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STUDIES ON GUINEA PIG SKIN CELL CULTURES[1]

II. *Effect of a Pig Skin Extract on DNA Synthesis*

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Abstract. Adult guinea pig epidermal keratinocytes and dermal fibroblasts have been grown as separate primary coverslip cultures. An aqueous extract prepared from split-thickness minipig skin flaps (PSAE) containing epidermis and dermis, inhibited DNA synthesis in both cultures. In fibroblast and keratinocyte cultures the inhibition differed significantly ($p<0.05$). This suggests that the PSAE contained both epidermal and dermal inhibiting factors. The specificity of the inhibition exerted by the PSAE on keratinocyte and fibroblast cultures was then tested by comparison with that on guinea pig cultures of lung, kidney, bone marrow, spleen, and bladder. The inhibition of fibroblasts was found significantly ($p<10^{-2}$ to 10^{-6}) greater than that of all other cultures. As regards keratinocytes, they were inhibited significantly more ($p<10^{-2}$ to 10^{-6}) than all other cultures except those from the bladder.

Epidermal keratinocytes and dermal fibroblasts can be grown as separate cultures (10).

During the first week of cultivation, these two cell types can be characterized on the basis of their morphology (6), cytoenzymatic reactions (8), and growth capacity (10).

Biological evidence demonstrates that the growth of a given tissue is likely to be controlled in vivo by tissue-specific, species-non-specific, inhibiting substances (1).

In culture, it has been shown that skin extracts do inhibit DNA synthesis in epidermal cells (3, 9, 2). However, this inhibition was said to be tissue-specific in a system using human skin extracts and human cells in culture (2) whereas it was found to be non-specific in experiments involving newborn rat epidermis extracts and guinea pigs and/or human epidermal cells (9).

In previous experiments at this laboratory, it has been shown that pig skin extracts inhibit the synthesis of DNA in cultured guinea pig keratinocytes (3) and that this inhibition is tissue-specific (4). The specificity was assessed in showing that the same pig skin extract reduced the incorporation of tritiated thymidine in keratinocytes, significantly more than in primary cultures of guinea pig lung fibroblasts and kidney cells.

In the present study, the effect of the same pig skin extract on DNA synthesis by dermal fibroblasts has been evaluated. In addition, this extract has been tested on cell cultures derived from guinea pig bone marrow, spleen, and bladder.

MATERIAL AND METHODS

1. *Adult guinea pigs* (body weight 250–300 g) of the Hartley strain, pathogen-free certified (EVIC-CEBA Blanquefort, France), have been used as cell donors.

2. *Tissue culture techniques* for keratinocytes and dermal fibroblasts have been described in detail in the first paper of this series (10).

Lung, kidney, spleen, and bladder tissues were chopped into fine fragments with Gillette B mounted blades and scissors. The fragmented tissues were rinsed in the glucose potassium sodium (GKN) solution of Hinz & Syverton (7) for 5 min with magnetic stirring. The resulting suspension was filtered through two layers of surgical gauze. Tissue fragments remaining on the gauze were then transferred to 0.1% collagenase (Worthington, crystallized) in GKN for 2 hours in a water bath at 37°C, with mechanical stirring (80 rpm) in an ordinary 50 ml Becher.

[1] This study was presented at the Joint Meeting of the American Society of Investigative Dermatology and the European Society for Dermatological Research, Amsterdam 17–18 May, 1972.

Table I. *Number of selected cultures*

	Epidermal keratinocytes	Dermal fibroblasts	Lung	Kidney	Spleen	Bone marrow	Bladder
No. of selected coverslip cultures	130	123	87	24	79	135	85

After filtration through one layer of surgical gauze, the collagenase suspension was spun down at 800 rpm for 5 min. The supernatant was discarded. The cells were resuspended in tissue culture medium (TCM), and distributed in Leighton tubes.

Bone-marrow cultures have been made by dissecting the femur bone. After having broken the diaphysis, small amounts of bone-marrow material were pipetted directly from the marrow and planted in Leighton tubes after gentle pipette dissociation in TCM.

TCM consisted of minimal essential medium (MEM, Eagle, 1959) supplemented with 25% horse serum for skin-derived cells. MEM supplemented with 10% calf serum was used for all other cultures. Two ml of TCM per coverslip cultures was used.

3. *Pig skin aqueous extract (PSAE).* One female minipig weighing 45 kg was anesthesized with Nembutal, intravenously. The hairs were removed by electric shaving followed by the application of a depilatory wax. After thorough rinsing in a water bath, the skin was brushed with soap and warm water, and dried. Split thickness skin flaps were then removed with an electric dermatome. Each flap was about 1 mm thick and had a surface area of around 10 cm^2. They were kept in dry ice. Some 422 g of skin were thus collected. After lyophilization, the skin was ground in an electric grinder and 129 g of fine powder was obtained. The powder was resuspended in 1 280 ml of iced distilled water and the suspension was mixed with a high speed electric mixer (TURRAX) under controlled temperature.

After high-speed centrifugation the yield was of 378 g of precipitate and 935 ml of supernatant. This supernatant was filtered on 0.22 Millipore filter and distributed in 1 400 ampoules containing 0.65 ml of aqueous extract each.

This extract was kept in sealed vials at $-20°C$ after lyophilization. Each vial contained 5 mg of equivalent protein as determined by the technique of Lowry.

4. *Thymidine pulses* were performed by adding tritiated thymidine[2] (spec. act. 14.9 Ci/mM) at a final dilution of 1 μCi/ml, for 30 min at 37°C.

5. *Evaluation of radioactivity (cpm).* This was done by direct counting of coverslip cultures in a liquid scintillator counter (Packard Tricarb) according to the technique of Fallot et al. (5).

6. *Evaluation of cell population.* The cell population of each coverslip culture was evaluated by measuring the optical densities (OD) of the cultures in a Chromoscan (Joyce & Loebl) after staining with Geimsa (3).

7. *Results were expressed* as cpm to OD ratio for each coverslip culture.

8. *Experimental procedure.* Six hundred and sixty-three coverslip cultures were selected for study in a series of 28 experiments. Selection of cultures was based on morphological appearance after 3 days of cultivation. Those which were contaminated or growing poorly were discarded. The remaining cultures were then called "selected cultures".

The number of selected cultures per tissue of origin is given on Table I.

Each experiment consisted in sacrificing 1, 2, or more animals, from which various cultures were prepared. The number of selected cultures per experiment and per tissue of origin is given in Table II. One can see, for example that in experiment 9, cultures were prepared from epidermis, lungs, kidney, spleen, and bone marrow.

Selected cultures from each tissue were randomly divided in two sets.

The first set was used as control. TCM was removed and new TCM added. After 3 hours of contact, tritiated thymidine was added and all cultures harvested after 30 min of contact with the radioactive tracer at 37°C.

The second set was challenged with PSAE. The TCM was removed and a new TCM containing 2.5 mg of protein equivalent per ml of lyophilized extract was poured into the tubes. Cell cultures were maintained in the tubes for 3 hours in contact with the extract-supplemented TCM at 37°C. Tritiated thymidine was then added and the cells kept for 30 additional minutes. Thus the total time of contact with the skin extract was 3 hours and 30 min.

The numher of first-set control cultures (C = control) and second-set treated cultures (T = treated) per experiment and per tissue is given in Table II.

All cultures were rinsed with three changes of Earle's saline solution, fixed for 30 min in acetic acid/methanol mixture 1 : 3, dehydrated through 3 baths of absolute ethanol of 5 minutes each, transferred to toluene (3 baths of 5 min) and air dried. They were then dipped in toluene + PPO + POPOP and placed in the scintillator counter.

After counting, the coverslip cultures were washed in toluene and ethanol and air dried. They were then stained all in the same bath of Giemsa stain and scanned in Chromoscan.

CPM as counted in the scintillator and OD as measured in the Chromoscan were recorded and cpm/OD ratios calculated.

[2] From the Commissariat à l'Energie Atomique (CEA, Saclay, France).

Table II. *Number of selected cultures per experiment*

C = Number of control coverslip cultures. T = Number of coverslip cultures treated with the Pig Skin Aqueous Extract (PSAE)

	Epidermis		Dermis		Lung		Kidney		Spleen		Bone marrow		Bladder	
Experiment	C	T	C	T	C	T	C	T	C	T	C	T	C	T
1	2	2												
2	10	10												
3	2	10			5	7								
4	5	10			4	10								
5	5	10			5	10								
6	4	8												
7	2	2			1	1	2	2	2	2	2	2		
8	7	8			5	6	7	7			7	8		
9	4	4			4	3	3	3	4	4	5	5		
10	4	4			1	1					8	3		
11											4	4		
12	6	6							1	1	4	4		
13	7	8									6	6		
14									1	1	5	5		
15					4	5					5	5		
16			3	3										
17			7	7	3	3			5	5	3	3		
18			7	7	4	5			3	3	8	8		
19			6	6										
20			6	7					4	5	7	7	4	5
21			6	7					4	5	8	8	4	5
22			6	5					5	4			4	5
23			7	8					5	5			5	5
24			9	7					6	4			6	4
25													5	5
26													5	4
27			5	4									5	5
28													5	4

9. *Statistical analysis.* The calculation of percentage incorporation and the comparison of the various cultures have been made in taking in account the fact that for each culture, several independent experiments had been performed. The block method with unequal numbers has been applied (12).

For each culture and each experiment i, the following calculations have been made:

(*a*) the ratio $p_i = (m_1 - m_0)/m_0$ and the difference $d_i = m_1 - m_0$ (m_1 and m_0 corresponding to the mean values of cpm/OD ratios in treated and control cultures respectively).

(*b*) weighted means p of p_i and d of d_i (the weights being $w_i = n_1 n_0/(n_1 + n_0)$ with n_1 and n_0 corresponding to the numbers of treated and control cultures respectively).

(*c*) the variance of d. $\sigma^2 d$.

The percentage of incorporation has been estimated by p. As regards the comparison of two given cultures, 1 and 2, it was made by testing the equality of d_1 and d_2 by $(d_1 - d_2)/\sqrt{(\sigma^2 d_1 + \sigma^2 d_2)}$, which is approximately Gaussian.

It has been found accepted that d_1 and d_2 were independent. This is not always true since one given experiment can be used to study two cultures. However, this effect is weak and the fact of disregarding it leads only to a lack of power.

10. *Control of toxicity.* In order to test for gross toxicity of the PSAE, dermal fibroblast cultures of varying density have been maintained in contact with TCM supplemented with PSAE for 3½ hours. Subsequently, they were rinsed three times with fresh TCM and kept in the fourth change for 20 hours at 37°C.

The growth capacity was then evaluated by tritiated thymidine incorporation as indicated above.

11. *Thermosensitivity of PSAE.* PSAE was heated at 37°C for 1, 4, 7, 10, 13, 16, 19, 21, and 24 hours. The effect of heated PSAE on dermal fibroblast coverslip cultures was evaluated according to the technique given above.

12. *Dose Response Curve.* Keratinocyte cultures were challenged with various doses of PSAE including 2.5, 5, and 50 mg of protein equivalent per ml and the incorporation of thymidine estimated as above.

RESULTS

Taking the cpm/OD values in control culture as representing 100% incorporation, the mean values of percentage incorporation in cultures treated with PSAE have been calculated. They are given in Table III. The incorporation in keratinocyte

Table III. *Mean values of percentage incorporation of thymidine in cultures treated with PSAE*

Type of cell culture	Per cent incorporation (Control = 100 %)	Comparison with keratinocytes	Comparison with dermal fibroblasts (p less than)
Keratinocytes	$28^{(10)}$	—	0.05
Lung	$56^{(10)}$	$p < 10^{-9}$	10^{-9}
Kidney	$45^{(3)}$	$p < 10^{-3}$	10^{-5}
Spleen	$39^{(11)}$	$p < 10^{-3}$	10^{-9}
Bone marrow	$34^{(13)}$	$p < 10^{-2}$	10^{-9}
Bladder	$26^{(9)}$	$p > 0.05$	10^{-2}
Dermal fibroblasts	$20^{(10)}$	$p < 0.05$	—

Numbers in parentheses correspond to numbers of experiments.

cultures has been compared with that in other cultures. It can be seen in Table III that it is significantly less than in lung, kidney, spleen, and bone marrow cultures. It is not significantly different from that in bladder cultures.

The incorporation of thymidine in cultures of dermal fibroblasts was also compared with that in other cultures. Table III shows that it is significantly less than in all other cultures including lung, kidney, spleen, bone marrow, bladder, and keratinocytes.

Experiments on gross toxicity have yielded the following information:

(*a*) Dermal fibroblast cultures did not exhibit any visible change, either immediately after contact with the PSAE, or 20 hours later.

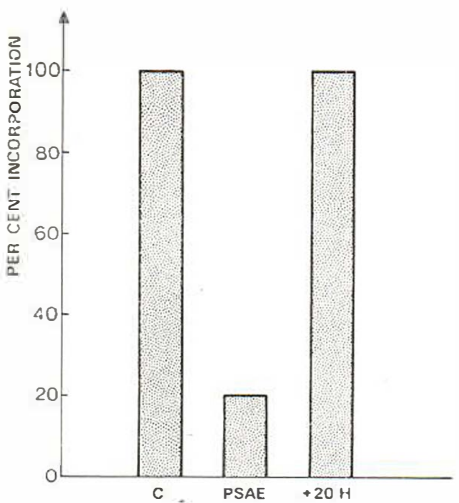

Fig. 1. Gross toxicity. The incorporation of thymidine (cpm) is reduced to 20% in PSAE-treated cultures. But 20 hours after the PSAE has been removed (+20 h) the cultures incorporate as much thymidine as the controls (*C*).

(*b*) Incorporation of thymidine decreased by about 80% during the time they were exposed to PSAE. Twenty hours after the PSAE has been removed and replaced by fresh TCM, thymidine incorporation returned to normal (Fig. 1).

Experiments on thermosensitivity are summarized in Fig. 2.

One can see that after 1 hour of incubation at 37°C the inhibitory effect of PSAE is less than that of the non-heated extract. The effect of heat is maximal after 4 to 7 hours of heating.

An example of dose–response curve is given in Fig. 3. One can see that the inhibition of DNA synthesis in keratinocyte cultures is dose-related.

DISCUSSION

The main finding of this study is that a skin extract inhibits the growth of both epidermal kerationcytes and dermal fibroblasts. One first consideration, however, is to decide whether or not this is simply a toxic effect.

In this regard, our experiments show that if the capacity of dermal fibroblasts in culture to incorporate tritiated thymidine is reduced by about 80% in presence of pig skin extract (PSAE), this capacity is restored to normal 20 hours after this exposure. This means that the cells recover from the inhibitory effect, and thus that no gross permanent damage has been inflicted on them. This speaks against a major toxic effect, and in favour of a drug-like action.

This latter interpretation is supported by the dose–response curve which presents itself with the classical shape of a drug dose–response curve.

However, the effect of heat on PSAE shows that the heat-labile components have only a lim-

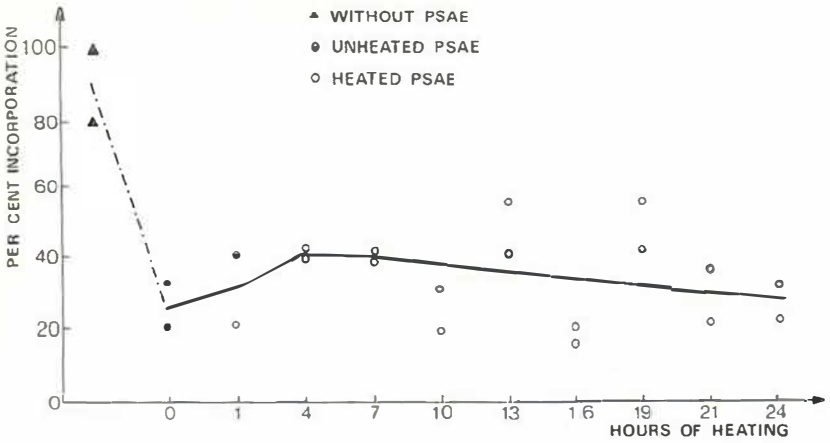

Fig. 2. Unheated PSAE reduces the incorporation of thymidine (- - -). When heated at 37°C for 4 to 7 hours, the effect of PSAE is less pronounced, indicating that part of its inhibitory action may be due to thermolabile factor(s).

ited inhibitory action. Therefore it is likely that the total inhibitory effect of PSAE is the result of combined factors.

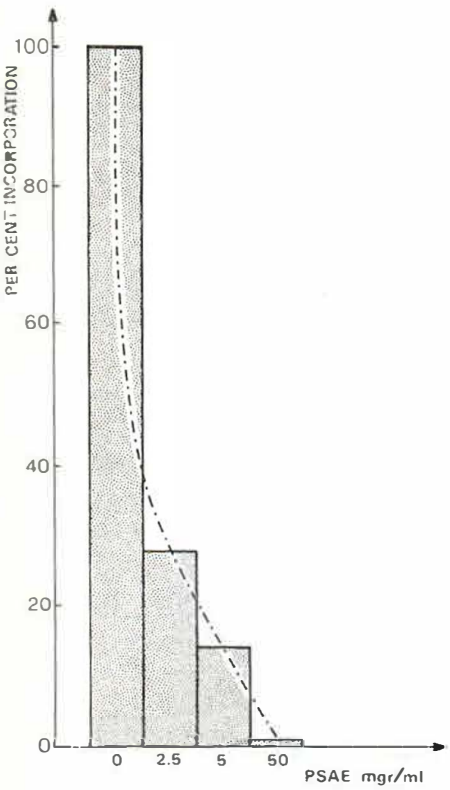

Fig. 3. The incorporation of thymidine is reduced by the PSAE; this depression is dose-related.

This is not surprising in view of the fact that the aqueous extract used in this study has not been purified and, therefore, represents a rather crude material.

One second task is to ascertain whether the inhibition is tissue-specific. One way to answer this question is to compare the effect of skin extracts with extracts from other tissues. Two conflicting results have been reported in the literature. In one, extracts of liver reduced the number of mitoses in cultures of guinea pig epidermal cells (9). In the other, liver extracts had no significant effect on epidermal cells in culture (2). In recent experiments which will be reported later, it has been found that tongue mucosa extracts of bovine origin also had some inhibitory effect on DNA synthesis by both epidermal keratinocytes and dermal fibroblasts. However, this inhibitory effect was not significantly stronger in guinea pig skin cells than in cells derived from lung or spleen of the same guinea pig. This militates in favor of the presence in the pig skin extract of specific factor(s) and against a non specific inhibitory effect due to the large quantity of non purified material introduced in the culture fluid (2.5 mg/ml).

Another way of assessing the specificity of a given extract is to test its effect on several different cell types. In the present study, the same pig skin extract has been tested on a wide variety of cultures. If one considers epidermal keratinocyte cultures, one can see that the inhibition

is significantly weaker in lung, kidney, spleen and bone marrow cells.

If the experiments had been limited to this series of cultures, it would have been logical to claim that the inhibition was tissue-specific. However, in extending the controls to bladder cells, it was found that the PSAE inhibitory effect was just as strong as in epidermal keratinocytes. Therefore, the specificity of the skin extract for keratinocytes in culture does not appear totally certain.

If one now turns to dermal fibroblasts, one is struck by their susceptibility to the PSAE. Indeed, they are more inhibited than either epidermal keratinocytes or bladder cells. This would indicate that the PSAE contains inhibitory factor(s) specific for connective tissue cells. In view of the thickness (1 mm) of the flaps of skin removed from the pig to prepare the extract, it is clear that the PSAE contains material from both epidermis and dermis, i.e. from ectodermal and mesenchymal derivatives. Therefore, it may be conceived that in the PSAE there is a combination of substances, some acting on epidermal cells and others on connective tissue cells such as those derived from the dermis.

One may then recall that the concept in which connective tissue cells could be kept under control by inhibitory material was suggested as far back as 1937 (11).

In this respect, it is of interest to note that the inhibitory effect on dermal fibroblasts is significantly stronger than that on all other connective tissue cell cultures tested in this study. This does suggest the presence in the dermis of some factor(s) acting preferentially on dermal fibroblasts.

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