Gap-Junctional Protein Connexin 43 Is Expressed in Dermis and Epidermis of Human Skin: Differential Modulation by Retinoids

Huiming Guo, Patrick Acevedo, F. Don Parsa, and John S. Bertram

Molecular Oncology Program, Cancer Research Center of Hawaii; and Department of Surgery (FDP), John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii U.S.A.

Retinoids are effective modulators of proliferation and differentiation of keratinocytes in vivo and in vitro. In mouse 10T1/2 cells, retinoid action on proliferation and neoplastic transformation is correlated with the upregulation of gap-junctional communication and expression of connexin 43 (Cx43). In the present study we have determined if retinoids induce similar effects on gene expression in human skin. Studies were conducted in intact skin and on cultured keratinocytes and dermal fibroblasts. In a clinical study, 2 weeks of treatment with 0.05% all-trans retinoic acid resulted in increased expression of Cx43 mRNA and protein in epidermis. Expression occurred predominantly in the suprabasal layer. Cultured cells exhibited a differential response to retinoic acid. In keratinocytes, increased expression of Cx43 occurred at low (10^{-11} M) concentrations, whereas inhibition occurred at high (10^{-7} M) concentrations; however, junctional communication, measured by dye transfer, was not altered over this concentration range. Dermal fibroblasts, in contrast, exhibited a dose-dependent increased expression of Cx43 at concentrations up to 10^{-7} M retinoic acid and proportionately increased their junctional communication over this dose range. These data indicate that control of Cx43 gene expression by retinoids in human skin cells is complex. The production of gradients of junctional channels could play a role in the control of growth and differentiation in epidermis. *J Invest Dermatol 99:460–467, 1992*

Retinoids, natural and synthetic compounds with vitamin-A–like activity, have found extensive use in dermatology. Their action in most cases appears due to an alteration in the program of squamous differentiation in normal or diseased epithelium. In reconstituted human skin in culture, changes in the expression of specific keratins occur in response to retinoic acid [1]; this action is consistent with its therapeutic effects on comedonal acne in humans [2]. In photoaged skin, retinoic acid increases the thickness of the epidermis in general, and the granular layer specifically [3]. Effects on dermal cells that could influence epidermal responses [4] have also been reported. The mechanism of action of retinoids on skin is not understood at the molecular level. However, the recent discovery of a family of nuclear receptors for retinoids (RAR), which have the capacity to modulate gene transcription by interactions with retinoid-responsive elements (RARE) in the regulatory regions of retinoid-responsive genes, provides a likely explanation for the diverse effects of retinoids in multiple cells [5]. One such RAR (RARγ), which is extensively expressed in human keratinocytes and dermal fibroblasts [6], has been described. It is not yet clear which genes are directly modulated by RARγ in skin.

Retinoids are also active in epithelial tissues as cancer-preventive agents. Premalignant lesions such as dysplasias of the uterine cervix [7] have been shown to regress after topical retinoic acid treatment. In patients at high risk for second primary malignancies of the head, neck, lung [8], and bladder [9], the recurrence rate decreases after systemic retinoid treatment.

For some years, we have been utilizing a model cell-culture system of mouse embryo fibroblasts (10T1/2 cells) to study mechanisms of action of carcinogens and of cancer-preventive agents. In this cell line, neoplastic transformation can be induced in a quantitative manner by chemical and physical carcinogens [10]. Moreover, retinoids can be shown to inhibit this process in vitro in a manner qualitatively and quantitatively similar to experimental animal systems [11]. In this model system, the ability of retinoids to suppress neoplastic transformation [12] and enhance growth control [13] is highly correlated with their ability to upregulate gap-junctional intercellular communication. In 10T1/2 cells, connexin 43 (Cx43) is constitutively expressed. This gene represents one member of a family of transmembrane proteins that are believed to assemble in a hexameric array to line the water-filled pore composing one-half (a hemiconnexon) of a junctional complex (a connexon) [14]. The other half of the connexon is donated by an adjacent cell. Gap junctions are found in most mammalian cells. Their functions are diverse and in general poorly understood. They are known to be responsible for the rapid transfer of ionic signals required for contraction of the heart and term myometrium [15]. There is evidence for their role in the transfer of nutrients and excretory products in the avascular lens and cornea and for positional control in embry-

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Reprint requests to: Dr. John S. Bertram, Molecular Oncology Program, Cancer Research Center of Hawaii, University of Hawaii, 1236 Lauhala Street, Honolulu, HI 96813.

Abbreviations:

Cx43: connexin 43
DMEM: Dulbecco's modified Eagle's medium
EDTA: ethylenediaminetetroacetic acid
FITC: fluorescein isothiocyanate
RAR: nuclear retinoic acid receptor
RARE: retinoic acid responsive elements
SDS-PAGE: sodium dodecylsulfate–polyacrylamide gel electrophoresis
TPA: tetradecanoyl phorbol acetate

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onic development and tissue homeostasis [16,17]. We and others have proposed that they play a role in growth control [18,19]. The principal evidence for such a proposal comes from experiments demonstrating that elevation of junctional communication is associated with enhanced growth control of normal or of neoplastic cells [18] and the inverse association of decreased communication with decreased growth control and malignancy [20]. As an example of the latter association, the potent skin-tumor promoter tetradecanoyl phorbol acetate (TPA) strongly inhibits junctional communication in cell culture [21], whereas, the rodent liver promoter phenobarbital decreases expression of Cx32 in liver, the predominant junctional protein found in this organ [22].

In mouse 10T1/2 fibroblasts retinoids increase junctional communication by upregulating expression of Cx43 at the protein and message level [23]. To determine if these results have relevance to retinoid action in humans, we have investigated the interaction of retinoic acid with connexin gene expression in human skin. Skin appears to be the major organ acutely affected by conditions of retinoid deficiency or excess [24], and is known to express RAR [25] and also gap junctions [26]. Here we report that human skin expresses Cx43 and that retinoic acid, applied in situ or to isolated keratinocytes or dermal fibroblasts, influences expression of this gene. These findings may have relevance to the proposed role of junctional communication in the control of growth and differentiation.

MATERIALS AND METHODS

Clinical Studies Patients scheduled for elective plastic surgery were recruited into the study. They were asked to apply 0.05% retinoic acid cream (Retin-A) daily to an area of skin scheduled for removal 2 weeks later, and an equal quantity of placebo cream to a contralateral portion of skin (breast or abdomen). Retin-A and placebo were kindly supplied by Johnson & Johnson. Patients gave their informed consent for this treatment. Consent forms and treatment protocols were reviewed and approved by the Human Subjects Committee of the University of Hawaii.

Excised skin was rapidly placed in a chilled sterile container and transported to the Cancer Center for processing.

Cell Culture

Human Keratinocytes: Normal human keratinocytes were isolated from newborn foreskin using a modified method by Wille et al [27]. The foreskin was cut into 5-mm pieces, and incubated in 25 units/ml dispase solution overnight at 4°C. Epidermal sheets were separated from dermis and digested in a 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution at 37°C for 30 min. The epidermal cells were suspended in serum-free keratinocyte medium (GIBCO) supplemented with epidermal growth factor (5 ng/ml), insulin (5 μg/ml), and bovine pituitary extract (35 μg/ml). The keratinocytes were fluid changed with fresh complete medium every 72 h. Second-passage cells were grown to confluence and then treated with all-trans retinoic acid at the stated concentration or with acetone as control.

Dermal Fibroblasts: Foreskins were chopped finely with scalpels and the small fragments were distributed in a series of culture dishes and cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 25 μg/ml gentamicin. After about 10 d, when fibroblast outgrowth was extensive, the fragments were removed and regions showing a regular fibroblastic morphology were removed by ring-cloning. The dermal fibroblast strains were cultured in the above medium and cryopreserved at passage 2 for future use. Experiments were performed on confluent cultures between passages 3 and 6.

Measurement of Junctional Intercellular Communication Gap-junction-mediated intercellular communication was measured by microinjection of 10% Lucifer Yellow CH in 0.33 M LiCl as previously described [23]. The extent of intercellular communication was determined by the number of fluorescent cells surrounding the dye-injected cell scored 10 min after dye injection. Measurements were carried out in duplicate cultures in which about 25 individual cells were probed/culture.

Immunofluorescence Portions of surgically removed skin samples were cut into 5-mm pieces and placed in cold 10% buffered formalin solution for fixation. Fixed tissues were embedded in paraffin, then subsequently sectioned (4 μm) and hydrated. Normal human keratinocytes and dermal fibroblasts were cultured on nonfluorescent plastic slides (Permanox, Nunc Inc., Naperville, IL) until confluent. They were then treated with retinoic acid for 4 d. Double-immunofluorescence microscopy was performed as described previously [23]. The slides were incubated for 30 min with a rabbit anti-Cx43 antibody (dilution 1:30) raised by immunization against a synthetic peptide representing the predicted final 15 residues of the C-terminal region of rat Cx43 [23], then exposed to a fluorescein isothiocyonate (FITC)-conjugated second antibody (goat-anti-rabbit IgG, Fab). Some sections were exposed to rabbit preimmune serum, or to immune serum preabsorbed against the immunizing peptide, then to the second antibody to determine the specificity of binding of the primary antibody. Epifluorescence microscopy was performed using a Zeiss Axioplan and images were recorded on Kodak T-MAX film exposed at 6400 ASA.

Total RNA Isolation RNA of control- and retinoic-acid-treated skin samples was isolated after washing in cold PBS and the epidermis excised from dermis with a fine surgical razor. The epidermal sheets were solubilized in 1 ml RNA Zol B solution (BioTecx) according to the procedures recommended by the manufacturer, with several strokes in a Tissumizer homogenizer. The homogenates were mixed with chloroform (10:1) and placed on ice, then centrifuged at 12,000 × g for 15 min. The upper aqueous phase containing RNA was precipitated with an equal volume of isopropanol at 4°C for for 45 min, followed by centrifugation at 12,000 × g for 15 min. The RNA pellet was washed with 75% ethanol twice and air dried. The purified RNA was dissolved in sterile water. Quantitation and purity of the RNA was determined by UV spectroscopy at 260/280 nm.

Northern Blotting Ten micrograms of total isolated RNA was subjected to Northern blot analysis as described previously [23]. RNA species were resolved by agarose gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was hybridized with $^{\alpha}[^{32}P]$-labeled full-length cDNA probe specific for rat heart connexin 43. Rat and human Cx43 exhibit 84% and 97% sequence homology at the nucleotide and amino acid level, respectively [28].

Western Blotting Extraction of connexin 43 protein from both skin samples and cultured cells was carried out as previously described [23]. For skin samples, the epidermis was dissected away from the dermis and lysed in lysis buffer (1% NP 40, 0.05 M iodoacetamide, 10 mM PMSF, 1 mM EDTA, 1 mM leupeptin, 2 mg/ml aprotinin, and 0.7 mg/ml pepstain in borate buffer, pH 8.0) at 4°C for 2 h. Cells were grown in 100-mm culture dishes, scraped off the dish in PBS (Ca^{++} and Mg^{++} free) with 1 mM NaF and 1 mM PMSF, and pelleted by centrifugation. Cells were lysed in the above lysis buffer at 4°C for 1 h. After clarifying by centrifugation, the skin or cell lysates, adjusted to contain equal protein concentrations by the BCA protein assay solution (Pierce), were subjected to 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto immobilon membranes (Millipore, Bedford, MA) by electroblotting, followed by blocking with 5% nonfat dry milk in PBS. The blots were incubated with anti-Cx43 antibody for 1 h, or as control, with immune serum pre-absorbed with the immunizing peptide, and washed in borate buffer. Antibody binding was detected with ^{125}I-labeled protein A followed by autoradiography.

RESULTS

Cx43 is Expressed in Human Epidermis Skin samples obtained during plastic surgery of adults or after routine neonatal circumcision were processed for molecular analysis or for immunofluorescence. Indirect immunofluorescence analysis performed

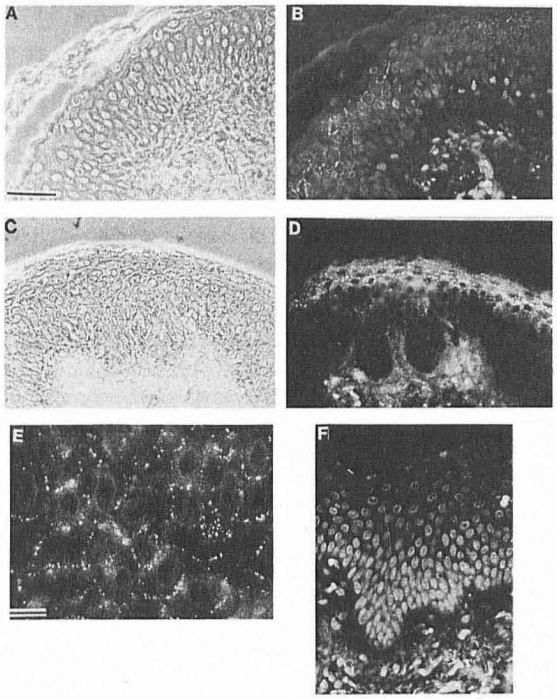

Figure 1. Immunofluorescent localization of Cx43 in control and retinoic acid–treated human skin. Retinoic acid 0.05% (Retin-A) or placebo cream was applied to an area of skin on a daily basis for 14 d prior to scheduled removal during reconstructive or cosmetic surgery. Skin was fixed and processed for indirect immunofluorescence using rabbit antiserum to the C-terminal domain of Cx43 [23]. *A*: phase-contrast control skin. *Bar,* 50 μ. *B*: same section under epifluorescent optics. Note the annular fluorescent plaques surrounding cells in the suprabasal region of epidermis. Little or no specific fluorescence was noted in the basal and lower spinous layers. *C*: phase-contrast treated skin. Note the increased thickness of the epidermis and lack of stratum corneum. *D*: epifluorescence view of C. Note the increased intensity of fluorescence in the suprabasal region. *E*: higher-power view of the Cx43-expressing region of a different retinoic acid–treated skin sample. Fluorescent plaques clearly define regions of cell/cell contact. *Bar,* 10 μ. *F*: epifluorescent micrograph of treated skin section labeled with pre-immune rabbit serum and FITC-labeled second antiserum. Note the background nuclear fluorescence in dermis and epidermis but lack of plaques in regions of cell contact.

using a polyclonal antibody to Cx43 demonstrated that this antibody recognized plaques in regions of cell/cell contact of supra-basal cells of normal human epidermis. We did not detect any specific immunofluorescence in the dermis. (Fig 1*A,B*). As a control, skin sections were incubated with primary antibody pre-absorbed with the immunizing peptide, to pre-immune rabbit serum, or to the secondary antibody only. No such intercellular plaques were observed in these controls (Fig *1F*). The primary antibody thus appears to specifically detect Cx43, a major portion of which is localized in regions of cell/cell contact, the expected location of gap-junctional plaques. By progressively focusing through the 4-μm skin section, it was observed that such plaques surrounded the antigen-positive supra-basal cells. No specific immunofluorescence was detected in basal or lower spinous keratinocytes in any of the multiple sections examined.

The absence of antibody recognition of intercellular junctions in the basal cells was surprising because basal cells in situ and keratinocytes in culture, which are believed to represent the in vitro equivalent of proliferating basal cells, are known to communicate. To determine if other members of the connexin family of molecules were expressed in basal cells, we performed indirect immunofluorescence using antibodies directed against mouse connexins 26 and 32, members of the connexin gene family known to be expressed in organs other than the eye [29,30]. As a control, we utilized adult mouse liver in which both proteins are expressed [31]. We were again unable to detect specific immunofluorescence in basal cells, nor were these connexins detected in other cell types found in skin. Both antibodies detected the expected punctate pattern of junctions expected in liver (data not shown).

Effects of Retinoic Acid on Expression of Cx43 We have previously reported that retinoids upregulate junctional communication and expression of Cx43 mRNA and protein in a line of C3H/10T1/2 mouse fibroblasts [23]. As skin is a major target of retinoids, we have investigated whether retinoids can also modulate expression of connexins in this organ.

In Situ Studies: Skin samples were obtained from patients undergoing plastic surgery. Several patients consented to apply a 0.05% retinoic acid cream (Retin-A) twice daily to the area of the skin to be surgically removed. Treatment began 2 weeks before surgery. In several cases, patients had multiple episodes of cosmetic surgery performed on both sides of the body. In these cases, retinoic acid was applied to one side and a placebo cream to the contralateral side.

Clinical Response to Retin-A: This short treatment duration and low concentration of retinoic acid (0.05%) produced no adverse reactions other than the expected mild erythema. In this respect, however, the treatments soon became unblinded to the patient. There were no reports of altered wound healing in retinoid-exposed areas. No patients withdrew from the protocol; on the contrary, several asked to again participate prior to subsequent surgery.

Expression of Cx43 Gene Products: Skin samples were placed on ice immediately after surgery and were processed for immunofluorescence and for Northern and Western blotting using specific probes to Cx43 mRNA and protein as described above. Samples were coded so as not to reveal treated or control status. For molecular studies only the epidermis was examined.

Cellular Location of Cx43: Portions of skin were removed prior to molecular analysis and processed in parallel with control samples for immunofluorescence. Retinoic-acid–treated skin consisted of more epidermal layers (5–8 in controls to over 10 in treated skin) and had lost the stratum corneum, as expected [32] (Fig 1*C*). Moreover, in the region of epidermis in which fluorescent intercellular plaques were visible fluorescence was more intense, plaques were in general more frequent, and more layers of epidermis expressed plaques (from about 2 in controls to 3–4 in treated skin) (Fig 1*D*). Specific immunofluorescence in the dermis was again not observed.

Western Blotting of Epidermis: Equal amounts of protein derived from control and retinoic acid–treated skin were separated by SDS-PAGE under reducing conditions, and the gels subjected to Western blotting using the same polyclonal anti-Cx43 antibody used for immunofluorescence. As shown in Fig 2, treated skin contained greater amounts of immunoreactive protein than control skin; in-

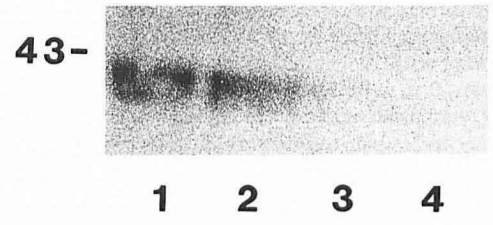

Figure 2. Increased expression of Cx43 in retinoic acid–treated human skin. Equal amounts of protein extracted from the same treated and control skin samples analyzed in Fig 3 were separated by SDS-PAGE and transferred to an immobilon membrane for Western blotting. The blot was incubated with polyclonal anti-Cx43 antibody, then with ^{125}I-labeled protein A and autoradiographed. *Lanes 1, 2,* treated skin; *lanes 3, 4,* control skin. Position of a molecular weight marker in kD is shown.

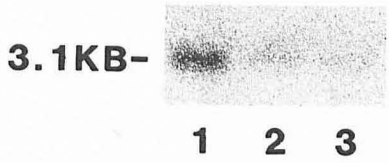

Figure 3. Effects of retinoic acid on gene expression of Cx43 in human skin. RNA was extracted from retinoic acid–treated and control skin. Equal amounts of RNA (10 μg per lane) were electrophoresed, blotted, and hybridized against ^{32}P-labeled Cx43 cDNA. *Lane 1*, retinoic acid–treated skin; *lane 2,3*, control skin from two separate locations on the same patient. The RNA from the second treated site was lost. Comparable results were seen in the three other patients studied. *Marker,* calculated position of the 3.1-kD expected transcript size of Cx43 mRNA.

deed, in controls, Cx43 was difficult to detect under the same conditions of loading and labeling that yielded strong immunoreactive bands in treated skin. Commassie blue staining of blots for total protein revealed similar patterns and intensities (data not shown). This increased expression of Cx43 was consistently detected between subjects, and within an individual subject in one case where samples were obtained from discrete anatomic regions (usually breast and abdomen) after surgery separated by several months.

Expression of Cx43 mRNA: Total RNA was extracted from treated and control skin, and identical amounts were separated by electrophoresis, prepared for Northern blotting, and probed with the full-length cDNA for rat heart Cx43. Retinoid-treated skin exhibited a 3.1-kb message that strongly hybridized to the Cx43 cDNA probe under stringent conditions (Fig 3). This is the expected transcript size for Cx43 mRNA [14]. Other bands were not detected. RNA from placebo-treated skin gave only a weak signal, suggesting that in those cells expressing Cx43, the message is only transiently expressed or is very labile.

These results demonstrated the expression of Cx43 gene products in human skin and the increased molecular abundance of gene products in the epithelial layer after only 2 weeks of treatment with retinoic acid. However, because the immunofluorescence studies indicated that the histologic region of epidermis in which Cx43 was expressed was also proportionally increased, increased Cx43 expression on a per cell basis could not be firmly established. Furthermore, the response of dermal fibroblasts was difficult to establish in the intact dermis where fibroblasts are distributed throughout a collagen matrix. To overcome these difficulties, we initiated cultures of keratinocytes and dermal fibroblasts and investigated retinoid effects on Cx43 gene expression in these cultures.

Expression of Cx43 in Cultured Skin Cells

Keratinocytes: Cultures were obtained from neonatal foreskin using established techniques and cultured in semi-defined medium lacking an exogenous source of retinoids. When confluent, cells were treated with retinoic acid or acetone as control. Whole-cell lysates were separated by SDS-PAGE and prepared for Western blotting using the Cx43 antibody. As seen in Fig 4, control cultures exhibited a low basal level of expression of an immunoreactive 43-Mr protein presumed to be Cx43. Expression of this protein exhibited a biphasic response to retinoic acid. High concentrations (10^{-7}–10^{-8} M) resulted in a decrease in expression of Cx43 protein, whereas lower concentrations (10^{-10}–10^{-11} M) caused a dose-responsive increase in expression. Staining of these blots with Coomassie Blue did not reveal any changes in the amounts of total protein in relevant regions of the gel, nor variations in the efficiency of protein transfer to the membrane (Fig 4, *bottom*). Pre-absorbed serum did not label this region of the blot. At the concentrations of retinoic acid used, no obvious changes in cell morphology resulted.

Dermal Fibroblasts: Cultures of fibroblasts were established from neonatal foreskins, grown to confluence, then treated with retinoic

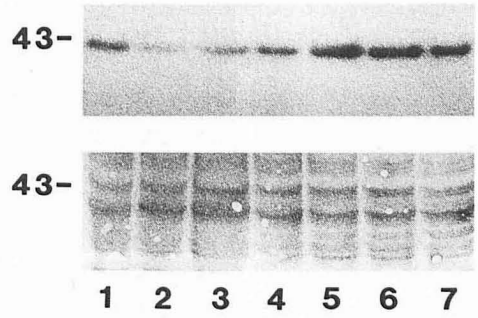

Figure 4. Induction of Cx43 by retinoic acid in cultured human keratinocytes, dose response studies. Confluent cultures of human keratinocytes were treated with retinoic acid (RA) or with acetone as control for 4 d. Cells were harvested and equal amounts of total cell protein subjected to SDS PAGE and Western blotting as in Fig 3. Top, *lane 1,* acetone control; *lane 2,* RA 10^{-7} M; *lane 3,* RA10^{-8} M; *lane 4,* RA 10^{-9} M;*lane 5,* RA 10^{-10} M; *lane 6,* RA 10^{-11} M; *lane 7,* RA 10^{-12} M. *Bottom,* same immobilon membrane stained with Coomassie Brilliant Blue to confirm that the protein loadings and transfer efficiency was approximately the same for each lane. Position of a molecular weight marker in kD is shown. Use of immune serum that had been preabsorbed with the immunizing peptide resulted in no labeling of the relevant 43-kD region of the blot (data not shown).

acid or with acetone as control. After treatment with retinoic acid, the Cx43 bands increased in intensity. Staining of the blots with Coomassie Blue and subsequent digital analysis revealed that protein loading and electroblotting resulted in comparable (within 20%) amounts of protein in each lane. Figure 5B shows a Western blot that more clearly demonstrates the presence of two immunore-

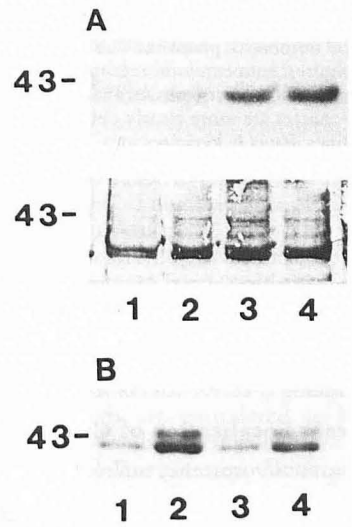

Figure 5. *A*: Induction of Cx43 by retinoic acid in cultured human fibroblasts, dose response studies. Confluent cultures of human dermal fibroblasts were treated with retinoic acid or with acetone as control for 4 d using an identical protocol as employed in Fig 4. Equal amounts of total cell protein were subjected to SDS-PAGE and Western blotting as described above. *Top, lane 1,* acetone control; *lane 2,* RA 10^{-11} M; *lane 3,* RA 10^{-9} M; *lane 4,* RA 10^{-7} M. *Bottom,* same blot after staining with Coomassie Brilliant Blue as above. Quantitation of the heavily stained (approximately 35-kD) protein bands by digital analysis demonstrated that staining intensity did not differ by more than 20%. Position of a molecular weight marker in kD is shown. Use of pre-absorbed serum, as above, resulted in no labeling of the Cx43 region of the blot. *B*: Western blot of cultured human dermal fibroblasts clearly demonstrating that Cx43 ran as a doublet on SDS-PAGE gel. *Lanes 1 and 3,* acetone control; *lanes 2 and 4,* retinoic acid 10^{-7} M. Treatment protocol as above.

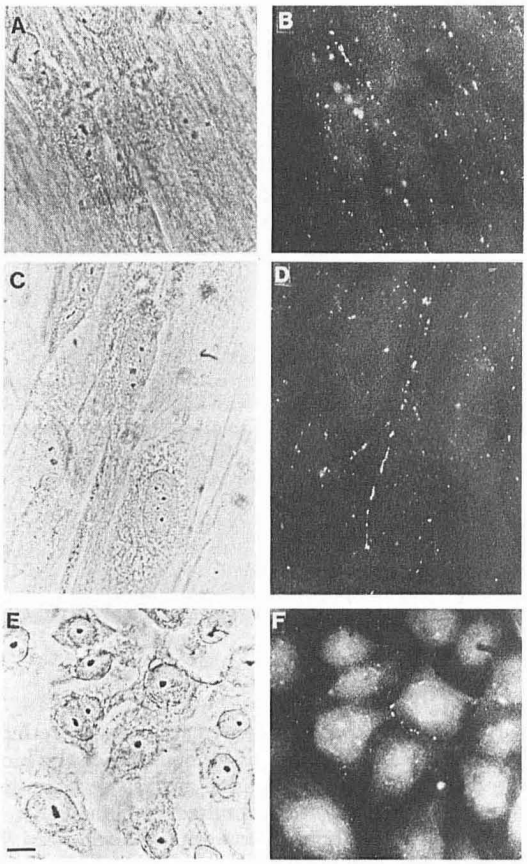

Figure 6. Expression of Cx43 in cultured human dermal fibroblasts and epidermal keratinocytes. Cells from neonatal foreskins were cultured as described, treated with retinoic acid 10^{-7} M or with acetone as control for 4 d, and subjected to indirect immunofluorescence staining essentially as in Fig 1. *A and B,* control fibroblasts, phase and fluorescence, respectively. Note occasional immunofluorescent plaques in regions of cell/cell contact. *C and D,* retinoic acid–treated fibroblasts, phase, and fluorescence, respectively. Regions of cell/cell contact are more clearly defined and junctional plaques are often fused into lines. *E and F,* keratinocytes, acetone control. *Central cell,* very infrequent junctional plaques observed in these cells. *Bar,* 10 μ.

active protein bands in the 43- and 45-Mr region. The higher Mr form of the protein is believed to represent a phosphorylated form of Cx43. As previously observed in mouse 10T1/2 cells [23], the upper, presumably phosphorylated, species was greatly increased by retinoic acid treatment.

Immunofluorescent Localization of Cx43

Keratinocytes: Immunofluorescence microscopy demonstrated that immunoreactive protein was localized in regions of intercellular contact, consistent with the location of gap-junctional plaques. However, in control cultures, only about 10% of keratinocytes exhibited detectable plaques (Fig 6*E,F*), whereas treatment with retinoic acid (10^{-7} M) reduced this frequency to about 2%, an observation consistent with the Western blots (data not shown).

Fibroblasts: Control cultures of confluent fibroblasts exhibited frequent immunoreactive plaques in regions of cell/cell contact (Fig 6*A,B*). Retinoic acid (10^{-7} M) caused an increase in the number and apparent size of these plaques. Whereas, in controls, plaques were punctate and discrete, plaques in treated fibroblasts tended to coalesce into lines that demarcated cellular peripheries (Fig 6*C,D*).

Retinoic Acid Effects on Junctional Communication: Both types of cells communicated well in monolayer culture as measured by dye-transfer studies (Fig 7). However, cells differed in their response to retinoic acid; whereas dermal fibroblasts were found to increase the extent of dye-transfer over the dose range tested (10^{-11}–10^{-7} M), keratinocytes, in contrast, did not change over this concentration range. Plotted in Fig 7 are estimates of Cx43 expression in these two cell types as influenced by retinoic acid (data obtained by densitometry of Western blots shown in Figs 4,5). It is apparent that in fibroblasts the molecular measurements of Cx43 expression and its modulation by retinoic acid are consistent with functional measurements of gap-junctional communication; however, in keratinocytes this relationship does not hold. For example, the decrease in expression of Cx43 observed at 10^{-7} M retinoic acid (Fig 4) was not accompanied by a proportional decrease in dye transfer. Photomicrographs of representative cells probed by microinjection of Lucifer Yellow are shown in Fig 8. The increase spread of dye in 10^{-7} M retinoic acid–treated fibroblasts (Fig 8*B* versus 8*D*) contrasts with the unaltered dye transfer seen in treated keratinocytes (Fig 8*F* versus 8*H*). These results suggest the amount of Cx43 is not a rate-limiting step for dye transfer in keratinocytes. A possible

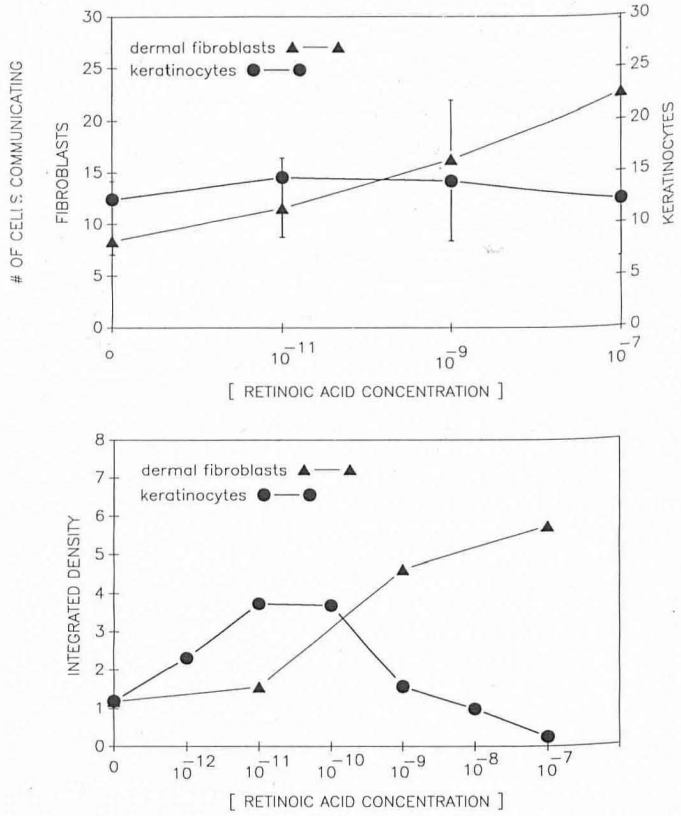

Figure 7. *Top,* effects of retinoic acid on gap-junctional communication in keratinocytes and dermal fibroblasts. Keratinocytes and dermal fibroblasts were grown to confluence as described in *Materials and Methods,* and treated with retinoic acid or acetone as control for 4 d. Individual cells were then probed by microinjection of the junctional permeable fluorescent dye Lucifer Yellow CH as described, and the number of adjacent cells that became fluorescent within approximately 10 min of injection were counted. Consistent results were obtained on two batches of cells. Results show a representative experiment in which about 45 cells in each dosage group were microinjected. *Data points,* means ± SD. For fibroblasts, all treatment groups were significantly different from acetone-treated controls ($p < 0.001$). Retinoic acid induced no significant changes in keratinocyte cultures. *Bottom,* quantitation of Cx43 in retinoic acid–treated cultures of dermal fibroblasts and keratinocytes. Data, expressed as arbitrary units of optical density, were obtained by digital quantitation of the Western blots shown in Figs 4 and 5. In both experiments, cultures were exposed to retinoic acid using identical protocols. The concordance between Cx43 expression and junctional communication in fibroblasts contrasts with the apparent lack of correlation between these two parameters in keratinocytes.

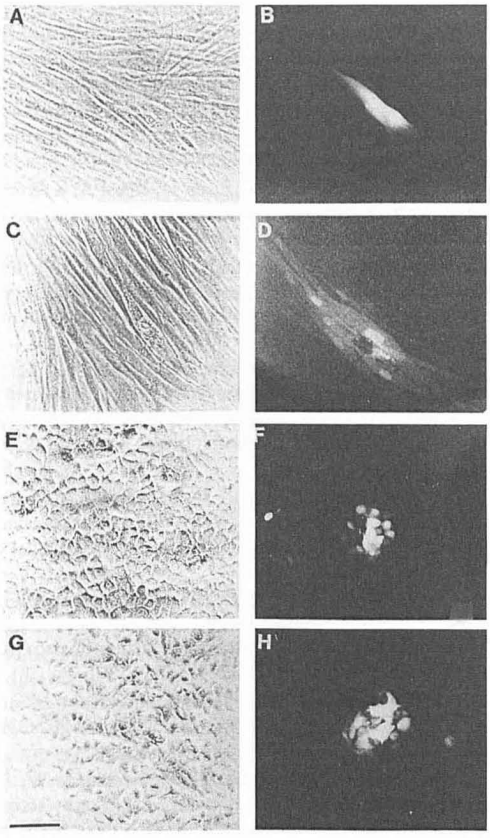

Figure 8. Junctional communication assays in human fibroblasts and keratinocytes. *Left,* phase-contrast images; *right,* epifluorescent images after microinjection of Lucifer Yellow. *A and B,* fibroblasts, acetone control. *C and D,* fibroblasts, retinoic acid 10^{-7} M. *E and F,* keratinocytes, acetone control. *G and H,* keratinocytes, retinoic acid 10^{-7} M. Treatment protocol as for Fig 7. *Bar,* 50 μ.

explanation for these observations is that Cx43 is not assembled in the cell membrane of most keratinocytes and thus not functional and not detectable by immunofluorescence because of the high background fluorescence of these cells (Fig 1*F*).

DISCUSSION

Retinoids are effective modulators of proliferation and differentiation of keratinocytes in vivo and in vitro [33]. This activity is presumably responsible for their clinical usefulness in a wide range of diseases of the skin characterized by abnormal differentiation [34]. The epidermal cells respond to retinoids by suppressing terminal differentiation. This has been characterized at the molecular level as a decreased expression of keratins associated with normal terminal differentiation. Little is known of the mechanisms by which keratinocytes regulate their differentiation, and less of how retinoids affect this complex process. Most evidence points to the existence of a gradient of signals, from the basal cells up, which triggers the sequential differentiation of these cells. Ca^{++} has been shown to possess the required characteristics; a gradient exists in the epidermis [35] and cultured keratinocytes undergo terminal differentiation in response to high Ca^{++} levels [36]. How might a gradient of this or other psysiologic regulator be maintained and controlled? Similar questions face investigators with interest in morphogenesis and growth control. The diffusion of regulators via gap junctions from cytoplasm to cytoplasm appears to offer many advantages of control, both in concentration and direction, over diffusion via the extracellular space. The observations that during embryogenesis junctional communication is compartmentalized to cell groups with similar destinies [37], that gradients of junctions exist in the developing chick limb bud [17], and that junctional communication in mouse and human skin appears restricted to vertical compartments [26,38] all suggest a role for junctional communication in the local control of growth and differentiation.

In previous studies using 10T1/2 cells as a model system to investigate retinoid action, we demonstrated that natural and synthetic retinoids that possessed the capacity to maintain the normal differentiation of hamster tracheal cells (i.e. those with vitamin-A–like activity) would suppress chemically induced neoplastic transformation [12] and enhance growth control [13]. These two phenomena were later found to be tightly correlated with the ability of retinoids to upregulate gap-junctional intercellular communication [39], driven by a major increase in Cx43 expression [23]. In the present study we have extended these observations to human cells and have demonstrated that retinoic acid, applied either topically in vivo or to cultured skin cells in vitro, elevated Cx43 expression in normal human keratinocytes and dermal fibroblasts. In the latter cells, just as in the model mouse cells, retinoic acid also elevated gap-junctional communication.

Gap junctions are ubiquitous, are found in virtually all adult and embryonic tissues in mammals, and constitute a family of proteins whose members are still being described [40]. These connexin molecules differ primarily in the length and sequence of their cytoplasm-orientated C-terminal regions. This report is the first to demonstrate expression of a specific connexin (Cx43) in the intact human epidermis and modulation of its expression by retinoids, although Cx43 was recently found in cultured mouse keratinocytes [41] and a novel connexin (Cx31) has been reported in whole mouse skin [42]. However the existence of gap junctions in skin has been known for some time, since Elias and Friend [43] revealed their presence by electronmicroscopy and showed that retinoic acid altered their distribution. In human squamous cell carcinoma, retinoic acid was reported to cause a proliferation of gap junctions [44], a finding that may be germane to the present observations of increased expression of Cx43 in suprabasal cells, because this tumor represents the malignant conversion of such cells. Junctional transfer of microinjected dye has been demonstrated by others [26] in intact human skin obtained after removal by keratome. In accord with predictions of the present study, suprabasal cells were much more extensively coupled than were basal cells, although both cell layers were coupled. No evidence for coupling between basal and dermal cells was found, although dermal cells were highly coupled. It is of interest that no statistically significant change in dye transfer between keratinocytes was recorded in skin samples obtained from retinoid-treated skin; however, mean values in skin treated topically with retinoic acid were twice as high as controls [26]. Madhukar et al [45] showed that TGF-β blocked gap-junctional communication in cultured human epidermal keratinocytes and induced terminal differentiation. The disruption of cellular communication led to the isolation of differentiated cells from as-yet undifferentiated cells. Gap-junctional communication in human keratinocytes is also inhibited by tumor promoters [21]. Retinoids are considered to be antipromoters [11,46,47], and they also inhibit calcium-induced differentiation of human keratinocytes [48]. Furthermore, the effects of retinoids and TGF-β on hyperproliferation of human skin cells are antagonistic. [49]. These data are all suggestive that one pathway for retinoic acid action on keratinocyte differentiation is mediated through gap-junctional communication.

In skin treated topically with retinoic acid, the increased expression of Cx43 was present mainly in the suprabasal layer of the epidermis. This was associated with the thickening of the epidermal layer and the disappearance of the keratinized layer (Fig 1). The thickening of the epidermis appears due to the effects of retinoic acid on inhibiting differentiation [1] and/or promoting proliferation [4,50]. The increase in Cx43 expression detected at the molecular level (Figs 2,3) could result from an increase in the Cx43 level per cell, or, alternatively, from an increase in the number of cells expressing Cx43. On the basis of our observations in intact skin, we were not able to differentiate between these alternatives. To do so we examined the effects of retinoic acid on expression of Cx43 and

on junctional communication in cultured skin cells. Although Cx43 was detected in both keratinocytes and fibroblasts, these cells differed qualitatively and quantitatively in their responses to retinoic acid. Cultured keratinocytes, when maintained in low-calcium medium, retain proliferative capacity and the majority do not terminally differentiate. In these respects, they resemble basal cells in situ [36]; the infrequent expression of Cx43 in both situations strengthens this interpretation. The reported ability of retinoic acid to inhibit keratinocyte differentiation [48] suggests that those 10% cells in which Cx43 was detectable by immunofluorescence were undergoing terminal differentiation and that 10^{-7} M retinoic acid inhibited expression of Cx43 (Fig 4) by inhibiting this process. At very low concentrations (10^{-11} M) retinoic acid increased Cx43 expression, whereas higher concentrations (10^{-7}–10^{-8} M) decreased expression (Fig 4). However, we could find no change in communication over the entire dose range tested (Fig 7). In contrast, dermal fibroblasts increased their expression of Cx43 and their transfer of dye at high, but not low, concentrations of retinoic acid. These results suggest that skin cells may differentially express specific high-affinity receptors for retinoic acid. In contrast to the frequent immunofluorescent plaques seen in cultured dermal fibroblasts, such plaques were not detected in situ. We attribute this to the low probability of sectioning through a region of intercellular contact in the dermis.

It has been shown that retinoic acid can directly bind to nuclear receptors (RAR) [51, 52], which in turn can control the transcription of differentiation-specific genes by binding to specific DNA-response elements (RARE). Expression of two retinoic acid receptors (RARα and γ) has been described in human skin [6]. RARγ is the predominant receptor expressed in both dermis and epidermis. RARα is also constitutively expressed in both tissues and is located mainly in the suprabasal cells of the epidermis [53]. It is of interest that RARβ is not constitutively expressed in either cell type but is inducible by retinoic acid in dermal fibroblasts but not in keratinocytes in culture [6]. This cell type specificity for induction mirrors the current observation for Cx43 induction. This difference between dermal and epidermal cells in their sensitivity to retinoid treatment may be due to: i) lower expression, or differential induction, of RAR in dermal cells than in epidermal cells; ii) differential expression of cellular receptors for retinoic acid (CRABP) [54], which modulate levels of unbound drug; or iii) the rich blood supply in the dermis, the only source of retinoids for the epidermis, which may also in part contribute to this differential sensitivity by producing a concentration gradient of retinoids in skin from dermis to epidermis.

Although Cx43 is a major gap-junctional protein presumably responsible for gap-junctional intercellular communication in suprabasal epidermal cells [41], the evidence indicates that other members of the connexin family of junctional proteins may be expressed in basal cells and cultured keratinocytes, which in proliferative capacity and differentiated phenotype closely resemble basal cells [36]. Our observation of no significant change in intercellular communication in cultured keratinocytes in response to retinoid treatment, in spite of major changes in Cx43 expression, in a positive and negative direction, and our failure to detect Cx43 expression in basal cells of intact skin both point to this conclusion. Other connexin proteins, such as Cx31, may be involved in gap-junctional communication in basal keratinocytes. These proteins too may be regulated by retinoids, adding further complexity to the modulation of epidermal differentiation.

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