Chin Dent J (中華牙誌) 16(3) : 148-155 September 1997

The cancer-promoting effects of 12-O-tetradecanoyl-phorbol-13-acetate and collagenase in hamster buccal pouch carcinogenesis

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The aim of this study was to investigate the cancer-promoting effects of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and collagenase in hamster buccal pouch carcinogenesis. Forty-eight non-inbred male adult Syrian golden hamsters were randomly divided into three main groups that were further subdivided into various subgroups. The right buccal pouch of each animal was painted three times per week with various combinations of 7,12-dimethylbenz[a]anthracene (DMBA), TPA, and collagenase. Squamous cell papillomas were induced in animals which had 4 weeks of DMBA initiation and 8 weeks of TPA promotion, and also in those which had 4 weeks DMBA initiation and subsequently 8 weeks collagenase promotion. Exophytic squamous cell carcinomas were contracted in hamsters treated for 8 weeks with DMBA followed by 4 weeks of TPA promotion, and in those treated for 8 weeks with DMBA followed by 4 weeks of collagenase promotion. Use of DMBA alone for 4 weeks failed to yield visible tumor outgrowths; only hyperkeratosis and epithelial hyperplasia were found histologically. TPA or collagenase used alone for 12 weeks revealed only hyperkeratosis and epithelial hyperplasia microscopically. This study constitutes an experimental model for use in further investigations to understand the mechanisms of action of the synergism of TPA and collagenase in carcinogenesis.

Key words: TPA, collagenase, promotion, hamster, DMBA-carcinogenesis.
TPA, 膠原酵素, 促癌, 倉鼠, DMBA-癌化.

Suitable chemicals may be used to initiate and promote the formation of tumors. 7,12-dimethylbenz[a]anthracene (DMBA) has been used extensively as an initiator. Covalent binding of DMBA to form DMBA-DNA adducts is considered to be essential in initiating the carcinogenic process[1]. 12-O-tetradecanoylphorbol-13-acetate (TPA), isolated as an active principal from croton oil by Hecker[2], has been an important promoter in studies of the cellular mechanisms of co-carcinogenesis[3]. TPA can trigger protein kinase C activity, resulting in cell proliferation in the promotion stage[4]. Type I collagenase, also known as interstitial collagenase, degrades the fibrillar collagens which compose much of the extracellular matrix in the interstitial space[5]. Messenger RNA of type I collagenase has been found in stromal cells immediately adjacent to tumor masses in the oral cavity[6]

and it also has been identified in the stromal cells of head and neck squamous cell carcinomas and in tumors scattered throughout the neoplastic islands[7]. Thus, type I collagenase may play a role in oral carcinogenesis by enhancing the progression of the early cancerous lesions[8].

The hamster buccal pouch model, first introduced by Salley[9] and further developed by Morris[10], is the most widely accepted experimental model of oral carcinogenesis, producing lesions closely resembling those found in humans[10]. Using this model, a number of studies[11,12] describing two-stage carcinogenesis in pouch mucosa were performed. However, little is known regarding the synergism of TPA or collagenase in two-stage carcinogenesis in the hamster pouch, using DMBA as the initiator and TPA and collagenase as the promoters. The aim of the current study was to investigate the cancer-promoting effect of TPA, and collagenase on hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS

Chemicals

All chemicals, procured from the Sigma Chemical Company (St. Louis, Missouri), were of the highest purity. A 0.5% DMBA solution in heavy mineral oil, stored at room temperature, was employed[9,10]. A solution of 2.5 μg TPA in 0.2 ml acetone was stored at 4 ℃ until use[13]. A 0.1% type I collagenase needed for painting[14].

Hamsters (Table 1)

Forty-eight non-inbred male adult Syrian golden hamsters (8 to10 weeks old), each weighing approximately 100g at the commencement of the experiment, were obtained from the National Taiwan University Breeding Laboratory. The hamsters were housed under constant conditions (22℃,12-hour light/dark cycles), fed with tap water and standard Purina laboratory chow ad lib. After allowing one week to acclimate to the new surroundings, the hamsters were randomly divided into three main groups A, B and C. Each main group was then further divided into four to six subgroups (Table 1).

TPA and collagenase treatments (Table 1)

The treatment regimen involved the 48 hamsters over a 12-week experimental period. The protocol ensured humane practices. The right buccal pouch of each animal was painted with different combinations of DMBA, TPA and collagenase at 9:00 a.m. each Monday, Wednesday and Friday using a No. 4 sable-hair brush. Approximately 0.2 ml of each solution of chemicals was topically applied to the medial walls of the right pouch in each painting. The left pouch of each animal was left unpainted and served as a built-in control. At the end of 12 weeks, all animals in the three main groups A to C with the exception of groups B-1 (killed at 4 weeks) and C-1 (killed at 8 weeks) were euthansized by inhaling lethal dose of diethyl ether at the same time of the day (9:00 a.m.). The killed animals were fixed in supine positions with pins. Bilateral buccal pouches were exposed by dissection, cut from their oral openings to their caudal ends along the middle of their lateral walls and examined grossly. The number of tumor growths were counted. Subsequently, both pouches were excised and placed on hard paper board to prevent distortion of the specimens. Representative specimens, taken from each pouch, were routinely processed for light microscopy by immersion in 10% neutral buffered formalin, dehydrated in ascend-

Table 1. The experimental components of two-stage carcinogenesis in hamster buccal pouches treated with TPA and collagenase

Group No.	Treatment protocol for 12 weeks
A-1	Mineral oil (12 weeks)
A-2	DMBA (12 weeks)
A-3	TPA (12 weeks)
A-4	Collagenase (12 weeks)
B-1	DMBA (4 weeks) → Killed
B-2	DMBA (4 weeks) → No treatment (8 weeks)
B-3	DMBA (4 weeks) → TPA (8 weeks)
B-4	DMBA (4 weeks) → Collagenase (8 weeks)
B-5	TPA (4 weeks) → DMBA (8 weeks)
B-6	Collagenase (4 weeks) → DMBA
C-1	DMBA (8 weeks) → Killed
C-2	DMBA (8 weeks) → No treatment (4 weeks)
C-3	DMBA (8 weeks) → TPA (4 weeks)
C-4	DMBA (8 weeks) → Collagenase (4 weeks)
C-5	TPA (8 weeks) → DMBA (4 weeks)
C-6	Collagenase (8 weeks) → DMBA (4 weeks)

ing alcohols, cleared in xylene and embedded in paraffin. Serial sections of each sample were cut 5 μm thick, stained with hematoxylin-eosin, and examined under a light microscope.

RESULTS

Gross observations (Table 2)

The numbers of visible exophytic tumors induced in the corresponding experimental groups are presented in

Table 2. Total and average numbers of tumors as well as histological features of lesions induced in each hamster group after buccal painting with various combinations of TPA and collagenase

Group No.	Tumor numbers	Hyperkeratosis & epithelial hyperplasia	Squamous cell papilloma	Squamous cell carcinoma
A-1	0**	-	-	-
A-2	10#(3,4,3)[3.33 ± 0.58]	-	-	+(3/3)*
A-3	0	+(3/3)	-	-
A-4	0	+(3/3)	-	-
B-1	0	+(3/3)	-	-
B-2	0	+(3/3)	-	-
B-3	5(2,2,1)[1.67 ± 0.58]	-	+(3/3)	-
B-4	4(2,2,1)[1.33 ± 0.58]	-	+(3/3)	-
B-5	4(2,2,1)[1.33 ± 0.58]	-	-	+(3/3)
B-6	3(1,1,1)[1 ± 0]	-	+(2/3)	+(1/3)
C-1	3(1,2,0)[1 ± 1]	+(1/3)	+(2/3)	-
C-2	5(2,2,1)[1.67 ± 0.58]	-	+(2/3)	+(1/3)
C-3	6(3,2,1)[2 ± 1]	-	+(2/3)	+(1/3)
C-4	9(4,3,2)[3 ± 1]	-	+(2/3)	+(2/3)
C-5	0	+(3/3)	-	-
C-6	0	+(3/3)	-	-

**: total number of tumors induced
-: negative finding
+: positive finding
#: number of tumors induced in each animal numbers in brackets indicate [mean ± standard deviation]
*: numbers in parentheses indicate (numbers of hamsters with positive findings/total numbers of hamsters in subgroup)

Table 2. Gross examination of all the treated pouches of group A-1 (mineral oil alone for 12 weeks) and the untreated pouches of each experimental group revealed no apparent changes. All the painted pouches in group A-2 (DMBA alone for 12 weeks) showed exophytic tumors. Thickened mucosa with a rough surface and whitish granular appearance was found in the treated pouches of groups A-3 (TPA alone for 12 weeks) and A-4 (collagenase alone for 12 weeks).

Occasional erythematous areas were seen in the treated pouches of groups B-1 (DMBA for 4 weeks, then killed) and B-2 (DMBA for 4 weeks; then untreated for 8 weeks). Papillomatous tumor out-growths were noted in the painted pouches of both groups B-3 (DMBA for 4 weeks, then TPA for 8 weeks) and B-4 (DMBA for 4 weeks, then collagenase for 8 weeks). Exophytic tumors were discernible in the treated pouches of groups B-5 (TPA for 4 weeks, then DMBA for 8 weeks) and B-6 (collagenase for 4 weeks, then DMBA for 8 weeks).

Visible tumors were observed in the treated pouches of group C-1 (DMBA for

8 weeks, then killed). Similar findings were found in the treated pouches of group C-2 (DMBA for 8 weeks, then untreated for 4 weeks). Overt tumor outgrowths were shown in the painted pouches of both groups C-3 (DMBA for 8 weeks, then TPA for 4 weeks) and C-4 (DMBA for 8 weeks, then collagenase for 4 weeks).

Treated pouches of group C-5 (TPA for 8 weeks, then DMBA for 4 weeks) showed white plaque-like lesions. However, reddish, irregular surfaces were observed in the painted pouches of group C-6 (collagenase for 8 weeks, then DMBA for 4 weeks).

Histological findings (Table 2)

Detailed microscopic findings of the treated pouch of each experimental group are summarized in Table 2. No significant histological changes were seen in any of the treated pouches of group A-1, whereas well-differentiated squamous cell carcinomas were observed in group A-2. Hyperkeratosis and epithelial hyperplasia were found in the painted pouches of groups A-3, A-4, B-1, and B-2 as well as in those of groups C-1, C-5, and C-6. Exophytic squamous cell papillomas, squamous cell carcinomas or both were observed in the treated pouches of groups B-3, B-4, B-5 and B-6 and also groups C-1, C-2, C-3 and C-4. No obvious histological changes were observed in any of the untreated pouches of each experimental group.

DISCUSSION

Despite the considerable evidence that TPA is a potent tumor promoter in the mouse skin model[15], the synergistic effect of TPA[16] or collagenase[8] in a DMBA initiated hamster buccal pouch model has been inadequately reported. These effects are described in the present study.

The findings of groups B-5, B-6, C-2, C-3 and C-4 indicate that for a 12-week experimental regimen, in order to induce an overt squamous cell carcinoma in a hamster buccal pouch carcinogenesis model, DMBA should be applied for at least 8 weeks regardless of the treatments with TPA or collagenase. Additionally, only squamous cell papillomas and no squamous cell carcinomas were induced in groups B-3 and B-4. This suggests that the promotion potency of TPA or collagenase may not be very strong, at least under the present experimental conditions. Further study using higher concentrations of the chemicals or a prolonged period of treatment is warranted. Nonetheless, this study demonstrates that the promotion potencies of TPA and collagenase are similar to each other. The synergistic effect of TPA in hamster buccal pouch mucosa appears to be comparable to that observed in the mouse skin model[15].

Tumor promoters elicit a wide spectrum of biochemical and morphological changes when applied in the mouse skin model. These changes include the induction of the synthesis of RNA, DNA and protein, and the development of edema, inflammation and pronounced epidermal hyperplasia[17]. Whether a similar cascade of changes can be observed in the hamster buccal pouch carcinogenesis model is worth for further investigation.

The disparity between groups C-3, C-4 (8 weeks of DMBA initiation) and groups B-3, B-4 (4 weeks of DMBA initiation) may be attributed to a longer initiation period in the C groups. Further studies with either a longer promotion period (>8 weeks) after 4 weeks of DMBA initiation or a prolonged observation period without further treatments after 4 weeks of DMBA initiation and subsequently 8 weeks of TPA or collagenase promotion would elucidate this

difference. The different outcomes between groups C-5, and C-6 and groups B-5, and B-6 may be due to the strong carcinogenic effect (8 weeks of DMBA application) acting on the pouches of groups B-5 and B-6. This also supported the finding that TPA or collagenase alone failed to induce tumors in hamster buccal pouches despite a period of 4 weeks of DMBA initiation.

Although epidermal hyperplasia has clearly been demonstrated in the skin model upon treatment with TPA[17], whether TPA has also been a hyperplasiogenic agent in hamster cheek pouch mucosa is seldom reported[18]. In the present study, both TPA (group A-3) and collagenase (group A-4) were demonstrated to be effective hyperplasiogenic agents in hamster buccal pouch mucosa. However, this is in contrast to a previous report[18], which found that the degree of hyperplasia decreased with time. On the other hand, the hyperplasiogenic effect of both TPA and collagenase seems to be comparable to that caused by 50% turpentine in mineral oil[19], which has been regarded as the prototype hyperplasiogenic agent in this experimental model. Under the synergistic effect of either 8 weeks of TPA (group B-3) or 8 weeks of collagenase (group B-4) to the 4-week DMBA-initiated pouches, the numbers of tumors induced appear to be similar to those found in groups treated with 8 weeks of DMBA alone (groups C-1 and C-2). Therefore, the sum of tumors induced is surmised to be comparable to that of those in the 12-week DMBA treated pouches in which the promotion period was extended to 12 weeks. Furthermore, the additional synergistic effect of 4 weeks of TPA or collagenase to the 8-week DMBA-initiated pouches may explain the higher number of tumors compared to the pouches treated with DMBA alone for 8 weeks (groups C-1 and C-2). Additionally, the similar outcomes of groups B-5, B-6 and groups C-1, C-2 and C-3 suggest that TPA or collagenase could decrease the latency period for tumor development in the pouch mucosa.

In conclusion, the number of hamsters in each main group of this study is generally adequate (12 animals in main group A, 18 animals in each of main groups B and C), with a moderate number of animals in each killing period. In order to obtain a greater promotion effect of TPA or collagenase in a DMBA-initiated hamster buccal pouch carcinogenesis model, a higher concentration of chemicals or a longer period of application is recommended. Nevertheless, this study constitutes a useful experimental model for further understanding the mechanisms of the synergistic action of TPA or collagenase in a DMBA-initiated cancer model.

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12-O-tetradecanoylphorbol-13-acetate 與 collagenese 於倉鼠頰囊袋癌化中之促癌作用

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本研究目的是探討 12-O-tetradecanoylphorbol-13-acetate 與 collagenase 於倉鼠頰囊袋癌化中之促癌作用。四十八隻雄性倉鼠取機分爲三大組，每一大組再細分爲若干小組，每週三次塗 7,12-dimethylbenz[a] anthracene (DMBA)，TPA 與 collagenase 之各種組合於倉鼠右側頰囊袋能實驗結果顯示 DMBA 塗抹四週後，再塗抹 TPA 八週，或 collagenase 八週，動物產生乳突瘤；塗抹 DMBA 八週，再塗抹 TPA 四週及塗抹 DMBA 八週後，再塗抹 collagenase 四週之動物，可產生外生性鱗狀上皮細胞癌；只塗抹 DMBA 四週，沒有產生任何腫瘤，而組織學檢查只見角化層增厚及表皮增生；塗抹 TPA 或 collagenase 十二週，顯微鏡下僅見角化層增厚與表皮增生。因此本研究結果可作爲探討 TPA 或 collagenase 促癌機轉之動物模式。

Received: June 16, 1997
Accepted: September 25, 1997
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