Development of an in Vitro Analogue of Initiated Mouse Epidermis to Study Tumor Promoters and Antipromoters

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CANCER RESEARCH 50, 4794-4800, August 1, 1990

8 cells. In this model system, the actions of promoters and inhibitors of normal keratinocytes. Simultaneous treatment with 12-0-tetradecanoylphorbol-13-acetate produced when the cells are grafted to the backs of athymic mice. Coculture of a small number of these initiated cells with confluent normal primary keratinocytes resulted in the inhibition of growth of colonies of 308 cells. Addition of fresh keratinocytes weekly was required to sustain the inhibition for 3-4 weeks. Inhibition of 308 cell colonies required culture medium with a calcium concentration of 1.2 mM; normal keratinocytes did not inhibit 308 cells in medium with 0.05 mM calcium. Growth of 308 cells was not inhibited by coculture with confluent fibroblasts or by 1.2 mM calcium medium conditioned by either keratinocytes or fibroblasts. During continuous exposure of the cocultures to tumor promoters, 308 cell colonies became apparent within 2-3 weeks. A limited number of promoters were tested in this model system and 12-O-tetradecanoylphorbol-13-acetate, 12-O-retinoylphorbol-13-acetate, mezerine, and benzoyl peroxide were all active. The number of colonies which developed during promoter exposure in cocultures showed a dose-response curve which differed from the dose-response curve for stimulation of growth of 308 colonies in the absence of normal keratinocytes. Simultaneous treatment with 12-O-tetradecanoylphorbol-13-acetate and known inhibitors of skin tumor promotion, such as retinoic acid, fluocinolone acetonide, and bryostatin 1, blocked the stimulatory effects of 12-0-tetradecanoylphorbol-13-acetate, 12-0-retinoylphorbol-13-acetate, mezerein, and benzoyl peroxide were all active. The number of colonies which developed during promoter exposure in cocultures showed a dose-response curve which differed from the dose-response curve for stimulation of growth of 308 colonies in the absence of normal keratinocytes. Simultaneous treatment with 12-O-tetradecanoylphorbol-13-acetate and known inhibitors of skin tumor promotion, such as retinoic acid, fluocinolone acetonide, and bryostatin 1, blocked colony formation of 308 cells in cocultures but not in cultures with only 308 cells. In this model system, the actions of promoters and inhibitors both appear to be mediated by normal keratinocytes.

INTRODUCTION

Considerable data indicated that mouse skin papillomas induced by initiation-promotion protocols develop by the clonal expansion of single initiated cells (1). Promotion of papilloma formation, the expression of the latent tumor phenotype resulting from initiation, requires selection for the growth of initiated cells. With the phorbol ester tumor promoters, the selective mechanism appears to involve the induction of terminal differentiation in one subpopulation of normal epidermal cells and proliferation in another, which includes initiated cells (2). The uniform resistance of cultured initiated cell lines to phorbol ester-induced differentiation (3) suggests a mechanism for the enrichment of the initiated cell population in the epidermis with each promoter application.

Since initiated cells do not develop into papillomas in the absence of promoter treatment, the clonal growth of initiated cells appears to be inhibited by the surrounding normal cells (4). If this is the case, then tumor promoters may overcome the presumed growth-inhibitory effects of the surrounding normal cells either by their differential effects on induction of differentiation or by other mechanisms. The selective isolation of initiated cells in vitro (5), the development of initiated keratinocyte cell lines (3, 6), and the ability to culture normal keratinocytes (7) allow the study of the interactions of normal and initiated cells in culture. We wished to determine whether initiated epidermal cells are inhibited in cocultures with normal keratinocytes and whether exposure to a tumor promoter would overcome the inhibition. Such an in vitro analogue of initiated epidermis would be valuable to investigate the mechanisms of promoter action and might serve as a model to screen for potential promoters or antipromoters.

In fibroblast models in culture, inhibition of transformed cells by coculture with normal cells (8) requires cell-cell contact (9, 10) and heterologous junctional communication between the two cell types (11, 12). Exposure of cocultures of fibroblasts to a phorbol ester tumor promoter results in the growth of foci of transformed cells (13). Few coculture studies have been reported with epithelial cells. Some lines of transformed rat liver epithelial cells were found to communicate with their nontransformed counterparts, while others did not (14). Heterologous communication was also observed between normal keratinocytes and cell lines developed from mouse epidermis at several stages during skin carcinogenesis (15). In those reports, neither inhibition of growth nor the effects of tumor promoters were determined.

In this report, cocultivation of a small number of cells of some initiated keratinocyte lines with a large number of normal keratinocytes resulted in the inhibition of growth of colonies of initiated cells. Treatment with tumor promoters induced the growth of foci of initiated cells. This selection for the growth of initiated cells is analogous to promoter action in vivo. Known inhibitors of mouse skin tumor promotion reduced or prevented the response to tumor promoters in this culture model of initiated epidermis.

MATERIALS AND METHODS

Cell Culture. Primary cultures of keratinocytes were prepared from newborn BALB/c mouse skin by a trypsin flotation procedure (16). Cells were cultured in calcium-free Eagle’s minimal essential medium (MA Bioproducts, Walkersville, MD) with nonessential amino acids and 8% Chelex (Bio-Rad, Richmond, CA)-treated fetal bovine serum (Armour, Kankakee, IL), at 36°C in a humidified incubator with 7% CO2 (7). The calcium concentration of the medium was adjusted from a stock solution of CaCl2 and was verified by atomic absorption spectrometry. High (standard) calcium medium contained 1.2 mM; low calcium medium contained 0.05 mM. Line 308 (3, 6) was derived from calcium-resistant foci of keratinocytes from adult BALB/c mouse skin which had been initiated in vivo with 7,12-dimethylbenz[a]anthracene. In contrast to primary keratinocytes, which cease proliferation and terminally differentiate in high calcium medium, cells of line 308 proliferate in this medium. The derivation of other lines tested is shown in Table 1.

In typical experiments, 100-200 cells of line 308 were co-plated with 4×104 primary keratinocytes in high calcium medium in 60-mm plastic culture dishes. Fresh primary keratinocytes were added each week. When lines other than 308 were tested, the number of neoplastic cells plated was adjusted to provide a final colony number between 10 and 50. In groups of dishes treated with a tumor promoter, modifier,

Received 7/13/89; revised 4/5/90.

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IN VITRO ANALOGUE OF INITIATED MOUSE EPIDERMIS

Table 1  Effect of coculture with primary keratinocytes with or without TPA on the clonal growth of keratinocyte cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number plated</th>
<th>TPA effect on colony size</th>
<th>Alone</th>
<th>Coculture</th>
</tr>
</thead>
<tbody>
<tr>
<td>308</td>
<td>150</td>
<td>+</td>
<td>50 ± 5</td>
<td>62 ± 1</td>
</tr>
<tr>
<td>PD16</td>
<td>150</td>
<td>0</td>
<td>16 ± 0</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Pam 212</td>
<td>500</td>
<td>–</td>
<td>32 ± 1</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>PB</td>
<td>200</td>
<td>0</td>
<td>10 ± 1</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>LC 14</td>
<td>300</td>
<td>+</td>
<td>23 ± 5</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>SP-1</td>
<td>50,000</td>
<td>+</td>
<td>188 ± 26</td>
<td>393 ± 21</td>
</tr>
</tbody>
</table>

*Line 308 was developed from 7,12-dimethylbenz[a]anthracene-initiated adult BALB/c mouse epidermis (3); lines PB, PD (25), and SP-1 (6) were derived from 7,12-dimethylbenz[a]anthracene-initiated TPA-promoted papillomas on SEN-CAR mice; lines LC-14 (6) and Pam 212 (31) were derived from newborn BALB/c mouse epidermis cultured long term in low calcium (LC-14) or high calcium medium (Pam 212). With the exception of SP-1, all lines grow clonally in either low or high calcium medium. *+, positive effect of TPA on colony size of each line plated in the absence of primary keratinocytes; –, negative effect; 0, no effect.

RESULTS

Reconstitution Model. For a valid culture model of initiated epidermis, the clonal expansion of colonies of initiated cells must be inhibited by normal keratinocytes and the inhibition should be overcome by treatment with a tumor promoter. Six calcium-resistant keratinocyte lines were tested for colony formation when cocultured with primary keratinocytes in high calcium medium. Clonal growth of cells of lines 308, PD, Pam 212, and SP-1 was prevented for 14 days when the cells were cocultured with normal keratinocytes (Table 1). The number of PB or LC 14 colonies was reduced but not eliminated by coculture with normal keratinocytes. Continuous treatment of cocultures with the phorbol ester tumor promoter TPA (100 ng/ml) induced colonies of 308, PD, and Pam 212 cells within 2–3 weeks. In contrast, no colonies were seen in TPA-treated cocultures of SP-1 cells and primary keratinocytes.

TPA treatment of clonal cultures of the cell lines in the absence of primary keratinocytes increased the numbers of colonies of lines PB, LC 14, and SP-1 but did not affect colony numbers of the other lines (Table 1). A TPA-induced increase in colony size was found only with lines 308, LC 14, and SP-1, and a decrease was found with Pam 212 cells. Thus, the ability of TPA to induce colonies in cocultures could not be predicted by the effects of the tumor promoter on clonal cultures of the cell lines plated alone. The two basic criteria for the desired culture model were met by cocultures of cells of lines 308, PD, or Pam 212 with normal primary keratinocytes. Because of its relatively high clonal growth rate and the large number of colonies found in TPA-treated cocultures, line 308 was used for most subsequent experiments.

The clonal growth of 5–500 cells of line 308 was inhibited for 14 days by confluent primary keratinocytes. However, small colonies of 308 cells were evident by 20 days in DMSO solvent-treated cocultures (308 + K at Time 0 in Fig. 1), although always fewer and smaller than in the TPA-treated dishes. If continuous confluence is required for inhibition, this result was not surprising, since high calcium culture of primary keratinocytes remain confluent for only 7–10 days and then differentiate and slough from the culture dish (7). The addition of keratinocytes each week (308 + K weekly in Fig. 1) maintained the inhibition of 308 cell colonies and still allowed the emergence of colonies when cocultures were treated with TPA. Subse-

Fig. 1. Clonal growth of 308 cells: inhibition by coculture with confluent primary keratinocytes and stimulation by TPA. One hundred fifty cells of line 308 were plated in 60-mm dishes in high calcium medium either alone or with 4 × 10^5 primary keratinocytes (K). In half of the cocultures, 4 × 10^5 primary keratinocytes were added each week. Beginning 1 day after plating, dishes were treated with either TPA (100 ng/ml) or 0.1% DMSO. Quadruplicate dishes were stained with rhodamine at 20 days. When primary keratinocytes were added only at the time of plating, the number of colonies ± SE in the DMSO control dishes was 10.0 ± 1.2 compared to 37.0 ± 4.2 with TPA treatment. With weekly addition of keratinocytes, no colonies developed in DMSO-treated dishes compared to 21.25 ± 1.9 in TPA-treated dishes.
Fig. 2. Effects of phorbol ester-type tumor promoters on 308 colony size and on induction of 308 colonies in cocultures. Seventy-five cells of line 308 were plated in 35-mm dishes in high calcium medium either alone or with 2 x 10⁶ primary keratinocytes (K). Fresh primaries were added each week. Exposure to TPA (a), RPA (b), or mezerein (c) at doses of 0.1, 1.0, 10, or 100 ng/ml was begun at day 1 and continued with each feeding. Four to six dishes in each group were stained with rhodamine or crystal violet at 27 days in a and 20 days in b and c. Shown are single dishes of 308 cells plated alone and pairs of the cocultures. a. With TPA, no 308 colonies were seen at doses of 0.1 or 1 ng/ml, 3.0 ± 0.9 (mean ± SE) at 10 ng/ml, and 8.8 ± 1.0 at 100 ng/ml. b. With RPA, no 308 colonies were found at 0.1 ng/ml, 1.0 ± 0.7 at 1 ng/ml, 4.4 ± 1.2 at 10 ng/ml, and 3.8 ± 0.8 at 100 ng/ml. c. With mezerein, no 308 colonies were seen at 0.1 ng/ml, 6.4 ± 1.3 at 1 ng/ml, 5.6 ± 1.2 at 10 ng/ml, and 8.0 ± 1.2 at 100 ng/ml.
quently, fresh primaries were added weekly in all protocols.

Tests of Phorbol Ester-type Tumor Promoters. The dose-response relationships for the direct effect of TPA on 308 colony size and for the induction of 308 colonies in cocultures are shown in Fig. 2a. TPA concentrations of 1, 10, and 100 ng/ml caused equivalent enhancement of colony size in 308 cultures, while 0.1 ng/ml was ineffective. In contrast, no colonies were found with 0.1 or 1 ng TPA/ml in the cocultures, while 10 ng/ml yielded 3 colonies/dish and 100 ng/ml yielded 8.8 colonies/dish.

With RPA, a weaker promoter than TPA in vivo (18), 308 colony size was increased equally with doses of 1, 10, and 100 ng/ml, with a slight effect at 0.1 ng/ml (Fig. 2b). In the cocultures, one 308 colony/dish was found with 1 ng/ml, with about 4 colonies/dish at both 10 and 100 ng/ml. No colonies were observed at 0.1 ng/ml.

A different pattern of response was found with mezerein, a weak promoter which is more toxic in vivo (19) than either TPA or RPA. Doses of 1 and 10 ng/ml were equally stimulatory to the growth of 308 cells alone, with a reduced effect at 100 ng/ml, perhaps due to toxicity at this dose (Fig. 2c). In the cocultures, doses of mezerein of 1, 10, and 100 ng/ml were nearly equally effective, producing 5.6–8.0 colonies/dish.

Test of Benzoyl Peroxide, a Non-phorbol Ester Tumor Promoter (20). Benzoyl peroxide was tested at doses of 10^{-4} and 10^{-3} M in the 308 cell-keratinocyte culture model. Treatment of clonally plated 308 cells with 10^{-4} M benzoyl peroxide reduced the number of colonies from 58 ± 5 (mean ± SE) to 26 ± 1. In cocultures treated with 10^{-4} M benzoyl peroxide, a few rapidly growing 308 cell colonies (14 on 4 dishes) appeared 21 days after plating. The lower dose of benzoyl peroxide did not induce foci in cocultures or alter colony number in 308 cultures.

Tests of Antipromoters. Retinoic acid and fluocinolone acetonide inhibit skin tumor promotion when given concurrently with TPA in vivo (21, 22). When added to clonal cultures of 308 cells, neither inhibitor acted directly to reduce colony number, colony size, or the TPA-induced increase in colony size (Fig. 3). However, the number of 308 cell colonies/dish in cocultures treated with either inhibitor along with TPA was reduced compared to TPA alone (Table 2). In an earlier experiment in which primary keratinocytes were added only at the time of plating, neither retinoic acid nor fluocinolone acetonide inhibited TPA-induced 308 colony formation (not shown). Thus, the reduction in number of 308 colonies by these agents in cocultures appears to be indirect, altering the interactions of TPA with 308 cells and confluent normal keratinocytes.

Bryostatin 1, which activated protein kinase C, is an inhibitor of skin tumor promotion by TPA in vivo (23). As shown in Fig. 4, bryostatin 1 did not affect the colony size of clonally plated 308 cells and, when given along with TPA, caused a reduction in colony size without a change in colony number. In 308-keratinocyte cocultures, no 308 cell colonies were seen in the groups treated with either bryostatin 1 alone or bryostatin 1 with TPA. Thus, this antipromoter in vivo was completely effective in blocking promoter-induced colony formation in vitro.

Mechanistic Studies. In order to determine whether the inhibition of 308 cells by primary keratinocytes was cell type specific, cocultures of 308 cells with primary dermal fibroblasts were prepared. The number of fibroblasts was chosen to produce confluence within 1–2 days after plating, in analogy to the reconstitution experiments with keratinocytes. As shown in Fig. 5, neither the number nor the size of the 308 colonies was affected by coculture with dermal fibroblasts.

The inhibition of growth of 308 cells by primary keratinocytes in high calcium medium could require contact of the two cell types or, alternatively, could be mediated by factors secreted into the medium. To test the latter possibility, clonal growth of 308 cells in standard medium was compared to growth in medium conditioned by confluent primary keratinocytes. Conditioned medium from primary dermal fibroblasts was tested in parallel. As shown in Fig. 6, neither type of conditioned medium inhibited clonal growth of 308 cells. Thus, the inhibition of growth of 308 cells by keratinocytes is not the result of stable extracellular factors but appears to require cell contact or other cellular interactions. However, the effect could be mediated by an unstable extracellular factor which did not remain active in the conditioned medium or a factor which requires activation by normal keratinocytes.

In initiated mouse epidermis, the existence of dormant initi-
Fig. 4. Clonal growth of 308 cells alone and in cocultures with or without TPA: effect of bryostatin 1 (BRYO). Fifty cells of line 308 were plated in 35-mm dishes in high calcium medium either alone or in cocultures with 2 x 10⁶ primary keratinocytes (K). Fresh keratinocytes were added each week. Triplicate dishes were stained with rhodamine at 22 days. Shown are single dishes of 308 cells alone, keratinocytes alone, or 308 cells cocultured with keratinocytes.

Fig. 5. Clonal growth of 308 cells alone and in cocultures with or without TPA: effect of coculture with dermal fibroblasts (F). Seventy-five cells of line 308 were plated in high calcium medium in 35-mm dishes, either alone or with confluent dermal fibroblasts. Fibroblasts were prepared from newborn BALB/c mouse dermis as described (32) and were plated at 2 x 10⁶/dish. Triplicate dishes were stained with rhodamine at 20 days.

Fig. 6. Clonal growth of 308 cells: lack of effect of medium conditioned by fibroblasts (F) or keratinocyte (K). Conditioned medium was prepared by growing confluent fibroblasts or keratinocytes in high calcium medium and collecting medium at days 3, 6, and 8 after plating (33). Seventy-five 308 cells were plated in 35-mm dishes. Conditioned or standard calcium medium was used from day 1 until staining with crystal violet at 20 days.

Fig. 7. Clonal growth of 308 cells: lack of effect of medium conditioned by fibroblasts (F) or keratinocyte (K). Conditioned medium was prepared by growing confluent fibroblasts or keratinocytes in high calcium medium and collecting medium at days 3, 6, and 8 after plating (33). Seventy-five 308 cells were plated in 35-mm dishes. Conditioned or standard calcium medium was used from day 1 until staining with crystal violet at 20 days.

DISCUSSION

We have developed a cell culture model which mimics the biology of initiated epidermis in vivo. The clonal growth of a small number of initiated cells (line 308) is suppressed by coculture with a large number of normal keratinocytes in high calcium medium. Exposure of these cocultures to the tumor promoters TPA, RPA, mezerein, or benzoyl peroxide induces the formation of foci of 308 cells in a dose-dependent manner. Retinoic acid, fluocinolone acetonide, and bryostatin 1, inhibitors of promotion in vivo, reduced the number of promoter-stimulated colonies of initiated cells.

Studies were conducted to determine if a TPA-mediated increase in colony size was required to produce foci in cocultures. Cells of line PD at early passage (passage 7) responded to TPA exposure with an increase in colony size (not shown). However, by passage 17, PD cells no longer displayed this direct response to TPA (Table 1). When PD cells at passage 17 were cocultured with primary keratinocytes, TPA treatment produced PD colonies (Table 1) with the same efficiency as in experiments with the earlier passage (not shown). Therefore, colonies are produced even when there is no direct effect of TPA on PD cell growth, suggesting that TPA is primarily influencing the normal keratinocytes.

The properties of a keratinocyte cell line which determine its responsiveness to inhibition by coculturing with primary keratinocytes are not understood. Neither the benign or malignant phenotype of the cell line nor the activation of the ras gene correlates with activity in this model. The lines most useful in the coculture assay, lines 308 and PD, differ in phenotype when the cells are grafted to the backs of athymic mice. Line 308 cells, grafted along with normal fibroblasts, produced papillomas at the graft site, while line PD cells resulted in carcinomas (6). Other lines tested in the culture model included lines PB and Pam 212, which produce carcinomas in grafts, and lines LC 14 and SP-1, which produce primary keratinocytes under low calcium conditions (not shown), suggesting that gap junctional communication is not involved in the inhibition of 308 colony formation.

H. Hennings, unpublished data.
colonies after this time coincides with the lack of confluence of late-passage PD cells that did not respond directly to TPA, PD colonies were as frequent as in cocultures of early-passage PD cells, which proliferated in response to TPA. (b) Pam 212 cell colonies are induced by TPA in cocultures, even though TPA reduces colony size of Pam 212 cells alone. (c) The inhibitors retinoic acid and fluocinolone acetonide did not have a direct effect on the size or number of 308 colonies and did not modify the proliferative response of 308 cells to TPA. Although the mechanism by which normal keratinocytes function in this assay has not been defined, both promoters and antipromoters require the presence of confluent keratinocytes for their action in this model.

In coculture models utilizing fibroblasts, suppression of tumor cell growth by normal cells is mediated by gap junctional communication (11). Although gap junctional communication in both primary keratinocytes and 308 cells is increased 5-fold under low calcium conditions compared to high calcium, the growth of 308 cells was not inhibited by normal cells in low calcium medium. Therefore, a different mechanism may be involved in the keratinocyte model. Since 308 cells were not inhibited by conditioned medium from normal keratinocytes, the inhibition is likely to require cell contact. A requirement for cell contact, but not junctional communication, for maintenance of hepatocyte differentiated functions has been reported in cocultures of hepatocytes with biliary epithelial cells (26). In the 308-keratinocyte model, the dependence of the inhibition of 308 cell growth on the calcium concentration of the medium suggests the possibility of a calcium-induced change at the cell membrane, perhaps involving calcium-dependent cell surface molecules (27) or junctions (28). Since fibroblasts do not inhibited 308 cells in cocultures, the critical molecules may be specific to epidermal cells.

In the absence of evidence suggesting a role for inhibited intercellular communication in the action of tumor promoters in this culture model, how might they accomplish the selection for growth of initiated cell foci? Some promoters, like benzoyl peroxide, are more toxic to primary keratinocytes than to keratinocyte cell lines (29) and may promote by a selective toxicity. Cells of initiated or malignant transformed keratinocyte cell lines are characterized by a reduced response to stimuli of terminal differentiation, such as high calcium medium or phorbol ester-type tumor promoters. Thus, prolonged exposure to a tumor promoter such as TPA accomplishes a continued enrichment for the initiated population, even in the absence of a direct proliferative effect on the cells of the initiated line. Bryostatin 1 mimics many TPA effects but does not induce terminal differentiation and blocks TPA-induced terminal differentiation. The inactivity of bryostatin 1 in the promoter assay and its inhibition of TPA-induced colony formation suggest the importance of induction of terminal differentiation in the primary keratinocytes for colony formation of 308 cells. In further support of this idea, primary keratinocytes inhibit 308 colony formation for only about 2 weeks. The breakthrough of colonies after this time coincides with the lack of confluence resulting from the terminal differentiation of the primary keratinocytes by high calcium conditions.

The cell culture model for initiated epidermis presented here is valuable not only for mechanistic studies but as a bioassay for promoter or antipromoter activity. For example, bryostatin 1 was tested in this culture model prior to its test as an inhibitor in vivo; its activity as an inhibitor was predicted by the culture assay. Only a limited number of promoters and antipromoters have been tested. Of the promoters tested in the keratinocyte coculture model, benzoyl peroxide showed the lowest activity, both in vivo and in the culture model. Although TPA is a stronger promoter than RPA or mezerein in vivo, mezerein was the most active in 308-keratinocyte cocultures. Differences have been described between types of initiated cells in their potential for progression to malignancy and their sensitivity to weak promoters (30); promoters may differ in their ability to enhance the growth of each type. Thus, 308 cells may represent the type of initiated cell most responsive to mezerein. The classification of a promoter as strong in vivo is based not only on the number of papillomas promoted but also on a short latent period for papilloma induction. So-called "weak" promoters, such as mezerein or RPA, produce papillomas with a longer latent period than those promoted by TPA. However, if one looks at the number of papillomas or carcinomas present after a long duration of promotion, the differences between TPA, RPA, and mezerein are much less pronounced (18, 19, 30). The potential utility of this culture model for bioassay will require validation by testing of a number of appropriate compounds with known activity as promoters or antipromoters in vivo.

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