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Modulation of gap junction expression during transient hyperplasia of rat epidermis

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SUMMARY

Retinoids and phorbol esters have profound effects on proliferation and differentiation of epidermal keratinocytes when applied topically on rodent skin. Since both agents also modulate gap junction (GJ)-mediated cell-cell communication, we have examined the effects of all-trans retinoic acid (RA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) on the expression of α_1 (Cx43) and β_2 (Cx26) connexins, the two major gap junction gene products in mature rat epidermis. In fully differentiated, mature epidermis, α_1 is expressed in the lower, less differentiated portion, while β_2 is localized in upper, more differentiated layers. Dorsal skin of 21-day old rats was treated topically with a single dose of RA, TPA or vehicle alone and used for histological and molecular analyses at different time points. Keratinocytes in interfollicular epidermis were examined for proliferation and differentiation using specific antibodies for keratins (K10, K14) and proliferating cell nuclear antigen (PCNA). An increase in epidermal thickness was noticed within 4 hours after the application of RA or TPA. This increase, however, appeared to be primarily due to hypertrophy, since no substantial changes were observed in the proliferative index of epidermal keratinocytes. PCNA immunoreactivity significantly increased after 8 hours treatment of RA or TPA, suggesting a hyperproliferative growth response. Epidermal hyperplasia was confirmed by monitoring the expression patterns of K10 and K14 in RA- or TPA-treated skin. RA-induced hyperplasia lasted longer as compared to TPA induction. Changes in keratin phenotypes were paralleled by an increase in α_1 and β_2 connexin expression as well as their colocalization in same epidermal layers. Differences in hyperplastic growth response kinetics were also confirmed at the connexin level, with β_2 antigen sustained for longer and at higher levels in suprabasal layers of RA-treated skin. Overall, this type of connexin expression resembled that observed in the non-differentiated rat epidermis during embryonic development. An increase in α_1 and β_2 connexin abundance was also observed at the protein and RNA levels. At 96 hours after RA or TPA treatment, expression of both connexins was similar to that of the control epidermis. Taken together, these findings suggest that a higher level of GJ-mediated cell-cell communication, is required for the maintenance of homeostasis during periods of rapid epidermal growth and differentiation.

Key words: Gap junction, Connexin, Epidermis, Hyperplasia, Rat, Phorbol ester, Retinoid

INTRODUCTION

Growth control and maintenance of a homeostatic equilibrium in the mature mammalian epidermis is manifested morphologically by a constant thickness of epidermal cell layers. These processes depend on a fine balance between the proliferative, differentiative and migratory activities of epidermal keratinocytes that take place during their transit from the proliferative basal layer to the terminally differentiated, cornified layer (Watt, 1989; Fuchs, 1990). The homeostasis of the epidermis can be severely perturbed in hyperproliferative skin disorders, such as psoriasis (Bernerd et al., 1992), or in experimentally induced hyperplasia following topical application of retinoids or phorbol esters on rodent or human skin (Schweizer and Winter, 1982; Connor, 1986; Lichti and Yuspa, 1988).

The processes of epidermal growth, differentiation and migration are closely associated with dynamic remodeling of intercellular contacts between epidermal keratinocytes, including gap junctions (GJs). GJs are specialized regions of plasma membranes formed by aggregates of intercellular channels that provide pathways for the passage of regulatory molecules between adjacent cells in developing and mature organs and organisms (Warner, 1992). A functional GJ channel is formed by pairing two hemichannels, one contributed by each cell. A hemichannel is assembled by oligomerization of six polypeptides, termed connexins, which belong to a multigene family (for recent reviews, see Goodenough et al., 1996; Kumar and Gilula, 1996). Some members of the connexin family are co-expressed in the same cell and their expression can be developmentally regulated. In

rodent epidermis, the expression of α_1 (Cx43) and β_2 (Cx26) connexins, the two major GJ gene products in keratinocytes, is closely associated with the course of growth and differentiation (Risek et al., 1992, 1994; Kamibayashi et al., 1993; Butterweck et al., 1994; Goliger and Paul, 1994). Both connexins are initially co-expressed in the same keratinocytes during early stages of embryonic development, and subsequently segregated in a fully differentiated, mature epidermis. The multiple co-expression of connexins by the same keratinocyte (Brissette et al., 1991, 1994) and their co-localization in the same GJ plaque (Risek et al., 1994), suggest that the epidermis can form communication compartments with different regulatory properties. Epidermal communication compartments have been demonstrated in several species by functional studies using fluorescent dyes that are GJ channel-permeable (Kam and Pitts, 1988; Salomon et al., 1988; Serras et al., 1993; Goliger and Paul, 1995). The extent and directional preference of the dye spread, which is an indicator of communication compartments, appear to vary with the method used for the dye transfer. A restricted, compartmentalized and, preferentially, vertical transfer to only 20-40 cells has been shown by microinjecting the dye into individual keratinocytes of rodent (Kam and Pitts, 1988) and human skin (Salomon et al., 1988). In contrast, an extensive transfer has been demonstrated in rodent epidermis by using two GJ-permeable tracers, Lucifer Yellow and neurobiotin, in combination with a 'cut-loading' technique (Goliger and Paul, 1995). The findings of Goliger and Paul (1995), however, are more consistent with an extensive and, preferentially, layer-specific (horizontal) pattern of connexin expression in fully differentiated, mature rat epidermis. Together, these studies suggest that GJ channels are required for the proper functioning and maintenance of the epidermal homeostasis. Hence, it is postulated that perturbations of the homeostatic control mechanisms will be manifested, qualitatively and/or quantitatively, by alterations in the connexin expression program.

Changes in GJ abundance and connexin expression have been reported in several studies following topical application of retinoic acid (RA) or 12-O-tertadecanoylphorbol-13-acetate (TPA). While an increase has been consistently observed in embryonic chick and mature human epidermis following treatment with RA (Elias et al., 1980; Guo et al., 1992; Masgrau-Peya et al., 1997), there are conflicting results regarding the expression and formation of GJs in TPA-treated mouse epidermis (Kalimi and Sirsat, 1984; Budunova et al., 1995, 1996). On the basis of these considerations, the present study was conducted to examine the expression program of α_1 and β_2 connexins in rat epidermis during the course of experimentally induced transient hyperplasia at steady-state protein and RNA levels and to compare with that of the embryonic epidermis. We provide evidence that a fully differentiated, mature rat epidermis has the potential to switch to a connexin expression program during the hyperplastic growth response which resembles that of the less differentiated, embryonic epidermis. The controlled induction of the epidermal hyperplasia in rodent skin offers a unique model with which to study the role of GJs during growth and differentiation of epidermal keratinocytes in vivo.

MATERIALS AND METHODS

Chemicals, animals, treatment protocol and tissue collection

All-trans retinoic acid (RA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma (St Louis, MO). Immediately prior to use, both chemicals were dissolved in acetone (see below) and kept protected from light during topical treatment. Experiments involving animals were approved by the Institutional Animal Care Committee and were conducted in accordance with Institutional regulations. 21-day old Wistar rats (Simonsen, Gilroy, CA) were chosen for the present study since at this age the animals were approaching the resting stage of the hair growth cycle (Risek et al., 1992). Animals were housed individually under reduced lighting conditions and fed Purina chow diet *ad libitum*. The back skin of the animals was shaved 24 hours before applying 200 μl of acetone (control group) or acetone containing 100 nmol RA or 50 nmol TPA. The solutions were applied as evenly as possible over an area of approximately 2×2 cm. Three rats were used for each time point in each group. The doses used in the present study were in the range of those previously applied for the induction of epidermal hyperplasia in rodents (Balmain, 1976; Sisskin et al., 1982; Lichti and Yuspa, 1988). Animals were killed by cervical dislocation at defined time points (0, 4, 8, 24, 48, 72 and 96 hours) and skin was processed immediately for histological and molecular analysis. The middle portion of the skin, with an aproximate area of 0.5×0.5 cm, was used for histological and immunohistochemical analysis. Samples were embedded in Tissue Tek II OCT compound (Lab Tek Products, Naperville, Il) and frozen slowly in an isopentane/dry-ice bath. Particular care was taken for the proper orientation of the sample during the freezing step in order to obtain sections perpendicular to the surface of the epidermis. OCT-embedded samples were stored at −80°C for cryosectioning. The remaining part of the skin was divided in two parts, frozen in liquid nitrogen and stored at −80°C for RNA and protein analysis.

Histology and indirect immunohistochemistry

Fresh-frozen, OCT-embedded skin was cut on a Reichert-Jung cryostat (sections 5 μm thick), mounted on gelatinized slides and stored at −20°C until further use. One set of tissue sections was subjected to histological and morphometric analysis following staining with Hematoxylin and Eosin. A parallel set of slides was processed for indirect immunohistochemistry as previously described (Risek et al., 1992). Briefly, after incubation in blocking solution (PBS containing 3% bovine serum albumin, 3% normal goat serum) for 30 minutes, slides were incubated with a panel of five different antibodies specific for keratins (K10 and K14), proliferating cell nuclear antigen (PCNA) and connexins (α_1 and β_2; Milks et al., 1988; Risek et al., 1990). The expression of keratin K14 was monitored by using a rabbit polyclonal antibody (generously provided by Dr Dennis Roop, Baylor College of Medicine, Houston, TX) that specifically recognizes K14 protein in proliferating and less differentiated keratinocytes (Smith et al., 1990). The expression of K10 keratin was analysed with a commercially available mouse monoclonal antibody (RKSE 60; ICN Biomedicals, Inc., Costa Mesa, CA) that specifically interacts with K10 protein in differentiated keratinocytes (Herman et al., 1985). The proliferative activity of epidermal keratinocytes was assessed by employing a human polyclonal auto-antibody (kindly provided by Dr Kevin Sullivan, The Scripps Research Institute, La Jolla, CA) that specifically recognizes a nuclear protein during the S-phase of DNA synthesis (Proliferating Cell Nuclear Antigen, PCNA; Miyachi et al., 1978). Slides were incubated for 1 hour with diluted antibodies (1:7,000 for K14; 1:100 for connexin and PCNA; 1:30 for K10; diluted with blocking solution), washed in PBS and subjected to an additional 1 hour incubation with a fluorescein-conjugated set of secondary antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL). Slides were then washed in PBS, air-dried and mounted in an antifade reagent (Slowfade, Molecular Probes, Inc., Eugene, OR).

Indirect immunofluorescence was examined with a Zeiss Axiophot microscope equipped with epifluorescence (Zeiss, Oberkochen, Germany). Control experiments were performed either with secondary antibodies alone (for PCNA, K10 and K14 staining), or in combination with preimmune sera. Typically, control slides did not show any staining that was noticeably above the intensity of the background.

Preparation of skin homogenates and immunoblot analysis

Pieces of frozen skin were subjected to extraction with 20 mM NaOH (Hertzberg, 1984). Following protein determination of alkali-insoluble homogenates (Lowry et al., 1951), 50 μg protein samples were solubilized in loading buffer by vortexing for 1 hour at room temperature and separated by 12.5% SDS-PAGE using the procedure of Laemmli (1970). Gels were either stained with Coomassie Brilliant Blue R-250 or silver nitrate to confirm equal loadings of proteins or transferred directly onto nitrocellulose membrane (0.2 μm; Schleicher & Schuell, Keene, NH) for immunoblot analysis (Milks et al., 1988). Signals were visualized by using an enhanced chemiluminescent substrate kit (Pierce, Rockford, Il). The immunoblots were exposed on a Kodak XAR-5 film for different time periods. Signal intensities were quantified by densitometry using an LKB laser scanner (UltroScan IL, Bromma, Sweden). The data were corrected by subtracting a 'background' value that was obtained from a non-specific chemiluminescence.

Extraction of ribonucleic acids, normalization of poly(A)$^+$ RNA and northern blot analysis

Frozen skin samples were pulverized under liquid nitrogen and homogenized in a buffer containing 4 M guanidine isothiocyanate, 25 mM sodium citrate, 100 mM β-mercaptoethanol and 0.5% sarkosyl, pH 7.0. Total RNA was sedimented through a $CsCl_2$ cushion by ultracentrifugation (Chirgwin et al., 1979), quantified by measuring absorbance at 260 nm, and normalized for poly(A)$^+$ RNA content by oligo(dT) hybridization (Harley, 1987). Hybridization conditions and [^{32}P]cDNA probes used for northern blotting were essentially the same as described previously (Risek et al., 1992). Signals were detected by autoradiography at −70°C using Kodak XAR-5 film with an intensifying screen.

Morphometry and statistics

The thickness of the noncornified interfollicular epidermis (IFE) was analyzed histologically and morphometrically by using Hematoxylin and Eosin-stained sections on a light microscope equipped with an ocular micrometer (Zeiss, Oberkochen, Germany). The thickness of IFE was determined by measuring the distance from the basal to the cornified layer in 10-15 microscopic fields of each rat. The measurements were performed only on those sites for which the basal layer was easily distinguishable and the plane of section passed through the longitudinal axis of basal cells. Three animals of the same group was analyzed for each time point. IFE of all three animals of the same group was also used for counting PCNA-positive cells. The percentage of PCNA-positive cells was calculated by counting cells in basal layer of IFE in 10-15 microscopic fields for each animal. Values for IFE thickness and percentage of PCNA positive cells were expressed as the mean ± s.d. Significant differences between groups were determined by Student's *t*-test (P<0.01).

RESULTS

Histologic and morphometric criteria of a transient hyperplasia in rat epidermis

Fig. 1 summarizes the results of histological and morphometric analysis of 21-day old rat skin following topical treatment with a single dose of 100 nmol RA, 50 nmol TPA or vehicle alone. There were no significant differences in the thickness of IFE of acetone-treated control animals during the entire 4-day study. However, a significant increase was detected at 4 hours following RA (35.1±4.2 μm; P<0.01) or TPA treatment (37.8±4.0 μm; P<0.01). In addition, TPA-treated skin was marked by swollen keratinocytes and enlarged intercellular spaces (data not shown). These findings, indicating the presence of hypertrophy, inflammation and edema, were consistent with those reported for TPA-treated mouse epidermis (Raick, 1973; Schweizer and Winter, 1982; Klein-Szanto et al., 1984). 24 hours later, keratinocytes of TPA-treated IFE were still swollen, intercellular spaces were greatly enlarged, and the thickness amounted to 57.7±6.4 μm. A significant expansion of the noncornified IFE was also observed in animals treated with RA (52.7±6.5 μm; P<0.01). The overall growth response resembled a hypertrophic/hyperplastic phenotype. In contrast to TPA, the RA-induced increase in the thickness of IFE was primarily due to an increase in total number of granular cell layers (see below). A maximum thickness of 62.2±6.3 μm was observed at 48 hours for RA, while IFE of TPA-treated skin amounted to 45.8±4.5 μm. At this time, expanded spinous and granular cell layers were characteristic for a hyperplastic epidermis of

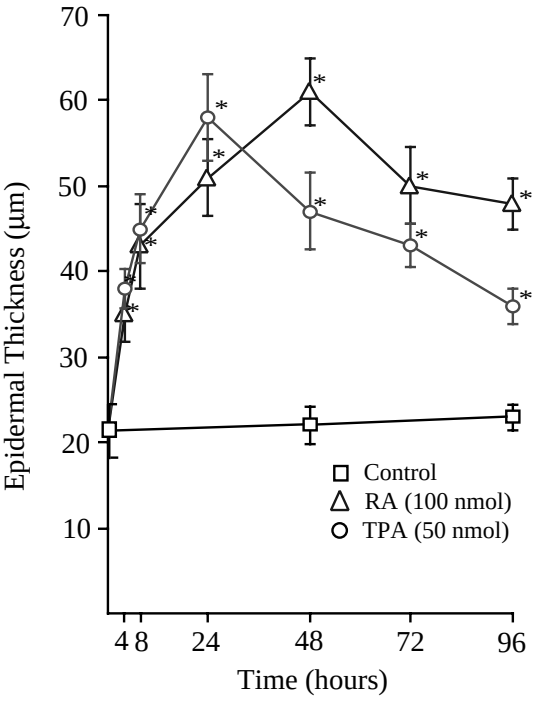

Fig. 1. Effect of a single topical application of RA or TPA on thickness of noncornified interfollicular epidermis (IFE) in 21-day old rats. The thickness of IFE was measured in Hematoxylin and Eosin-stained sections using a light microscope equipped with an eyepiece micrometer at a final magnification of 400×. Measurements were performed on 10-15 microscopic fields per rat. Three animals were used for each time point. Particular care was taken for the proper orientation of skin during the freezing step. Values represent mean ± s.d. Statistically significant differences between groups were determined by Student's *t*-test (*, P<0.01).

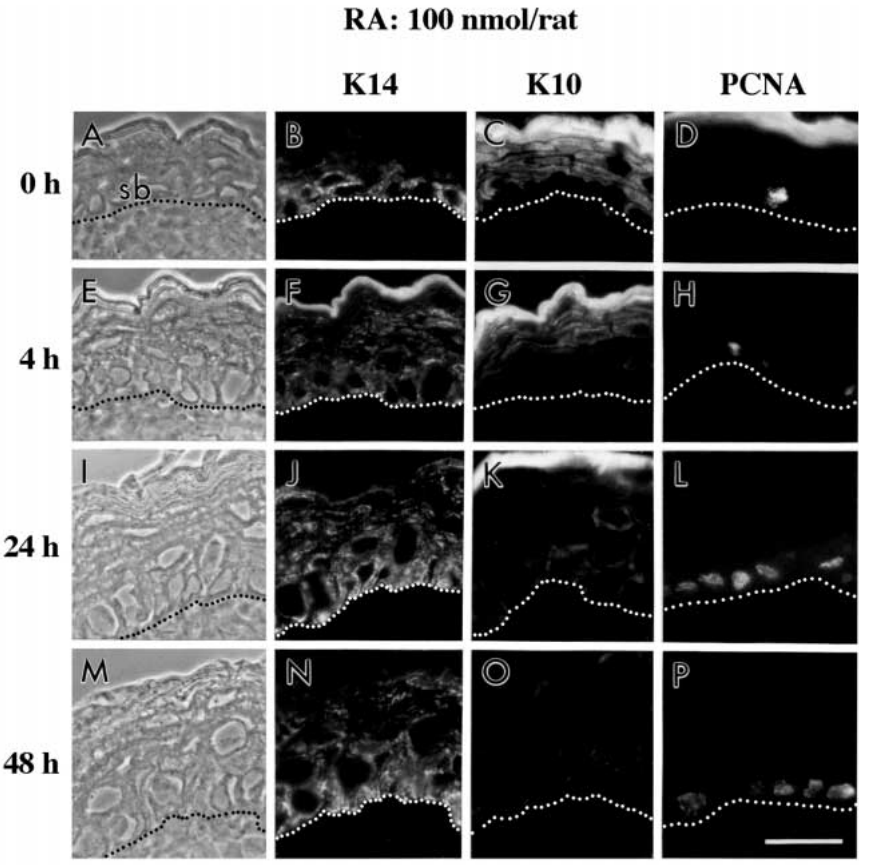

Fig. 2. Immunohistochemical analysis of a transient hyperplasia in rat epidermis following a single topical dose of 100 nmol RA. (A-D) Control. The appearance of the proliferation-associated K14 keratin throughout the epidermis was indicative of an early (4 hour, E-H) and late, 24 (I-L) and 48 (M-P) hours, growth responses of epidermal keratinocytes. The re-expression of K14 was paralleled by disappearance of differentiation-associated K10 keratin. The late growth response of the epidermis at 24 and 48 hours was associated with a marked increase in number of PCNA-positive cells in the proliferative basal layer. sb, stratum basale. Dotted line indicates epidermal/dermal junction. Bar, 25 μm.

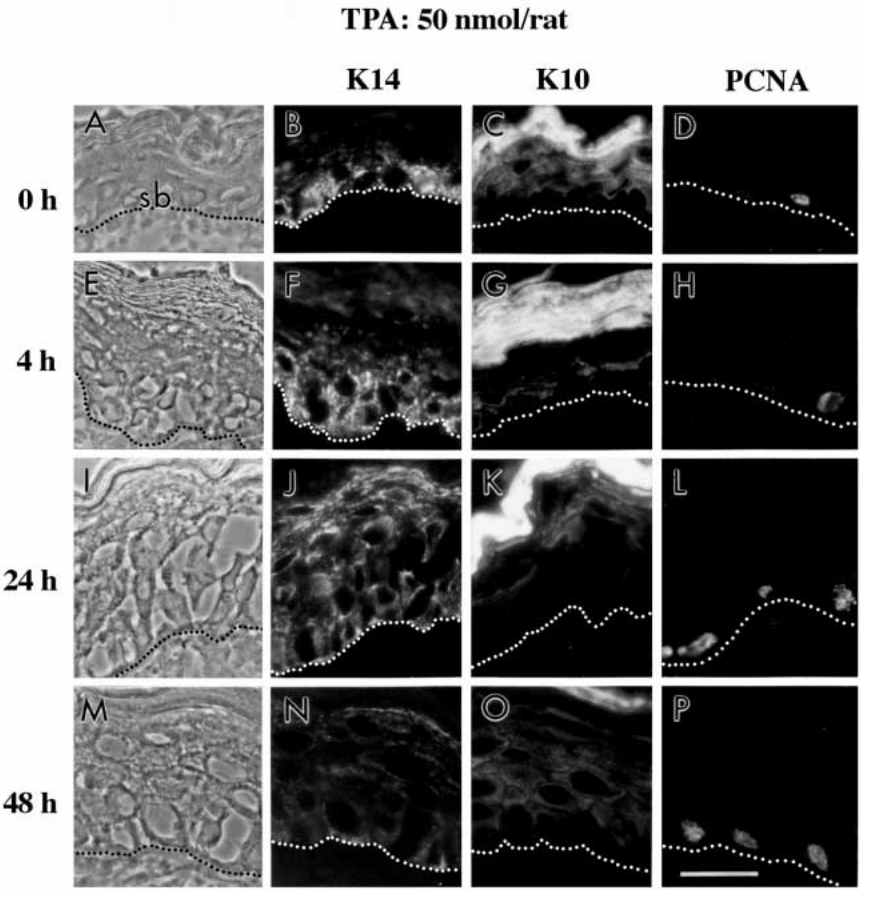

Fig. 3. Immunohistochemical analysis of a transient hyperplasia in rat epidermis following a single topical dose of 50 nmol TPA. (A-D) Control. Resumption of expression of proliferation-associated K14 keratin throughout the epidermis was indicative of an early (4 hours, E-H) and late, 24 (I-L) and 48 (M-P) hours, growth responses of epidermal keratinocytes. The expression of K14 was paralleled by an attenuation of differentiation-associated K10 antigen in noncornified IFE. Characteristic for the late growth response at 24 and 48 hours was a significant increase of PCNA-positive cells in the proliferative basal layer. sb; stratum basale. Dotted line indicates epidermal/dermal junction. Bar, 25 μm.

both RA- and TPA-treated skin. Hyperplasia gradually declined at the expense of spinous layer around 72 hours (RA, 50.7±5.3 μm; TPA, 42.5±3.9 μm), reaching minimum levels in thickness of IFE at 96 hours (RA, 43.8±3.6 μm; TPA 35.7±3.3 μm).

In summary, histological and morphometric criteria were employed to examine the onset, peak and decline of a transient hyperplasia in rat epidermis following a single application of RA or TPA on the dorsal skin. The overall growth response consisted of early changes at 4 hours that were primarily due to inflammation, edema and hypertrophy, and a late response at 24 hours and thereafter that was mainly due to hyperplasia.

Expression of keratins and proliferating cell nuclear antigen during transient hyperplasia in rat epidermis

Traditionally, the maturation of the epidermis is monitored by analyzing the phenotypes of proliferative/undifferentiated keratinocytes, expressing keratin 14, and differentiated keratinocytes, expressing keratin 10 (Kopan et al., 1987). Expression of keratins and PCNA was examined immunohistochemically in sections derived from the same skin material that was subjected to histological and morphometric analysis. The results, demonstrating early (4 hour) and late responses (24 hours and thereafter) of the epidermis following RA and TPA treatment, are presented in Figs 2 and 3, and the proliferation index in Fig. 4.

During the initial period of 24 hours, the temporal modulation of keratin and PCNA expression was similar in both RA- and TPA-treated epidermis. In the control epidermis, K14 antigen was localized in the proliferative basal and the adjacent, less differentiated spinous layer (Figs 2B, 3B), whereas K10 was expressed in keratinocytes of differentiated suprabasal layers (Figs 2C, 3C). PCNA staining, observed exclusively in the proliferative basal layer (Figs 2D, 3D), indicated a constant proliferation rate of approximately 15% in the control epidermis (Fig. 4). Concomitantly with the onset of morphological alterations at 4 hours after RA or TPA treatment, notable changes were observed in keratinization phenotypes (Figs 2F,G and 3F,G). Characteristic of early changes was an increase in K14 antigen at the expense of K10 in the spinous layer. Although the number of PCNA-positive cells increased in both groups at 4 hours as compared to the control, these changes were not significant (Figs 2-4). In contrast, a significant increase (Fig. 4; $P<0.01$) was observed at 8 hours following RA (39.0±7.4%) or TPA (32.7±3.9%) application. Increase in proliferative activity at 24 and 48 hours after RA or TPA treatment was also associated with abundant expression of K14 in the entire IFE at the expense of K10 antigen (Figs 2, 3). At 48 hours, however, differences were noticed in keratin expression between RA- and TPA-treated skin. While K10 was not detectable in the RA-treated group, it was re-expressed in TPA-treated epidermis. The modulation of K10 expression was paralleled by an inverse expression profile of K14. These changes indicated different kinetics in epidermal growth response, suggesting a longer lasting hyperplasia in RA-treated epidermis. At 72 and 96 hours, the expression patterns of K14 and K10 gradually approached that of the control group (data not shown), despite a significantly elevated number of PCNA-positive cells in the proliferative basal layer (Fig. 4).

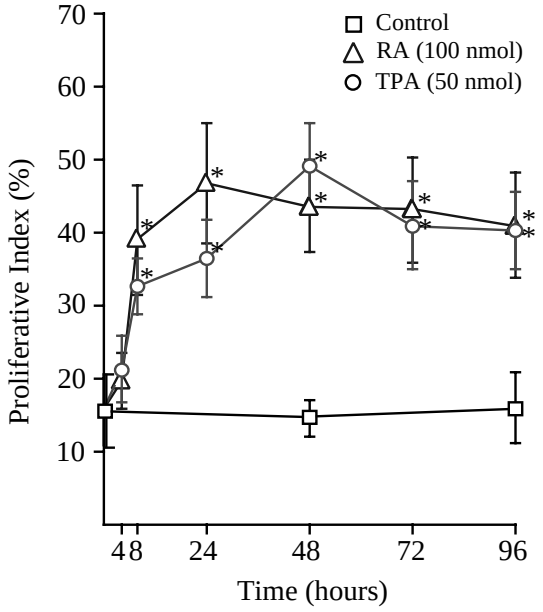

Fig. 4. Effects of single topical application of RA and TPA on proliferative activity of interfollicular epidermis (IFE) in 21-day old rats. Proliferative index of basal layer IFE was calculated by dividing the number of PCNA-positive cells by the total number of basal layer cells in a given microscopic field at a final magnification of 400×. 10-15 microscopic fields were scored in sections from a single animal. Three animals were analyzed for each time point. Values represent mean ± s.d. Statistically significant differences between groups were determined by Student's *t*-test (*, $P<0.01$).

Expression of α_1 and β_2 connexins during transient hyperplasia of rat epidermis

Expression profiles of α_1 and β_2 connexins are summarized in Figs 5 and 6. In epidermis of control animals, the localization of α_1 and β_2 connexins was consistent with previous observations (Risek et al., 1994). α_1 was expressed by keratinocytes in basal and the less-differentiated, adjacent spinous layer (Figs 5B, 6B), while β_2 connexin was expressed by keratinocytes of the differentiated upper spinous and granular layer (Figs 5C, 6C). This profile was markedly altered for both connexins in RA- and TPA-treated skin at 4 hours. Characteristic of early changes was the detection of α_1 and β_2 connexins throughout the epidermis (Figs 5E,F and 6E,F). The increase in abundance of α_1 and β_2 antigen in spinous and upper granular layers was consistent with elevated protein and transcript levels (see below). In addition to elevated connexin expression in individual cells, an increase in cell number expressing connexin gene products could also have contributed to an overall increase in connexin abundance. Elevated levels of both connexins were also observed at 24 hours and 48 hours (Figs 5H-L and 6H-L). At 48 hours, however, there were noticeable differences in connexin expression between RA- and TPA-treated epidermis. While both connexins were still coexpressed in lower and upper parts of RA-treated epidermis, their expression profile was more separated in the TPA-treated group. The onset of segregation in connexin expression, with presence of α_1 in lower and β_2 in upper regions, indicated a decline of TPA-induced hyperplastic growth response. Thus, the differences in kinetics of chemically induced transient

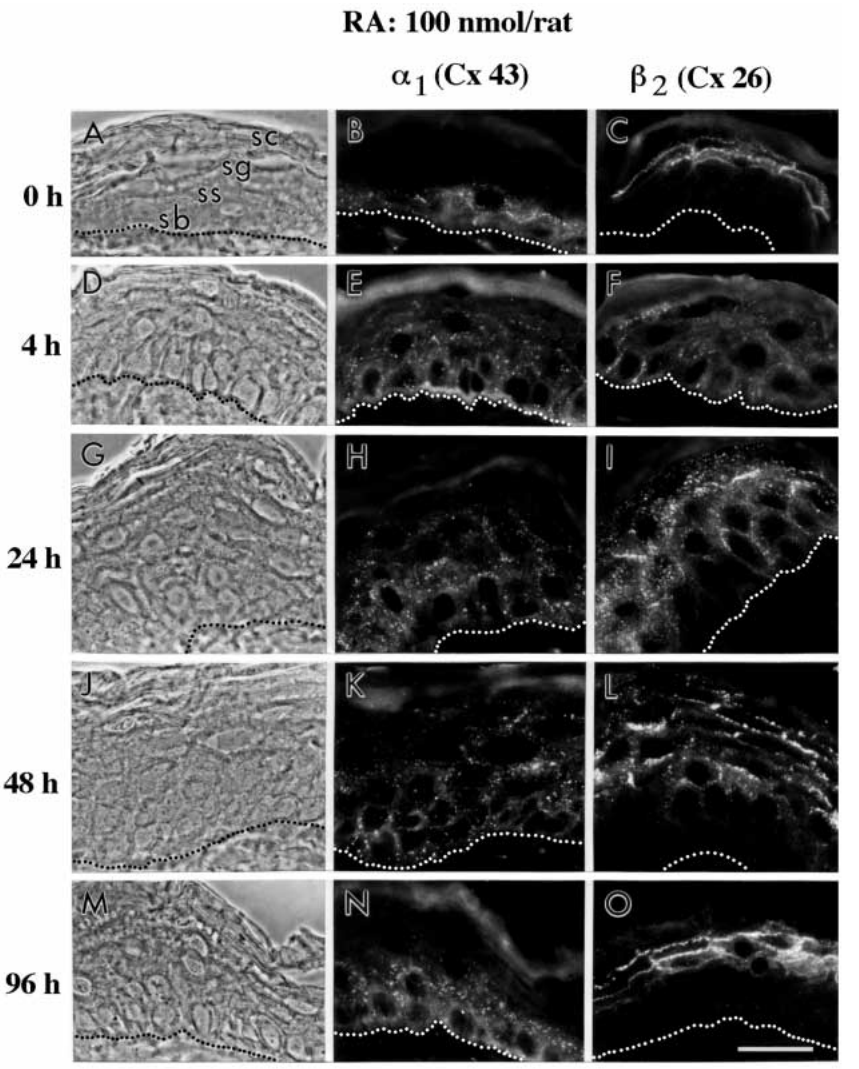

Fig. 5. Expression profiles of α_1 and β_2 GJ antigens in rat epidermis treated topically with a single dose of 100 nmol RA. In mature epidermis, α_1 antigen is expressed in the basal and the adjacent spinous layer (B). β_2 antigen, in contrast, is expressed in more differentiated keratinocytes of the upper spinous and granular layers (C). The uppermost cornified layer is devoid of connexin staining. Note the presence of β_2 antigen in some cells of the basal layer at 4 and 24 hours. 96 hours after RA treatment, the expression of both connexins resembled that of the control group epidermis. sb, stratum basale; sg, stratum granulosum; ss, stratum spinosum; sc, stratum corneum. Dotted line indicates epidermal/dermal junction. Bar, 25 μm.

hyperplasia were confirmed at the connexin level. At 96 hours, the connexin expression pattern was partly restored in both treatment groups, resembling that of control epidermis.

In summary, the transient hyperplasia of rat epidermis was described histologically and morphometrically by using Hematoxylin and Eosin-stained sections of dorsal skin, as well as immunohistochemically by analyzing the expression pattern of keratins, PCNA and connexins. The time course and the expression pattern of keratins and connexins revealed different kinetics of hyperplastic growth response, with a longer lasting hyperplasia in RA-treated skin.

Modulation of α_1 and β_2 connexins at the protein and mRNA levels during transient hyperplasia of rat epidermis

In order to assess whether early changes in connexin expression were due to *de novo* synthesis of GJ gene products, western and northern blotting were performed on skin samples isolated from control (0 hours) or from animals treated with RA or TPA for 4 hours and 24 hours (RNA only). The results of protein analysis using alkali-insoluble material are illustrated in Fig. 7. Silver staining of SDS-PAGE separated proteins revealed no obvious differences in total protein profile of the control (lane 0) or treated (lane 4) animals (Fig. 7A). Increased levels, three- to fourfold, of both α_1 and β_2 proteins were detected at 4 hours in samples of RA- and TPA-treated animals (Fig. 7B-D). The size of the 43 kDa protein, detected in control as well as in RA- and TPA-treated skin, indicated a phosphorylated form of α_1 connexin. This band shifted in electrophoretic mobility to approximately 40 kDa on an 8% gel following treatment with alkaline phosphatase (data not shown). β_2 protein was detected with two molecular masses, 21 and 38 kDa, corresponding to the monomeric and dimeric subunits. These results suggest that both proteins were synthesized de novo in epidermis during the early growth response, and that α_1 protein existed predominantly in a phosphorylated form in epidermal keratinocytes.

The corresponding transcripts of α_1 and β_2 GJ gene products are illustrated in Fig. 8. Both transcripts (α_1, 3.3 kb; β_2, 2.8 kb) were markedly elevated in skin treated with RA or TPA for 4 hours. RA and TPA increased specifically the steady-state levels of connexins, since no changes were noticed for actin transcripts (α, 1.4 kb; β, 2.0 kb).

Collectively, the results of the present study demonstrate an upregulation of α_1 and β_2 GJ gene products at the antigen, protein and transcript levels during early and late growth

responses of rat epidermis treated topically with a single dose of RA or TPA.

DISCUSSION

The present study is the first report demonstrating modulation of GJ gene products during transient hyperplasia of mature rat epidermis. Furthermore, since this type of chemically induced growth response represents the most common benign injury of epidermis (Schweizer and Winter, 1982), these results provide further insights into the role of GJ-mediated control of cell growth and differentiation during perturbation of epidermal homeostasis.

Implications of GJs in control of epidermal homeostasis

Profound effects on the epidermal growth and differentiation were observed following a single topical application of RA or TPA on dorsal skin. These effects, manifested in early (4 hours) and late (24 hours and after) growth responses, were mainly due to hypertrophy (including inflammation and edema of TPA-treated epidermis), followed by hyperplasia. Different kinetics in the time course of RA- and TPA-induced transient hyperplasia were noticed at the level of keratin and connexin expression, demonstrating a longer lasting response in RA-treated epidermis. An increased expression of α_1 and β_2 connexins was associated with both types of epidermal growth response, with an expression profile similar to that observed previously in rapidly developing embryonic epidermis (Risek et al., 1992, 1994). The increase in connexin expression observed at the molecular level in hyperplastic epidermis could have been due to an increase in connexin level per cell, and/or resulting from an increase in the number of connexin-expressing cells. Immunohistochemical analysis of connexin expression suggests that both factors contributed to an overall increase in connexin abundance. Since no noticeable changes were observed in follicular and dermal components of the skin, the increase in protein and mRNA levels appears to be mainly derived from the epidermis. Collectively, these findings suggest that there is a requirement for the upregulation of GJ gene expression during periods of rapid epidermal growth and differentiation. Consistent with this view are studies reporting increased levels of GJs during wound healing of rodent epidermis, an additional experimental model of epidermal growth and differentiation (Gabbiani et al., 1978;

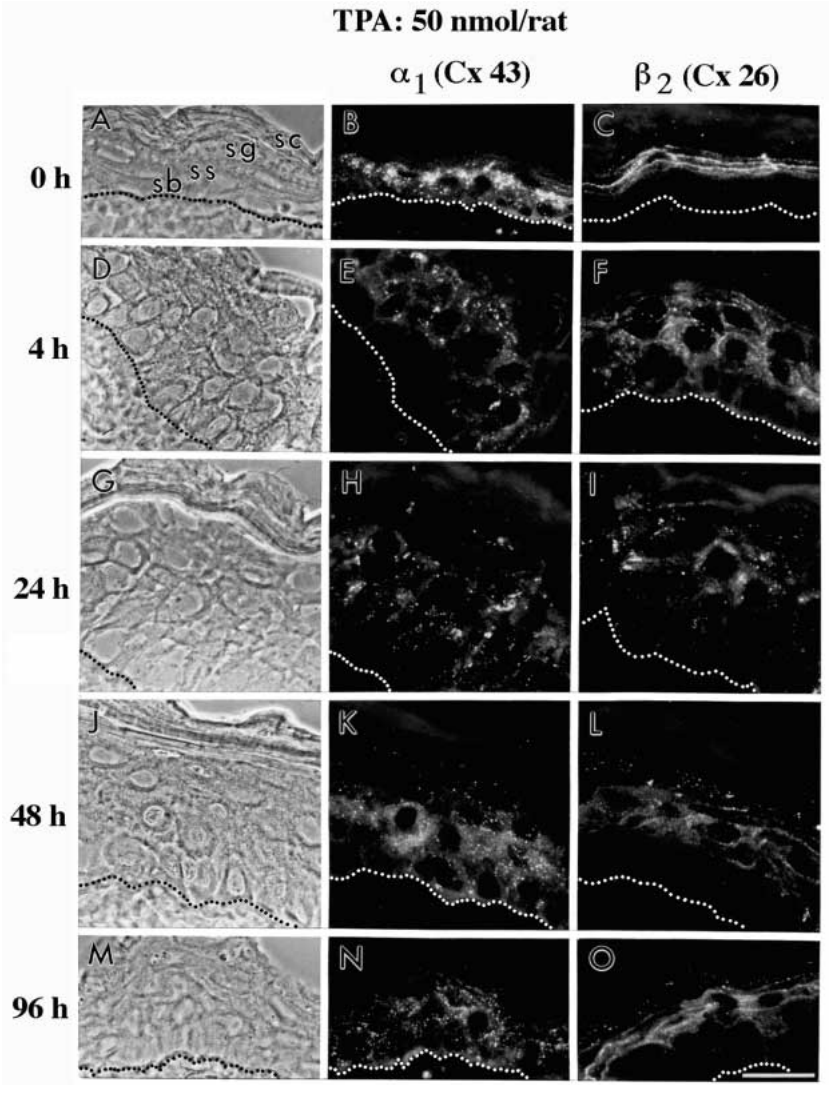

Fig. 6. Expression profiles of α_1 and β_2 GJ antigens in rat epidermis treated topically with a single dose of 50 nmol TPA. As described in Fig. 5, the expression of α_1 and β_2 antigens is restricted in mature epidermis as a function of its differentiation (A-C). Both α_1 and β_2 antigens are induced during early (4 hour) and late (24 and 48 hour) epidermal growth responses. At 96 hours of TPA exposure, the expression of both connexins was similar to that of the control group. sb, stratum basale; sg, stratum granulosum; ss, stratum spinosum; sc, stratum corneum. Dotted line indicates epidermal/dermal junction. Bar, 25 μm.

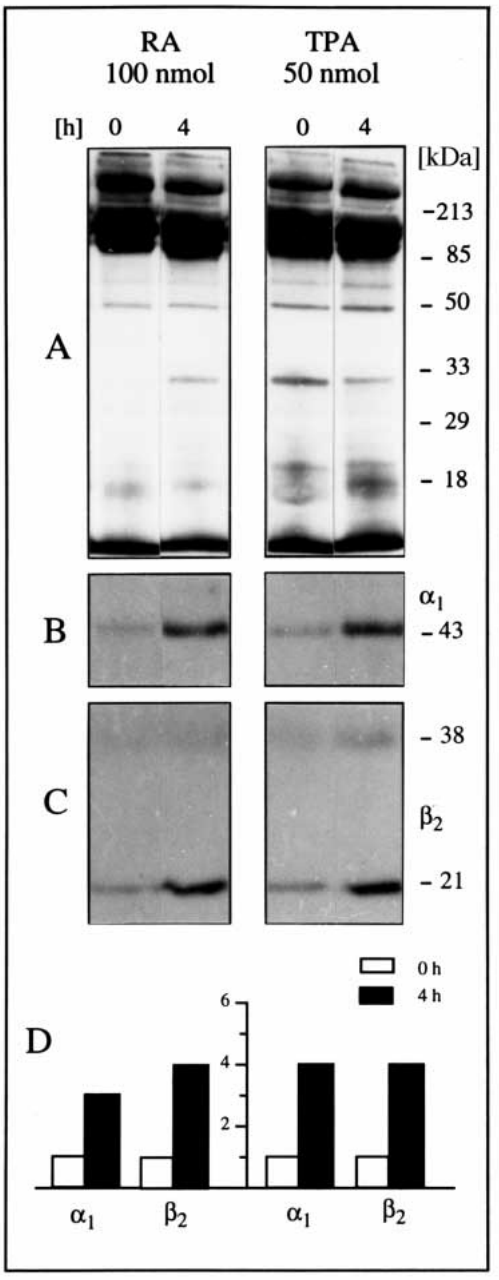

Fig. 7. Induction of α_1 and β_2 GJ proteins during transient hyperplasia of rat epidermis following a single topical application of RA or TPA. (A) Silver staining of SDS-PAGE separated proteins (50 μg/lane; 12.5% gel) indicating a similar profile of the total protein between control (0 hours) and treated (4 hours) animals. (B,C) Western blots of a similar gel to that shown in A following transfer to nitrocellulose membranes and incubation with α_1 and β_2 connexin-specific peptide antibodies. Increased levels of both α_1 (43 kDa monomer) and β_2 proteins (21 kDa monomer, 38 kDa dimer) were detected in skin samples treated with RA or TPA for 4 hours, respectively. The 43-kDa band represents a phosphorylated form of α_1 protein. This band was shifted to 40 kDa following digestion of total proteins with alkaline phosphatase and separation on a 8% gel (data not shown). (D) For relative comparison of the protein abundance, signal intensities were analyzed by densitometry and normalized for the value of the control group. The data presented here are representative of several blots with similar results. Three animals per group were analyzed at each time point.

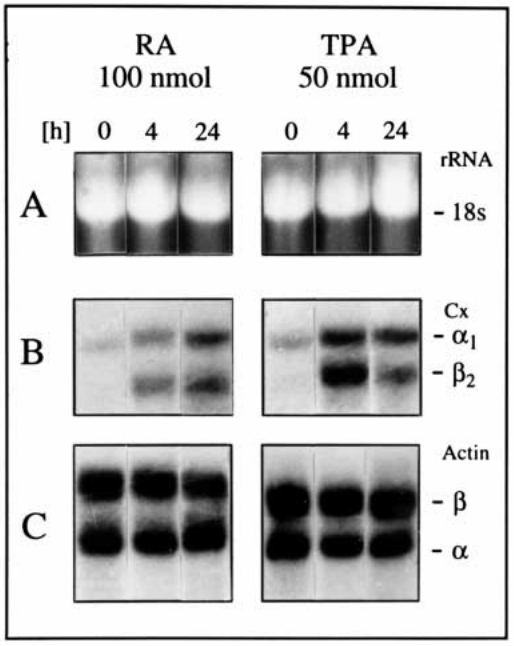

Fig. 8. Induction of α_1 and β_2 GJ mRNA during transient hyperplasia of rat epidermis following a single topical application of RA or TPA. (A) Following normalization of total poly(A)$^+$ RNA by oligo(dT) hybridization, comparable amounts of total poly(A)$^+$ RNA were separated by agarose/formaldehyde gel electrophoresis, transferred onto nylon membranes and probed for connexin and actin transcripts using specific [^{32}P]cDNA probes. (B) Increased levels of α_1 and β_2 GJ mRNA were detected at 4 hours and 24 hours. (C) The abundance of actin transcripts (α, β) was not affected either by RA or TPA. The data shown here are representative of several blots with similar results. Three animals per group were analyzed at each time point.

Goliger and Paul, 1995). Although the role of GJs in avascular tissues such as epidermis remains to be elucidated, these studies suggest that GJs might be important in the maintenance of tissue growth and homeostasis by providing direct intercellular pathways for cell signaling and synchronization of metabolic activities between discrete cell populations. Recently, several studies demonstrated a correlation between perturbation in GJ gene expression with different degrees of hyperplasia and neoplasia in mouse epidermis (Budunova et al., 1995, 1996; Kamibayashi et al., 1995; Sawey et al., 1996). The complexity of GJ-mediated cell-cell communication, including its regulation and perturbation in epidermis, is already evident in the diversity of epidermal GJ gene products. In addition to α_1 (Cx43) and β_2 (Cx26) connexins, rodent epidermis also expresses α_4 (Cx37), α_5 (Cx40) and α_6 (Cx45), as well as β_3 (Cx31) and β_4 (Cx31.1) connexins. However, some restrictions apply in formation of functional channels between adjacent cells. These limitations include pairing compatibilites between homotypic (both hemichannels are comprised of only one connexin type) or heterotypic GJ channels (hemichannels comprised of different connexins, each one provided by a different cell). Possible implications of such limitations on cell physiology and intercellular communication, with a particular reference to rodent epidermis, were discussed recently by Goliger and Paul (1994, 1995).

The elevated expression of α_1 and β_2 connexins in TPA-

treated rat epidermis is consistent with increased levels of both connexins in TPA-induced hyperplastic mouse epidermis (Budunova et al., 1995, 1996). However, our results contrast with findings of Kalimi and Sirsat (1984), who observed a complete elimination of GJs by electron microscopy in TPA-treated mouse epidermis. Although the reasons for this discrepancy are not fully understood, use of different species, treatment protocols (including the dose and the exposure time of TPA to rodent skin) as well as detection methods could, at least in part, account for the conflicting results. In this context, one of the obvious species-specific differences is the absence of β_2 signal in normal human epidermis (Guo et al., 1992; Masgrau-Peya et al., 1997). The increased expression of α_1 and β_2 connexins in TPA-treated skin also contrasts with suppressive effects of TPA on the same gene products in cultured epidermal keratinocytes (Brissette et al., 1991). These differences most likely reflect the complex control of epidermal growth in vivo, including the influence of extracellular matrix as well as the interactions between epidermal and dermal components of the skin (Watt, 1989; Fuchs, 1990). The increased expression of α_1 and β_2 connexins in RA-treated rat epidermis is consistent with their induction in human epidermis following topical application of RA (Masgrau-Peya et al., 1997). Moreover, there were no differences detected in phosphorylation states of α_1 protein in RA- or TPA-treated rat epidermis. A phosphorylated form of 43 kDa on a 12.5% SDS gel was a predominant species in rat epidermis.

Although the present study does not provide information on the possible mechanisms by which connexin gene expression is controlled during hypertrophic and hyperplastic growth in RA- or TPA-treated epidermis, their closely coordinated expression at the protein and mRNA levels indicates transcriptional and/or post-transcriptional control. Further studies are required, however, to address cellular and molecular mechanisms by which RA and TPA exert their effects on the regulation of connexin expression and the formation of functional GJ channels. Ongoing studies analyzing promoters of connexin genes and their regulatory elements should facilitate the identification of factors which regulate their expression. The discovery of RA-specific nuclear receptors in the epidermis (reviewed by De Luca, 1991) provides a possible basis for describing hypertrophic and hyperplastic growth effects at the cellular and molecular levels. Thus, it is conceivable that physiological levels of retinoids contribute to the maintenance of epidermal growth and differentiation (homeostasis) by controlling local expression and the stability of GJ gene products. One of the mechanisms by which RA controls the expression level α_1 is by enhancing the stability of mRNA (Clairmont and Sies, 1997). The mechanism/s of TPA action leading to epidermal hypertrophy and hyperplasia are less well understood. One of the possible mechanisms involves the activation of protein kinase C (Nishizuka, 1988). This enzyme regulates members of AP-1 group of transcriptional factors that play a critical role in cell cycle control (Cantley et al., 1991). The presence of AP-1 binding site in the promoter region of α_1 connexin gene (Sullivan et al., 1993) indicates that this gene is a potential target of a signaling cascade involving a concerted action of protein kinase C/AP-1 binding proteins. In addition, the discovery of TPA response elements in intron 1 of human β_2 gene provides an evidence for a direct regulation of this gene by phorbol esters (Kiang et al., 1997).

The results of the present study demonstrated changes in GJ expression levels during early and late responses of the epidermal growth. These results suggest that experimentally controlled induction of epidermal growth may provide an ideal model with which to study the role of GJs during the transient disruption of homeostasis that occurs during the period of epidermal injury and repair.

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