EPIDERMAL PROLIFERATION OF NUDE MOUSE SKIN, PIG SKIN, AND PIG SKIN GRAFTS

Failure of Nude Mouse Skin to Respond to the Tumor Promoter 12-*O*-Tetradecanoyl Phorbol 13-Acetate*

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The description of transplantation of human skin to the congenitally athymic (nude) mouse by Reed and Manning (1) presented the potential of an animal model through which the pathobiology of human skin can be studied. The rationale for acceptance of this system for the study of such mechanisms in the skin include considerations of whether the graft retains the characteristics of the donor, or acquires those of the host, in terms of differentiation and proliferative phenomena. The possibility exists that the graft serves as a skeleton for the growth of mouse epidermis. Experimental data that support the notion that the transplanted tissue remains of donor origin include: (*a*) xenogeneic grafts are rejected after thymic reconstitution (2, 3); (*b*) markers unique to the graft, e.g., hair, feathers, and specific histologic features, persist for the life of the graft (2); (*c*) histologic features unique for the skin disease, e.g., psoriasis and lamellar ichthyosis, persist (4, 5); (*d*) tumors that produce unique peptides, e.g., insulin and calcitonin, continue secretion when transplanted to the nude (6, 7). These experiments demonstrated that xenogeneic tissues retain donor characteristics.

To study the effect of host factors on graft proliferation, we have devised a system whereby readily available domestic pig skin can be transplanted to the nude mouse. Pig skin has features in common with human skin, including its histology, permeability to topically applied agents, turnover time, and epidermal cell cycle kinetics ([8–11]; and B. W. Bennett, J. O. Ahrchambeau, and J. Hisato. Personal communications.).

This paper reports that the topical application of 10 ng of an agent, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)[1] enhances epidermal proliferation in mice heterozygous for nude, in domestic pig skin, and in grafts of pig skin to nude mice. Surprisingly, the topical application of this dose of TPA does not show a similar enhancement in mice homozygous for nude.

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[1] *Abbreviations used in this paper:* D/A, dimethyl sulfoxide:acetone ratio; DMSO, dimethyl sulfoxide; LI, labeling index; Δ LI, increases in the LI; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate.

Materials and Methods

The nude mice used in these experiments were derived from a pathogen-free colony initiated from animals that were a gift from Dr. Norman Reed, Montana State University, Bozeman, Mont. The colony was begun and expanded by mating pathogen-free female BALB/c mice, heterozygous for nude, with BALB/c males, homozygous for nude. The mice were housed in a germ-free environment provided by laminar airflow stations (Lab Products, Garfield, N. J.) and maintained on an artificial circadian rhythm of 12 h darkness and 12 h of light, at a room temperature of 26°C. The average lifespan of nude mice, reared under these conditions, was 14–18 mo. The colony displayed no evidence of early wasting.

At 2–3 mo of age, nude mice were grafted with pig skin obtained by shaving the pig's hair and removing a split-thickness skin graft, 0.6 mm thick by 20 mm wide, with a Castroviejo electrokeratome (Storz Instrument Co., St. Louis, Mo.), as previously described (5). Later modifications include holding the grafts in place until a coagulum forms. The grafted site (~1.5 cm in diameter) was covered with a bandaid that was clipped to the animal's ventral surface with a surgical staple (Clay Adams, Parsippany, N. J.). Little or no ghost or crust formed. All grafts were placed over the animal's lateral thoracic cage.

TPA and croton oil, inducers of epidermal proliferation, were applied in 0.1-ml vol to a 2-cm^2 area on the pig. For the mouse, and the pig skin graft, 0.05 ml of the agent was applied to a 1-cm^2 area. These topical treatments were carried out while the mice were lightly anesthetized by fluorathane inhalation. The proliferative agents were solubilized in a volatile vehicle. Fresh TPA was prepared in a solution of 50% dimethyl sulfoxide (DMSO), 50% reagent grade acetone (DMSO:acetone [D/A] ratio 1:1), unless otherwise indicated, and flushed with nitrogen and stored at −70°C. 5% croton oil in acetone was applied in a similar fashion. The proliferative agents were obtained from Sigma Chemical Co., St. Louis, Mo. Concentrations of TPA used to induce epidermal proliferation in mice in two-stage carcinogenesis experiments vary from 10^{-5} M (310 ng in 0.05 ml) to 10^{-9} M (0.031 ng in 0.05 ml) (12, 13). Because of the potential problem of transfer of agents among animals by direct contact, the treated areas were occluded with bandaids.

18 h after the initiation of proliferation, a small snip biopsy (2–3 mm wide, 4–6 mm long, non-full-thickness) was taken from the treated site, along with a biopsy from an untreated site on the opposite side of the animal. An additional control that consisted of either an untreated graft or nude mouse skin was also obtained. A small number of experiments measuring the labeling index (LI) of the contralateral untreated side showed an elevation over untreated animals. This activity disappeared when the treated site was occluded with a bandaid, suggesting that the increased activity could be explained by animal to animal transfer of the agents being tested. Control animals for these experiments were groups of totally untreated animals, or animals to which placebo vehicles had been applied. Data generated from the contralateral side of treated animals did not differ from controls, and were subsequently omitted from analysis.

D/A alone does cause a significant increase in the LI when applied to pig skin, relative to no vehicle or acetone. However, when D/A is applied to grafted pig skin and to non-grafted nude skin, no significant change in the LI occurs. A plausible explanation for the increased response by de novo pig skin could be related to the

observation that, when D/A is applied to skin of human subjects, an exothermic response is experienced (Unpublished observations.). Holding the epidermis of ears of mice at high ambient temperatures is reported to increase the LI (14). Whether D/A causes a similar exothermic response in nudes or pigs is currently unknown.

Biopsies were placed in a Petri dish containing 2 ml of RPMI-1640 and 2 μCi/ml of [^{3}H]thymidine (sp act 20 Ci/mM; New England Nuclear, Boston, Mass.) and incubated at 37°C for 2 h. The specimens were rinsed, placed in 10% buffered formalin, and prepared for histologic sectioning. 2-μm sections were placed onto slides and covered with NTB-3 emulsion (Eastman Kodak Co., Rochester, N. Y.) for 4 d. The slides were counterstained with hematoxylin and eosin, and the number of cells that contained five or more grains over the nuclei were determined by microscopic counting at 1,000 ×. The LI is the number of labeled cells in the basal layer/1,000 basal cells. The Student's two-tailed *t* test of means was used for statistical analysis.

Results

Effects of TPA and Croton Oil on Skin before Transplant. In that TPA is commonly used to induce epidermal proliferation, this agent was used to initiate comparative studies of epidermal proliferation of donor skin, pre- and posttransplant (12, 13). The wave of proliferation by germinative cells of normal mouse epidermis induced by the topical application of TPA is quite marked. Raick (12) has demonstrated that applying 1 ng of TPA in acetone to the skin of ICR mice increases the DNA synthesis in epidermal cells by threefold. In addition, repetitive applications of TPA at concentrations as low as 10 pg can induce epidermal hyperplasia (12, 13). In our experiments, eight nudes were challenged with dosages of TPA, 31 ng and 310 ng in acetone, to 1-cm^2 areas. Table I depicts the varied responses of the nude mouse to these dosages of TPA. No evidence of the proliferative response seen in the hairless mice was observed in the nude mouse. These results were unexpected. When parallel experiments using the same amounts of TPA in acetone on pig skin also failed to evoke an increase in the LI of the epidermis, we considered the possibility that TPA was not absorbed. In an effort to enhance absorption, DMSO was added to the acetone vehicle at a ratio of 1:1 (D/A). In this vehicle, 10 ng TPA did effect an increase in the LI of pig skin, but not of nude skin.

The above observation suggests that the epidermis of nudes may be relatively nonresponsive to agents that induce proliferation. Experiments were designated to investigate this possibility. As controls, we compared the LI of nude skin, untreated

TABLE I

Ratio of LI of Basal Cells of Skin of Individual Animals Treated with TPA to the Contralateral Side of the Animals, Nudes vs. Hairless

	n	Ratio of LI index	
		(1×10^{-5} M)*	(1×10^{-6} M)*
hr/hr	8	7.5 ± 2‡	5.9 ± 3
nu/nu	8	1.8 ± 1.1	1.8 ± 1.6

* TPA in acetone, 0.05 ml, is applied to a 1-cm^2 area, 1×10^{-5} M (310 ng), 1×10^{-6} M (31 ng).

‡ Values are the mean of ratios ± SD of the LI of treated side/LI of untreated side.

and treated, with the vehicle alone (see Fig. 1). No major differences were found. The baseline LI of ~45 in nude mice was the same as in normal mice (hairless and BALB/c heterozygous for nude) (data not shown). This demonstrated that baseline LI of nudes was like that of other mice.

The effects of the application of agents that induce epidermal proliferation when applied topically to skin are shown in Fig. 2. These data are presented as increases in

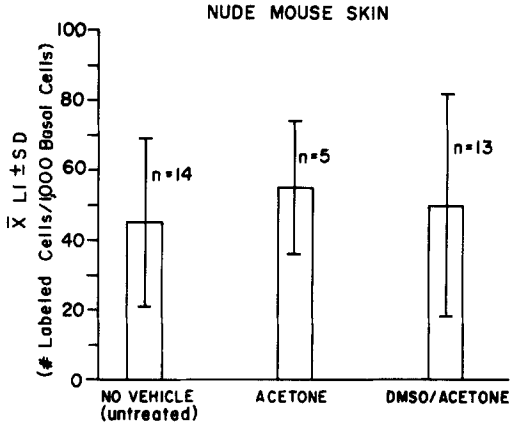

FIG. 1. Comparison of epidermal LI no treatment vs. vehicles. Bars represent mean LI ± SD; no significant differences exist between the groups; n = number of animals evaluated for that experiment.

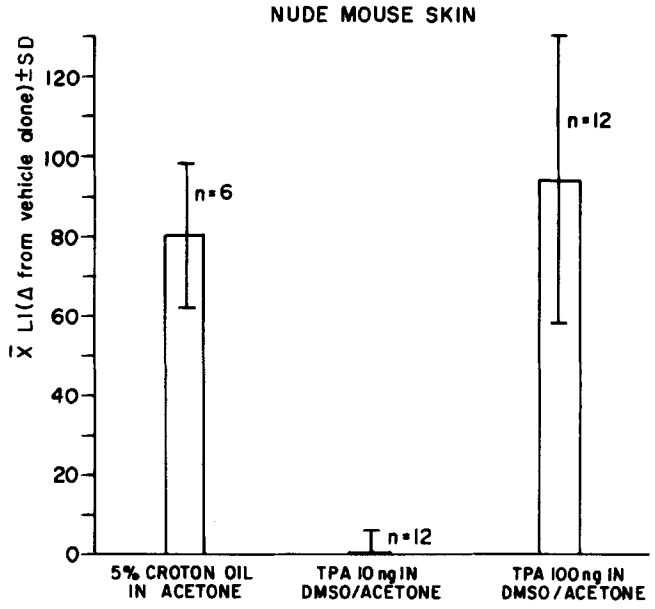

FIG. 2. Comparison of the Δ LI relative to vehicle alone by agents that induce epidermal proliferation. Bars represent the mean change in the LI evoked by various agents relative to the mean LI of the appropriate vehicle alone treated group. n for these vehicle treated groups ranges from 5–10. n listed beside the bars represents the number of animals treated with active agents. Significance: croton oil vs. acetone $P < 0.05$; TPA 10 ng vs. D/A = NS; TPA 100 ng vs. D/A = <0.005.

the LI (ΔLI) above that induced by the respective vehicles alone. The topical application of 5% croton oil in acetone results in a significant increase in the LI, as did the addition of a higher concentration of TPA, 100 ng.

The major component of croton oil that induces the proliferative response has been demonstrated to be TPA (13). In light of these observations, a dose response to TPA in D/A (1 ng, 10 ng, 100 ng, 1 μg, and 10 μg) was carried out. Only one concentration of TPA, 100 ng, yielded a response (see Fig. 2). At concentrations higher than 100 ng, the treated epidermis sloughed, whereas at dosages less than 10 ng, no response could be detected.

Effect of the Application of TPA and Croton Oil on the Proliferation of Normal and Transplanted Pig Skin, Compared with Host Skin. The base line untreated LI of pig skin was 42 ± 15, compared with 45 ± 14 for nude mice (c.f. Fig. 1 and Fig. 3). The skin's response to acetone was similar. However, pig skin was more responsive to D/A than was the similarly treated nude skin ($P < 0.05$) (c.f. Fig. 1 and Fig. 3).

As discussed previously, the application of a high dose of croton oil (5%) to nude mouse skin resulted in an increase in the LI of 80, relative to the vehicle. No erythema accompanied this proliferative response. When a similar dose was applied to pig skin, erythema was noted concomitant with an increase in the LI of 263 (see Fig. 4). Highly responsive pig skin transplanted to the less responsive nude mouse showed an intermediate response of 130.

The application of 10 ng of TPA in D/A to pig skin resulted in a significant increase in the LI (see Fig. 4). Because this dose of TPA increased the LI, one might expect that a similar dose to pig skin grafted on the nude mouse would result in a similar increase in the LI, unless the unresponsiveness of the host to 10 ng of TPA effects the ability of the graft to respond. The response of grafted pig skin to 10 ng TPA in D/A was similar, pre- and postgrafting, whereas the nude continued to be unresponsive (see Fig. 4).

Relationship of Genetics of the Nude Mouse to the Response to TPA. The foregoing experiments have demonstrated that, in addition to the persistence of anatomical

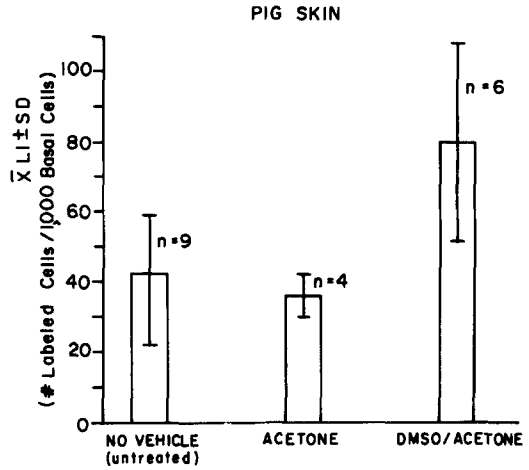

FIG. 3. Comparison of LI no treatment vs. vehicles. Bars represent mean LI ± SD. There is a significant increase in the LI of groups treated with D/A, $P < 0.005$. n = number of animals evaluated for that experiment.

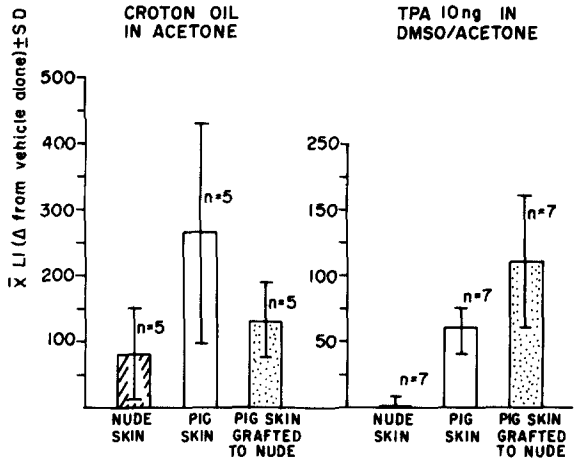

FIG. 4. Comparison of the Δ LI after the topical application of proliferative agents, host skin vs. donor skin pre:post transplant. Bars represent the mean change ± SD in the LI evoked by croton oil and TPA relative to the mean LI of the appropriate vehicle treated group on various types of skin. *n* for vehicle treated groups and active agent treated groups is the same, and is listed beside each bar.

	Significance (*t* test of means − 2 tail)		
	Comparison of agents on various types of skin		
Agent applied	Nude vs. pig	Nude vs. graft	Pig vs. graft
Nothing	NS	NS	NS
Acetone	NS	NS	NS
DMSO/acetone	NS	<0.02	NS
Croton oil	NS	NS	NS
TPA	<0.001	<0.01	NS

features, the xenogeneically grafted skin retained the characteristic donor responsiveness to agents that induce epidermal proliferation. Because the inability of the nude mouse skin to respond to lower dosages of TPA has ramifications beyond the viability of this system for studying skin biology, e.g., the characterization of two-stage carcinogenesis, further analysis of this phenomenon was undertaken. These experiments were directed at investigating the contribution of the mice's genetic background to their inability to respond to 10 ng TPA. Two different genetic groups were assayed in this context, mice heterozygous for nude from the same genetic background as the fully mutant nude, and nude on an outbred strain, ICR mice.

TPA (10 ng), as well as the vehicle (D/A), were applied to BALB/c haired mice, heterozygous for nude (nu/+). These nu/+ mice responded dramatically to TPA, the LI increasing by 4.5 times that of unresponsive mice homozygous for nude (nu/nu). Thus, mice heterozygous for nude, with the same genetic background as the unresponsive mice homozygous for nude, have the ability to respond to the lower dosage of TPA.

To further test the role of nude on the ability to respond to TPA, nude mice with a different genetic background, ICR, were subjected to experiments paralleling those described for the BALB/c nude. Preliminary experiments with nude mice of the ICR background showed that these animals had a consistent, but minimal, response to

TPA. Normal ICR mice, without the nude gene, respond to TPA in a manner similar to haired BALB/c mice, heterozygous for nude. During the course of these experiments, this locally derived ICR nude mouse colony was lost. Because no similar colony exists, these experiments had to be discontinued. However, similar experiments using nude on strains other than BALB/c are in progress, and will be the subject of a subsequent communication. In the main, the observations with the ICR mice support the contention that the genetic trait responsible for nude results in a diminished response to the tumor promoter, TPA. Comparing the response to TPA in normal mice and mice heterozygous for nude, with that by BALB/c and ICR nude mice, certainly suggests that TPA responsiveness follows genetic rules of a recessive trait.

A determination of whether or not responsive pig skin imparted responsiveness to the normally unresponsive nude (BALB/c) skin was also made. In this experiment, 10 ng TPA was applied to nudes having a pig skin graft on the contralateral side. These results were compared with the same procedures to ungrafted nudes. The response to TPA to nude skin was very similar in both groups of animals. Six grafted animals receiving TPA to the side contralateral to the graft had a mean Δ LI of 6.5 ± 7, over six similarly treated nongrafted nudes, an insignificant difference. Having a 1.5-cm^2 pig skin graft, capable of responding to TPA, does not confer responsiveness to the nude. An equivalent experiment, wherein unresponsive nude (BALB/c) skin was transplanted to responsive heterozygous littermates, demonstrated that the nude skin remained unresponsive to 10 ng TPA, Δ LI of zero. The mean Δ LI of similar nude grafts treated with 100 ng TPA was 63. These results confirm the observation that the unresponsiveness to 10 ng TPA is inherent to the skin of the nude.

Discussion

The work delineated in this study strongly suggests that the epidermis transplanted onto the nude mouse maintains its unique responsiveness to exogenous stimulation. The data presented support the concept that this transplantation system is a unique and useful one to study pathophysiologic responses of tissue maintained on a biologic support system (15). Further, our results imply that such studies can be carried out without interference from the host, e.g., in these experiments the nonresponsive skin of nudes does not evoke nonresponsiveness upon the foreign skin graft, and vice versa. The extent of such a generalization remains to be determined by specific experiments. Additional evidence that grafted skin retains donor responsiveness includes the experiments reported by Sharkey et al. (16). They demonstrated that rabbit skin susceptible to Shope papilloma virus, when transplanted to the naturally resistant nude mouse, continues to be susceptible to the virus, i.e., it develops the characteristic papilloma.

The observations reported herein demonstrate that the epidermis of the nude can be induced to undergo a proliferative response, the exception being the application of 10 ng TPA. Several possible hypothesis may explain why nudes do not respond in a normal way to the low dose TPA. One possibility is that nude skin has an inherently higher threshold of response. Holland and Perkins have noted that endogenous levels of cortisone are two to three times higher in nudes than in heterozygous littermates (J. M. Holland. Personal communication.). Glucocorticoids are potent inhibitors of the proliferation induced by TPA (17, 18). If enhanced levels of circulating glucocorticoids account for the nonresponsiveness, one would predict that a higher dose of

TPA should induce epidermal proliferation in the nude. Indeed, when 100 ng TPA in D/A is applied, a wave of proliferation ensues (see Fig. 2). However, the higher cortisone levels of nudes cannot explain all of the findings because nudes can respond to other means of enhancing proliferation, e.g., tape stripping (19).

A second possibility is that two mechanisms are involved in the initiation of epidermal proliferation, one functioning through inflammatory systems (agents that trigger inflammatory events and in a secondary way trigger epidermal proliferation), and one acting via a system independent of an inflammatory response (agents that trigger proliferative events directly). If true, it follows that nudes are relatively intact to one insult and not the other. If the inflammatory system is required at all dosages, and this system is impaired in the nude, the transplanted pig skin should also not respond to a 10 ng dose of TPA. TPA will directly enhance cell proliferation in in vitro cell culture systems, suggesting that inflammatory responses are not required (20, 21). Additional support for the notion that inflammatory events are not obligatory for proliferation includes the observation that 5% croton oil causes erythema when applied to pig skin in vivo, but not when applied to grafted pig skin or to nude skin. However, croton oil causes significant epidermal proliferation in all three cases.

A third possibility concerns the relative absorption of topically applied TPA in nude and normal mice. It is difficult to comprehend that the thinner stratum corneum of the nude could be more resistant to the passage of TPA in D/A than the thicker stratum corneum of pig skin. TPA in acetone alone evokes a rather dramatic response in the hairless mouse. A major difference between the skin of hairless mice and nude mice is that the nude mouse has poorly developed hair follicles throughout life, whereas the mature hairless mouse is devoid of hair follicles (22–24). It can be argued that topically applied medication is absorbed via follicles; however, no evidence is available to suggest that animals with follicles absorb less of a given agent than animals without follicles.

Recent experiments from this laboratory support the possibility that the stratum corneum of the nude mouse possesses increased resistance to percutaneous penetration. Application of 100 ng of TPA to nude skin or to pig skin grafts will cause a wave of proliferation. However, in experiments where this topical application is followed by 0.2 mg betamethasone dipropionate in an alcohol propylene glycol vehicle, inhibition of proliferation can be seen in pig skin grafts, but not in nude skin (preliminary data).

A fourth possibility relates to features such as the shortened growth phase of hair in nude mice (22). This observation suggests a shortened cell cycle in epidermal structures of nudes. That a shortened epidermal cell cycle might effect proliferative responses is currently speculative.

As already mentioned, the major component of croton oil which induces proliferation is TPA (13). The ability of nude mice to respond to croton oil is probably a function of the high amounts of TPA present in croton oil, mirroring the 100-ng quantities used in these experiments.

The mechanistically unclear selective responsiveness to TPA, reported in this paper, appears to provide insight into the reasons that initiation/promotion carcinogenesis experiments in nudes have not followed predicted patterns. Johnson and Reed, Montana State University, Bozeman, Mont., first examined this question (E. A. Johnson and N. D. Reed. Unpublished observations. [reviewed in reference 25]). They attempted to induce tumors by painting nudes with dimethylbenzanthracine (initia-

tion) and croton oil (promotion). Under these conditions, normal littermates did develop skin tumors but nudes did not. Implanting nudes with a thymus graft prior to initiation/promotion led to the development of tumors. Because nudes will respond to high dosages of croton oil and to high dosages of TPA, the experiments of Johnson and Reed should have led to tumors, provided that the proper dosage of croton oil was applied, and the nude was not resistant by other mechanisms. Schjerven et al. (26) also demonstrated, in preliminary studies, that the number of papillomas in surviving mice was less than expected in two-stage carcinogenesis. Holland et al. have also examined this question (27). To obviate the problem of wasting, the experimental animals were derived and maintained in a germ-free state. At 5 mo of age, the initiator (dimethylbenzanthracine) was applied and followed by the twice weekly application of TPA. After 20 wk, 95% of normal littermates had tumors, whereas only 4% of nudes had tumors. At 32 wk, all of the normal littermates had tumors, whereas only 66% of the nudes had developed tumors. This failure of TPA to promote carcinogenesis in nudes supports the contention that nudes are less responsive to TPA.

The observation of the induction of proliferation with TPA on responsive skin (pig) transplanted to nudes implies that initiation/promotion experiments under these conditions should lead to tumors. In a limited series, using human skin transplants, urathane as the initiator and 2.5 μg TPA in acetone twice weekly to the grafts as the promoter, Yuspa et al. (28) demonstrated increased numbers of tumors, both in the grafts and on the facial area of the grafted animals. Ungrafted animals treated in a similar manner did not develop tumors.

In conclusion, we feel that the experiments described herein argue that epidermis transplanted onto the nude mouse retains the proliferative properties characteristic of the donor animal. Lower concentrations of TPA applied to nude mouse skin showed an unexpected lack of the normal proliferative response. In contrast, pig skin responded normally to TPA, and retained that responsiveness even when grafted to unresponsive nude mice. The genetic nature of the TPA response seems to be recessively transmitted in BALB/c mice. Comparison of different genetic species of nude mice (BALB/c vs. ICR) suggests that the dimension of the TPA effects may also be controlled in a genetic manner.

Summary

Human skin transplanted to nude mice offers a possible experimental system for the study of normal epidermal proliferation and differentiation, and for their pathological counterparts. Crucial to the development of such a system is the demonstration that such grafts retain the responsive features of donor skin. To document that donor proliferative characteristics are maintained in the grafts, a comparative analysis of agents that induce proliferation was made on skin of mice homozygous and heterozygous for nude, on pig skin, and on pig skin transplanted onto nude mice. A wave of epidermal proliferation could be induced in pig skin and pig skin grafted onto nude mice, but not in nude mouse skin after the topical application of 10 ng 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). A 10-fold greater concentration of TPA or 5% croton oil induced proliferation in all species of epidermis studied. Mice, heterozygous for nude, showed a normal response to 10 ng TPA, suggesting that the ability to respond to TPA may be related, in part, to a recessive genetic trait. Nude mouse skin transplanted to a heterozygous littermate capable of responding to 10 ng TPA

does not respond. These observations argue that: the graft retains its donor proliferative characteristics when transplanted to the nude, and the inability of the nude mouse to respond to lower doses of TPA may be related to absorption, the nude gene(s), or an inherent threshold to response. The lack of response to the promoter TPA provides a plausible explanation for the decreased incidence of tumors arising in nude mice during two-stage carcinogenesis experiments.

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