks.

RESULTS

a. *Dermal effects of skin mitogens*

With the treatment schedule described above tumors developed after 6 weeks in 80% of the animals in the TPA group. With RPA we observed papillomas in 34% of the animals from the 8th week on. No tumors developed in animals treated with either A 23187 or 4-*O*-methyl-TPA (Table 1).

Collagen content and collagenolytic activity in the dermis were measured until the 15th application (8 weeks), because at that time tumor development in the TPA group was manifest and irreversible. The collagen content of acetone-treated control dermis ranged between 76 and 81 mg hydroxyproline per g dry wt of dermis (Fig. 1a). It was depressed by all mitogens, but to different extents. Both TPA and RPA led to a decrease of about 80% of the control values at the 1st to 5th applications. This reduction remained stable throughout the treatment so that the dermal collagen content of TPA- and RPA-treated skins never exceeded 90% of the collagen in control skins. With the non-promoting mitogens A 23187 and 4-*O*-methyl-TPA the collagen content dropped to about 90% of the controls established at the beginning of the treatment, but recovered later on and reached control levels at the 10–12th application for 4-*O*-methyl-TPA and at the 15th application for A 23187.

The reductions of collagen content were paralleled by the stimulation of collagenolytic activity (Fig. 1b). As the control values from individual experiments ranged from 0.1 to 0.5 ng collagen digested per mg dermis, reflecting a rather high degree of variability, increases were calculated as multiples of the corresponding control. The values given in the Fig. 1 represent the mean of three experiments, i.e. at least 15 skins per point. In the TPA and RPA groups the increase in collagenolytic activity was 2.5–4-fold with a stable mean level throughout the experiment.

After 4-*O*-methyl-TPA application values were highly variable especially after the 1st and 2nd applications, sometimes reaching a "TPA-like" level of 3-fold, but in general ranging between 1.5- and 2-fold. With A 23187 modest, but constant, increases of 1.25–1.75-fold were observed. At the end of the treatment with both substances, the activity returned to control level.

b. *Inhibitors of tumor promotion*

Both RA and FA delayed tumor development and reduced tumor rate as well as tumor yield (Table 2). There were, however, striking differences with regards to their effects on TPA induced alterations in the dermis. FA almost totally prevented TPA-stimulated collagenolytic activity and collagen decrease. In contrast, in the RA/TPA treated groups, collagenolytic activity and dermal collagen content were essentially the same as in the corresponding TPA treated samples (Fig. 2).

DISCUSSION

The dermal collagenous matrix is supposed to be involved in the mediation of regulatory signals to

Table 1. *Tumor promoting activity of skin mitogens in SWA females*

Agent*	Dose (nmol)	Number of animals	Time until first tumors appear	% tumor bearers at 15 weeks	Number of tumors/survivor at 15 weeks
TPA	10	60	6	80	6.2
RPA	10	50	8	34	1.3
A 23187	100	40	—	0	0
4-*O*-Me-TPA	400	20	—	0	0

*Starting 1 week after initiation with 100 nmol DMBA mitogen treatment was performed by twice weekly applications of mitogen doses indicated. The animals received a total of 20 applications.

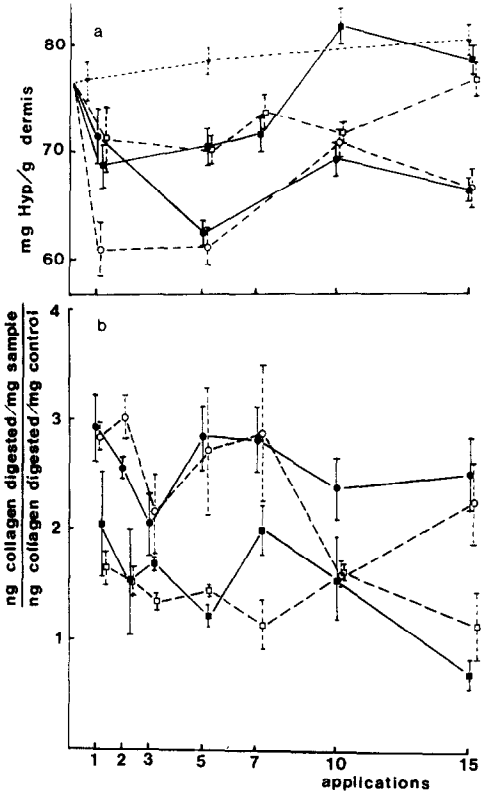

Fig. 1. *Collagen degradation during treatment with skin mitogens.* (*a*) *Collagen content: hydroxyproline content is determined from TCA extracts of dermis according to Woessner* [7]. *The values given in the figure represent the means of* 20 *individual determinations obtained in four independent experiments* ± *S.E.M.* (*b*) *Collagenolytic activity: collagenolytic enzymes are extracted from the dermis with* 5 M *urea, concentrated by ammonium sulfate precipitation, dialyzed and the activity measured in a fibril assay using tritiated type I collagen. For details see the Materials and Methods section. The increase of collagenolytic activity over the corresponding controls is calculated for each experiment separately. The figure shows the means of three experiments* ± *S.D.* ●—● *TPA*, ○—○ *RPA*, □—□ *A* 23187, ■—■ 4-O-*methyl-TPA*, ▼...▼ *control.*

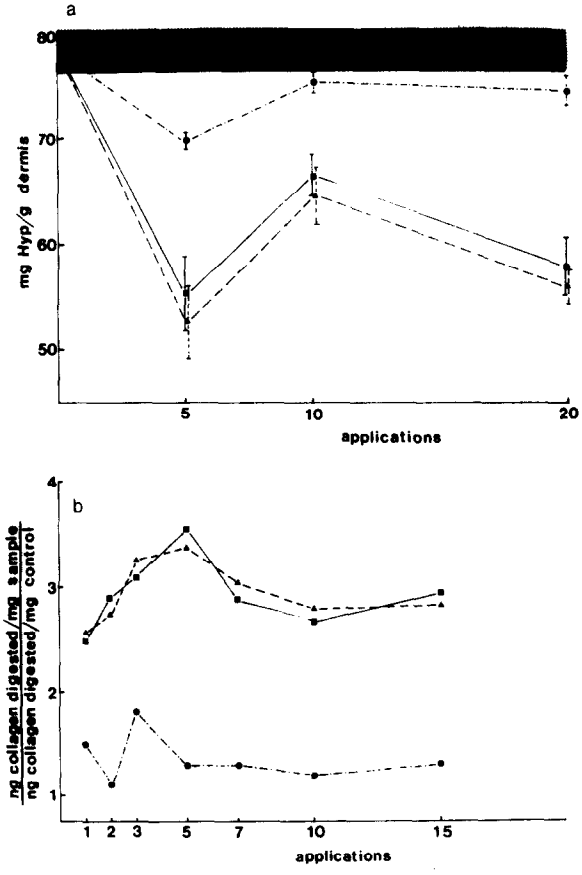

Fig. 2. *Effects of RA and FA on TPA-induced collagen degradation.* (*a*) *Collagen content: for details see legend to Fig.* 1*a. Values represent the means of* 10 *single samples* ± *S.E.M.* (*b*) *Collagenolytic activity: for experimental details see legend to Fig.* 1*b. Values represent the means of three determinations.* ■—■ *TPA*, ▲---▲ *RA/TPA*, ●—● *FA/TPA*, *shaded area: control.*

the epidermis [9, 10]. Therefore, the question to what extent alterations of the dermal matrix play a role in the process of tumor promotion has been investigated. Recently we have shown the secretion and activation of collagen degrading enzymes after a single application of skin mitogen correlates with the inflammatory effect of the drug [4]. This is ascertained by the present study. Moreover, 4-*O*-methyl-TPA, which has no such effect with 24 h of application [4], stimulates collagen degrading enzymes after 48 h. In the absence of an inflammatory reaction this indicates that collagenolytic activities may not only be associated with inflammation but also with the proliferation induced by the agents. Induction of cell proliferation *in vitro* by EGF and PDGF is also accompanied by a stimulation of collagenase secretion [11, 12] showing that the

Table 2. Inhibition of tumor promotion by RA and FA

Treatment	Number of animals	Time until first tumors appear	% tumor bearers at 15 weeks	Number of tumors/survivor at 15 weeks
TPA alone*	40	6	94	4.5
TPA + FA†	40	11	15	1.2
TPA + RA‡	20	9	23	0.35

*Mice were initiated with 100 nmol of DMBA. One week later promotion was started with twice weekly applications of 10 nmol TPA.
†Together with each TPA application mice received 1 μg FA.
‡1 h before each TPA application animals received 17 nmol RA.

proliferation response *per se* may also involve collagenolysis.

Thus, at the beginning of the treatment all skin mitogens used in this study produce a stimulation of collagen degradation and a reduction of the dermal collagen content, and differences between promoting and non-promoting mitogens only manifest themselves during chronic application. With tumor-promoters not only more collagenolytic enzymes are secreted than with non-promoting mitogens, but secretion is also maintained throughout the treatment. This results in a chronically reduced collagen content of the dermis.

In contrast, the non-promoting agents A 23187 and 4-*O*-methyl-TPA, which are not able to support chronic but only transient alterations in the epidermis, do not have any sustained effects in the dermis either. The level of collagenolytic enzymes returns to normal values and the dermal collagen content is restored. We have never observed tumor development without a stable reduction of the dermal collagen content. However, there is a correlation between the induction of a stationary hyperplasia and of chronic inflammation, which are both necessary conditions for tumor promotion, but not for tumor development. This is supported by the inhibition of tumor promotion with RA without affecting the mesenchymal alterations. RA prevents tumor formation but neither TPA induced inflammation nor stationary hyperplasia [5, 7]. If, on the other hand, the anti-inflammatory steroid FA is used to inhibit inflammation and hyperplasia as well as tumor development [6], the effects of TPA on dermal collagen are practically abolished.

Tumor development after RPA-application is slower and less efficient than with TPA in SWA mice, but one or two TPA applications preceding the treatment with RPA did not establish full promoting activity. Either our mice are especially resistent for the conversion (first stage) step or the difference in activity between TPA and RPA does not concern conversion as in NMRI mice [13] but promotion (second stage). Therefore, in our system we cannot distinguish between two stages of tumor promotion or observe any conversion activity of A 23187 or 4-*O*-methyl-TPA as described in SENCAR mice [14, 15]. In any case the differences in tumor development between TPA and RPA were not reflected in quantitative differences in the dermal effects of the agents, which were found to be essentially equal. This is also the case for the inflammatory reaction and the degree of hyperplasia.

The data suggest that prolonged collagen degradation and reduction of dermal collagen are related to the stationary hyperplasia and/or chronic inflammation caused by phorbol ester tumor-promoters and like them are a necessary but not sufficient condition of tumor promotion.

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