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Gap Junction Communications Influence Upon Fibroblast Synthesis of Type I Collagen and Fibronectin

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Abstract In rats polyvinyl alcohol sponge subcutaneous implants treated with gap junctional intercellular communications (GJIC) uncouplers showed reduced deposition of connective tissue. Do uncouplers inhibit the synthesis and deposition of a new connective tissue by fibroblasts? Confluent human dermal fibroblasts in serum-free medium received either endosulfan or oleamide, GJIC uncouplers. Collected media were subjected to Dot Blot analysis for native Type I collagen and fibronectin. Uncoupler-treated fibroblasts released less Type I collagen, while there was no change in fibronectin release. Collagen synthesis was restored to normal, when the uncouplers were removed, showing that these uncouplers were reversible and not toxic to cells. Northern blot analysis revealed procollagen α1 (I) mRNA was minimally affected by endosulfan. Oleamide-treated 17-day chick embryo calvaria explants were incubated with Type I collagen antibody, frozen, cryosectioned, and then subjected to rhodamine (Rh) tagged anti-mouse-IgG antibody, to detect newly deposited Type I collagen. Fluorescent antibody-collagen complexes were localized on the periphery of cells in control calvaria, but absent around cells in oleamide-treated calvaria. GJIC optimize collagen synthesis but not fibronectin synthesis. The lack of connective tissue deposited in granulation tissues treated with uncouplers appears related to the inhibition of collagen synthesis. These findings suggest that altering GJIC might control collagen deposition in scarring. J. Cell. Biochem. 98: 735–743, 2006. © 2006 Wiley-Liss, Inc.

Key words: collagen synthesis; gap junctional communications; oleamide; endosulfan; fibronectin synthesis

Type I collagen is the major connective tissue protein of skin and bone. Type I collagen is affiliated with a family of proteins that have at least 27 members [Myllyharju and Kivirikko, 2004]. The functions or specific roles of these various collagen types are uncertain. Type I and Type III are the major collagen types of dermis, where Type III represents about 20% and Type I about 75% of the collagens. In rigid tissues such as bone, tendon, and cartilage, there is little or no Type III collagen, while in soft tissues and organs greater proportions of Type III are found. In granulation tissue of healing wounds, Type III collagen increases to 30% of the collagen [Bailey et al., 1975]. Collagens are synthesized as larger precursor proteins, procollagen. Procollagen has peptides located on both its N and C termini. Procollagen stripped of its propeptides is referred to as tropocollagen, which is the fundamental building block of collagen fibers. There are specific steps in the synthesis of procollagen, its conversion into tropocollagen, and release into extracellular space. Collagen secretion involves its transport from its site of synthesis, the endoplasmic reticulum, through the Golgi, past the plasma membrane and release into the extracellular compartment [Ross, 1975]. Disrupting the transport of procollagen blocks the deposition of the newly synthesized extracellular matrix (ECM) [Ehrlich et al., 1974]. In contrast, fibronectin another ECM protein is released from fibroblasts by a mechanism independent of procollagen pathway [Hedman, 1980]. The transport of procollagen involves Golgi linked to microtubules and the transport of fibronectin is independent of Golgi linked to microtubules [Ehrlich and Bornstein, 1972; Ledger et al., 1980].

Gap junction intercellular communications (GJIC) allows direct passage of molecules

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between the cytoplasm of coupled cells. Small molecules of less than 1,200 molecular weight, such as nutrients like sugars, amino acids, and oxygen, or signaling molecules like cAMP are examples of molecules that directly pass between coupled cells through gap junction channels. Intercellular communications between cells through gap junctions is rapid and facilitates coordinated cellular responses between coupled cells [Goodenough et al., 1996]. Gap junction channels are gated structures, where the channel can be in a closed or open state [Kumar and Gilula, 1996]. The gap junction channel is made up of a pair of connexon structures, which are embedded in the cell's plasma membrane. The connexin (Cx) is composed of six Cx proteins. A pair of hexameric oligomer protein connexons anchored in the plasma membrane of neighboring cells come together and form the functional gap junction channel. In fibroblasts, the most common Cx is Cx-43, which makes up a major portion of the connexons found in gap junction channels of fibroblasts.

The secretion of proteins can be influenced by GJIC [Serre-Beinier et al., 2002]. In one study, stretched tendons produce more collagen. Treating stretched tendons with a GJIC uncoupler blocks increased collagen synthesis [Banes et al., 1999]. In another study inhibiting the expression of Cx-43 in transformed rat bone cells, blocks the transcription of the procollagen gene [Stains et al., 2003]. The reduced levels of connective tissue between uncoupler-treated PVA sponge implants could be related to the inhibition of collagen by inhibiting procollagen gene expression.

During wound re-epithelialization, the expression of Cx-43 on the surface of migrating keratinocytes is suspended [Kretz et al., 2003]. At the termination of keratinocyte migration and the completion of wound re-epithelialization, keratinocytes are reattached to the underlining basement membrane surface and these cells once again express Cx-43 on their surface. It is reported that GJIC between wound fibroblasts in granulation tissue influences the repair process. Promoting GJIC between wound fibroblasts increases the rate of collagen maturation in granulation tissue [Moyer et al., 2002]. Inhibiting GJIC between wound fibroblasts in granulation tissue with added GJIC uncouplers increases the density of wound fibroblasts [Ehrlich and Diez, 2003]. One possibility for increased cell density by the action of uncouplers is increased cell proliferation, which is associated with decreased GJIC [Lowenstein and Kanno, 1966]. Another possibility for greater cell density is less connective tissue deposited between uncoupled wound fibroblasts. Blocking GJIC with stretched tendon fibroblasts reduces the deposition of collagen [Stains et al., 2003]. There is transcriptional sensitivity of the procollagen $\alpha 1$ gene in rat transformed bone cells associated with a deficiency in Cx-43 expression [Serre-Beinier et al., 2002]. Here, we report that blocking GJIC in human dermal fibroblasts with added uncouplers reduces Type I collagen synthesis but not fibronectin synthesis, and the levels of mRNA for Type I procollagen is unaffected by added uncoupler.

METHODS

Cells

Human dermal fibroblast cultures were derived from neonatal foreskin explants and maintained in complete DMEM, Dulbecco's modification of Eagles medium, with 10% fetal bovine serum and 15 μg/ml of gentamicin. Fibroblasts were studied between their 8th and 12th passage.

Gap Junction Dye Coupling Assay

The effectiveness of uncoupler agents on GJIC in fibroblasts was evaluated by the scrape-loading technique [Pepper et al., 1989]. When fibroblasts in 35-mm dishes approached confluence, the cells were rinsed with phosphate buffered saline (PBS) and 1.0 ml of a dye solution, consisting of 20 mg lucifer yellow (LY) and 5 mg rhodamine (Rh)-dextran (Molecular Probes, Eugene, OR) in PBS was added. The dye-covered cell layer was scratched with a commercial glasscutter, returned to the incubator at 37° for 2 min. The dye solution was removed; the cells rinsed with warmed PBS, the cell layer fixed in buffered 4% paraformaldehyde (PFA), and cells in the scratched line viewed with a fluorescent microscope. Both LY and Rh-dextran accumulated within the scrape-injured cells. If a scrape-injured cell was coupled to a neighbor, LY dye would pass into the uninjured coupled cell. The Rh-dextran particles were too large to pass through gap junction channels and they were retained in the scrape-injured cells. The coupling index was

determined by counting the red and yellow–green fluorescent stained cells within five randomly selected microscope fields in two dishes from each experimental treatment group. The ratio of yellow–green LY fluorescent cells to red fluorescent cells was the reported coupling index. A coupling index of unity indicated no coupling, while a coupling index of 2 or greater indicates cell coupling. The uncoupler agents used in these experiment were endosulfan [Kenne et al., 1994; Matesic et al., 2001] (Chem Service, West Chester, PA) and oleamide [Guan et al., 1997; Boger et al., 1998] (Calbiochem, La Jolla, CA), which were dissolved in 70% ethanol as 1 mM solutions.

Fibroblast Treatments

To investigate in fibroblasts that the interference with the transport of protein from the rough endoplasmic reticulum (RER) to the Golgi would block the secretion of collagen and fibronectin, fibroblasts were treated with Brefeldin A [Fujiwara et al., 1988]. Confluent fibroblast cultures were incubated with 50 ng/ml Brefeldin A overnight in serum-free medium. Collagen released into the medium from Brefeldin A-treated cells was compared to untreated cells. Microtubular disruptive agents like colchicine were reported to inhibit procollagen synthesis and secretion [Ehrlich and Bornstein, 1972]. Confluent cultured fibroblasts were incubated with 10 μM colchicine overnight and the levels of Type I collagen and fibronectin were determined in the medium.

Dot Blots Analysis

The levels of released soluble collagen and fibronectin from cultured fibroblasts was determined by Dot Blot analysis, employing the Bio-Rad Dot Blot apparatus (Bio-Rad, Hercules, CA). Cultured medium from fibroblasts in 10-cm dishes was processed for Dot Blot analysis, when fibroblasts reached confluence. The medium was removed, the cells rinsed in PBS, then 5 ml of serum-free-DMEM supplemented with 50 μg/ml of ascorbic acid 2-phosphate sesquimagnesium (Sigma Chemical Co.), vitamin C and 10 μg/ml β aminoproprionitrile fumarate (Sigma Chemical Co.), BAPN were added. Vitamin C optimizes collagen synthesis and BAPN blocks collagen cross-linking, which promotes the accumulation of soluble collagen in the medium. The fibroblasts were incubated for 24 h, the media were collected, concentrated HCl was added to acidify the medium, which promoted collagen solubility and inhibited proteolytic breakdown of collagen. The collected media were stored at 4°C until analysis.

Protein blotted on a nitrocellulose membrane was subjected to immuno-blot detection for native Type I collagen, mouse monoclonal IgG1 clone COL-1, (Sigma Chemical Co.) and fibronectin, mouse monoclonal IgG1 clone FN-3E2 (Sigma Chemical Co.). The COL-1 antibody is specific for native Type I collagen and does not detect denatured collagen α chains, which limits collagen detection to Dot Blot analysis. The membrane was incubated overnight in a solution of 5% dried milk to block non-specific protein binding. The membrane was then incubated with a peroxidase-conjugated antibody directed to mouse IgG1 (Jackson Immuno Research Laboratories, West Grove, PA). Antibody antigen complex was detected on the membrane by the Super Signal West Dura Chemiluminescence detection system (Pierce Biotechnologies, Rockland, IL).

Chick Embryo Calvaria Explants

Documentation of inhibition of collagen secretion by GJIC uncouplers in tissue utilized chick embryo calvaria explant cultures. The cross-reactivity of the monoclonal IgG1 clone COL-1 antibody, which is directed to human Type I collagen, was evaluated for its reactivity with chick embryo Type I collagen. Briefly, acid soluble collagen was isolated from chick embryo. Two 17 day chick embryos were homogenized in 0.5 M acetic acid, the homogenate cleared by centrifugation, and the supernatant saved. The collagen was purified from the supernatant by salt precipitation, exhaustive dialysis, lyophilized, weighted and stored at 5 mg/ml in 1 mM HCl [Ehrlich and Rittenberg, 2000]. The isolated collagen was tested for its cross-reactivity with the antibody using the Dot Blot analysis. Chick embryo Type I collagen cross-reacted with the Type I collagen antibody, IgG1 clone COL-1.

This antibody was utilized to entrap newly released Type I collagen in an antibody-collagen complex within 17-day chick embryo calvaria. The technique was originally described to identify cathepsin D secretion on the surface of chondrocytes within cartilage explants [Poole et al., 1974]. Calvaria from a 17-day chick embryos were harvested under sterile conditions and placed in serum-free, vitamin C supplemented DMEM containing 15 μg of gentamicin per milliliter of medium. Each of four 35 mm-dish with 1.0 ml of

complete DMEM received a calvarium. Two control dishes each received 25 μg of mouse IgG, which served as the immunoglobulin control. The two antibody-treated dishes received 25 μg of mouse IgG1 clone COL-1 monoclonal antibody. Uncoupler-treated dishes received culture medium supplemented with 10 μM oleamide and control dishes received 10 μl of 70% ethanol as vehicle controls. The calvaria were placed in a 37°C incubator overnight. The calvaria were collected, fixed in 4% PFA for 5 min, and then washed in PBS. All calvaria were incubated at room temperature with donkey anti-mouse IgG conjugated with Rh (Jackson Immuno Research Laboratories) and Alexis phalloidin (Molecular Probes) for 1 h. The calvaria received four 5 min washes with PBS before incubation with DAPI (Molecular Probes) for 5 min to identify nuclei, rinsed three times with PBS, placed on a glass slide, submerged in mounting gel, and then covered with a glass coverslip. The slides were viewed with a fluorescent microscope equipped with appropriate filters and photographs taken with a Photometric Cool Snap digital camera.

Northern Blot Determination of Procollagen mRNA

Total RNA was collected from two 10-cm dishes incubated with 30 μM endosulfan and two dishes receiving 50 μl of PBS, using RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) following the suppliers instructions. Total RNA 5 μg per lane was fractionated on a 1% formaldehyde gel and transferred overnight to a charged nylon membrane by diffusion and linked to the membrane using a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Oligonucleotide probes for human Type I collagen and GAPDH were synthesized (IDT, Inc., Coralville, IA) and labeled, using the AlkPhos Direct Labeling kit and Detection System (Amersham Biosciences, Piscataway, NJ), following the procedure provided by the supplier. The membrane was hybridized, washed, exposed to X-ray film, and developed. To confirm uniform RNA loading of each sample, the membrane was reprobed with a GAPDH oligonucleotide probe. Type I collagen α1 mRNA and GAPDH mRNA were compared between fibroblasts treated with endosulfan or PBS.

RESULTS

By the scrape loading, the uncoupling action of endosulfan and oleamide on GJIC between our human dermal fibroblasts was confirmed. Dye localization within cells was recorded with 100–150 cells from each test group. The coupling index for Group 1, the control that received 10 μl of 70% ethanol, was 3.7 ± 0.2. The coupling index for the 30 μM endosulfan treatment group was 1.2 ± 0.1 ($P \leq 0.001$). The coupling index for the 10 μM oleamide treatment group was 1.2 ± 0.6 ($P \leq 0.001$). Endosulfan and oleamide were effective uncouplers of GJIC between human dermal fibroblasts.

To document collagen and fibronectin secretion from human dermal fibroblasts, known inhibitors of protein secretion were investigated. Brefeldin A blocked the transfer of proteins synthesized in the RER to the Golgi complex [Fujiwara et al., 1988]. Fibroblasts at confluence were incubated overnight in serum-free medium with 50 ng/ml of Brefeldin A. As shown in Figure 1A, Brefeldin A blocked the release of Type I collagen and fibronectin (Fig. 1B) from fibroblasts into the culture medium. These experiments were repeated twice. The findings support the release of fibronectin, and Type I collagen required the passage of newly synthesized protein to the Golgi. Colchicine, which inhibits the polymerization of microtubules, blocked the secretion of Type I collagen [Diegelmann and Peterkofsky, 1972]. As expected, human dermal fibroblasts treated with 10 μM colchicine released less Type I collagen (see Fig. 1A). However, colchicine did not interfere with the release of fibronectin (Fig. 1B). These experiments were repeated five times. The secretion of Type I collagen was dependent upon microtubules, but the release of fibronectin was independent of microtubules.

The harvested media from dermal fibroblasts at confluence incubated for 24 h with 70% ethanol vehicle control, 30 μM endosulfan, or 10 μM oleamide were analyzed by Dot Blot analysis. As shown in Figure 2A, 30 μM endosulfan middle blot and 10 μM oleamide bottom blot inhibited the synthesis of Type 1 collagen compared to control top blot. The inhibition of Type I collagen synthesis was confirmed in six separate experiments. The action of uncouplers on fibronectin release was also examined and the results shown in Figure 2B. Equivalent concentrations of fibronectin were released in the media from fibroblasts receiving ethanol vehicle control, endosulfan or oleamide (Fig. 2 panel B). Unlike Type I collagen

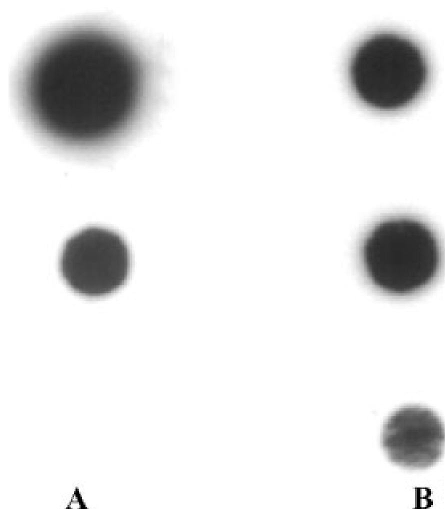

Fig. 1. Collagen and fibronectin synthesis with added Brefeldin A and colchicine. Dot Blot analysis showing the actions of Brefeldin A and colchicine on collagen and fibronectin synthesis. **Panel 1 A** is a Dot Blot of Type I collagen from culture medium collected from fibroblasts exposed to PBS (**top**), colchicine (**middle**), or Brefeldin A (**bottom**). **Panel 1 B** is a Dot Blot of fibronectin accumulation in culture medium collected from fibroblasts exposed to PBS (**top**), colchicine (**middle**), or Brefeldin A (**bottom**).

synthesis, which was inhibited by uncouplers, fibronectin synthesis was unaffected by the inclusion of uncouplers.

To show that endosulfan and oleamide, which are reversible uncouplers of GJIC, were not toxic to cells; the recovery of collagen secretion in previously treated fibroblasts was investigated.

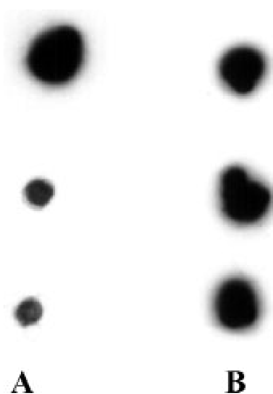

Fig. 2. Collagen and fibronectin synthesis with the uncouplers oleamide and endosulfan. Dot Blot analysis showing the actions of endosulfan and oleamide (GJIC uncouplers) on the synthesis of collagen and fibronectin from culture human dermal fibroblasts. **Panel A** shows the secretion of Type I collagen, where the fibroblasts treated with PBS is the top blot, endosulfan is the middle blot, and oleamide is the bottom blot. **Panel B** shows the synthesis of fibronectin, where fibroblasts treated with PBS (**top**), endosulfan (**middle**), and oleamide (**bottom**).

The medium from endosulfan and oleamide-treated cells was collected for Dot Blot analysis, the cells were then rinsed with PBS, and the fibroblasts incubated for 4 h in complete DMEM, which allowed the fibroblasts to recover from their initial treatment with uncouplers. After 4 h, serum-free DMEM supplemented with vitamin C and BAPN were added. As shown in Figure 3 panels A and C, the medium from cells initially incubated with endosulfan or oleamide showed little Type I collagen secretion (top of panels). Washing out the inhibitors increased the levels of Type I collagen secretion (bottom of Fig. 3 panels A and C). When endosulfan and oleamide were removed from cells, Type I collagen secretion was restored, showing that these uncouplers were reversible and not toxic. Also confirming the non-toxic action of these inhibitors, the level of fibronectin released from treated fibroblasts was the same with and without uncouplers, see Figure 3 panels B and D.

Type I COL-1 antibody was demonstrated to cross-react with chick embryo Type I collagen. Calvaria explants in organ culture were incubated for 24 h with or without 10 μM oleamide. The calvaria were homogenized and by Dot Blot analysis the homogenates were analyzed for Type I collagen. It was also shown that the synthesis of soluble Type I collagen from calvaria treated with 10 μM oleamide was inhibited (Fig. 4A). In addition to inhibiting the release of collagen from cultured human dermal fibroblasts, oleamide inhibited the release of Type I collagen from chick embryo calvaria. To visualize newly synthesized collagen released from cells, we utilized an antibody-antigen complex immunohistology technique previously reported for identifying secreted proteinases [Poole et al., 1974]. As shown in Figure 4B, human Type I collagen antibody reacts with newly released native collagen, but did not bind to established fibrillar collagen in the ECM. The incubation of 17-day chick calvaria with Type I collagen antibody showed the accumulation of collagen antibody complex at the periphery of cells, see Figure 4B. The collagen antibody-soluble collagen complex that formed with chick calvaria treated with 10 μM oleamide was minimal (Fig. 4C). The inclusion of the oleamide with chick calvaria explant cultures reduced the release of collagen from cells. These same findings were also found with 30 μM endosulfan substituting for oleamide (data not shown).

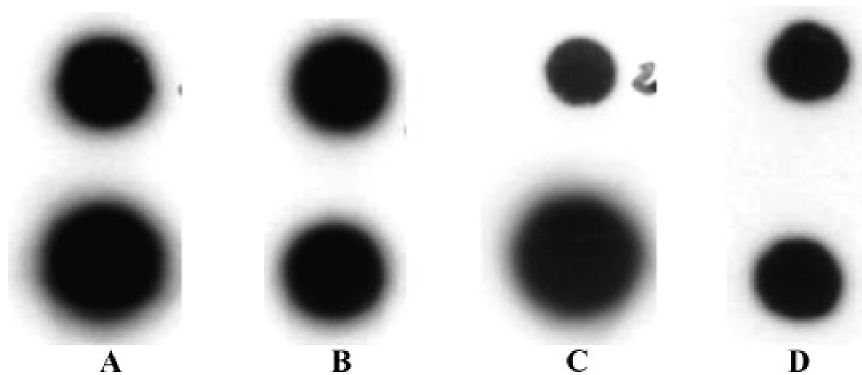

Fig. 3. Dot Blot analysis showing the reversibility of endosulfan and oleamide inhibition of collagen synthesis. Fibroblasts were initially exposed to oleamide or endosulfan and the collected media analyzed for Type I collagen and fibronectin released into the medium. **Panels A** and **B** are from oleamide-treated fibroblasts and **panels C** and **D** are from endosulfan-treated cells. Panels A and C are blots of Type I collagen and panels B and D are of fibronectin. The media from cells treated with uncouplers all appear as the top blot in each panel. The bottom blot in each panel represents the protein released from cells that had the uncouplers removed and the cells incubated for another 24 h.

Is the inhibition of Type I collagen synthesis due to an inhibition of the expression Type I procollagen mRNA [Stains et al., 2003]? By Northern blot analysis changes in the level of pro α1 (I) collagen mRNA were investigated in cultured dermal fibroblasts receiving 30 μM endosulfan or PBS control. As shown in Figure 5A, the level of pro α1 (I) collagen mRNA was equivalent in both control and treated fibroblasts. The treatment of fibroblasts with endosulfan did not change the expression of procollagen α1 (I) mRNA. To show equivalent loading of cell mRNA, levels of GAPDH mRNA were also evaluated by Northern blot analysis. The level of GAPDH mRNA in endosulfan-treated fibroblasts compared to PBS-treated fibroblasts

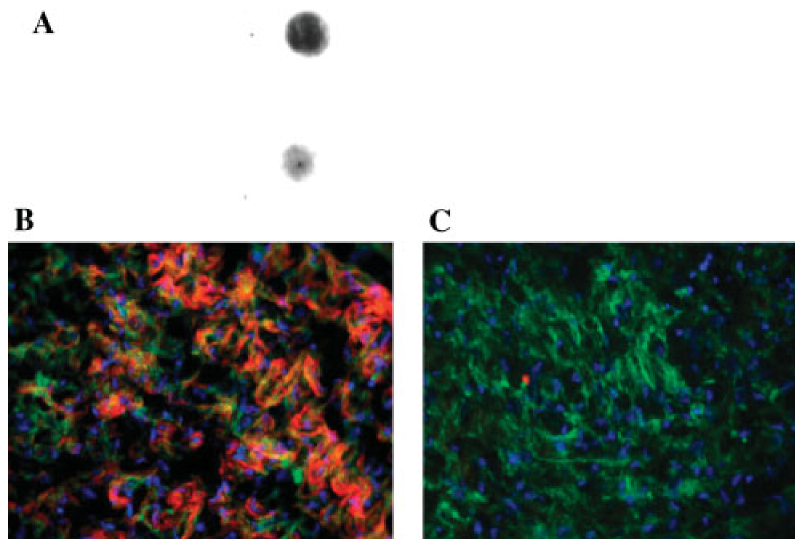

Fig. 4. Collagen secretion in chick embryo calvaria explants cultures. **Panel A**. Dot Blot analysis of oleamide-treated cultured 17-day chick embryo calvaria explants on the secretion of collagen. Calvaria were incubated with either PBS or 10 μM oleamide overnight, the calvaria were harvested, homogenized, and assayed for Type I collagen by Dot Blot analysis. The top blot is collagen from the PBS-treated explant homogenate and the bottom blot is from the oleamide-treated explant homogenate. **Panel B** and **C** are immuno-entrapment histology of 17-day chick embryo calvaria incubated with anti-Type I collagen antibody overnight then fixed and exposed to Rh anti-mouse IgG, counter stained with Alexis phalloidin for f-actin, and DAPI for nuclei. Collagen is in red, f-actin is in green, and the nuclei are in blue. Panel B is a calvarium incubated with 10 μl of PBS and Panel C is a calvarium incubated with 30 μM endosulfan.

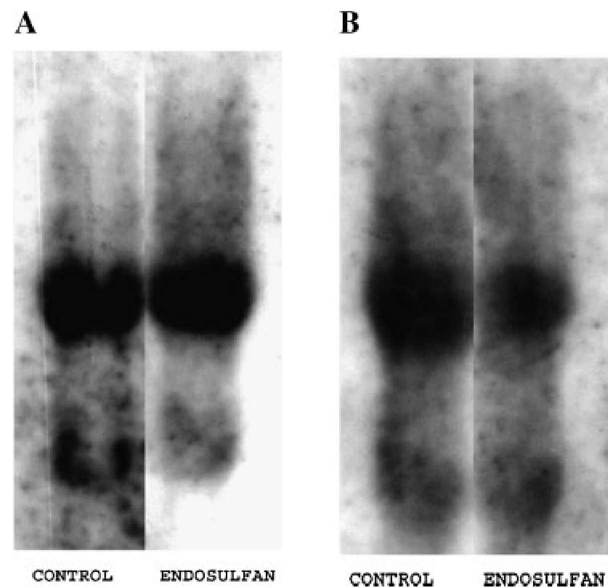

Fig. 5. Northern blots of procollagen α (I) mRNA and GAPDH mRNA. Human dermal fibroblasts were incubated overnight with 30 μM endosulfan or PBS, total RNA was collected and subjected to Northern blot analysis for determining pro α1 (I) mRNA and GAPDH mRNA. **Panel A** shows pro α1 (I) mRNA and **panel B** shows GAPDH mRNA.

was equivalent (Fig. 5B). The incubation of fibroblasts with uncoupler did not inhibit the expression of procollagen α1 (I) mRNA.

DISCUSSION

Rat granulation tissue exposed to GJIC uncouplers show less deposited connective tissue [Ehrlich and Diez, 2003]. Is the decrease in connective tissue accumulating between fibroblasts related to less connective tissue synthesis, enhanced fibroblast proliferation, or a combination of both? There is a relationship between increased cell proliferation and the inhibition of GJIC, where transformed cells that display uncontrolled proliferation lack GJIC [Vine and Bertram, 2002]. The focus here is to determine if interfering with GJIC alters the synthesis of connective tissue proteins collagen and fibronectin in human dermal fibroblasts and chick embryo calvaria explants. Mechanically loaded tendon cells show enhanced collagen synthesis. Blocking GJIC by added octanol eliminates that enhancement of collagen synthesis by these mechanically loaded tendon cells [Stains et al., 2003]. The concentration of octanol needed to inhibit GJIC is in the mM range, while the concentrations of endosulfan and oleamide are in the μM range.

There is a possibility uncouplers disrupt the translocation of collagen from its site of synthesis, the RER to its site of exocytosis at the plasma membrane. Monensin, an agent that prevents the translocation of protein entering the Golgi compartment from the RER, inhibits the secretion of collagen from cultured human dermal fibroblasts [Ledger et al., 1980]. Brefeldin A also blocks the translocation of protein from the RER to the Golgi [Hedman, 1980]. Here Brefeldin A, an agent that has the similar actions as Monensin, blocks the secretion of collagen from cultured human dermal fibroblasts. The secretion of collagen from chick embryo calvaria can be blocked by the depolymerization of microtubules [Ehrlich et al., 1974]. There is histological evidence showing the translocation of newly synthesized procollagen in vesicles associated with microtubules [Cho and Garrant, 1981]. The inclusion of colchicine, an agent that disrupts polymerized microtubules, inhibits the secretion of collagen from human dermal fibroblasts. To optimize the secretion of collagen, functional microtubules are required.

Added uncouplers, which block GJIC between cultured human dermal fibroblasts, inhibit the synthesis of Type I collagen but they do not affect fibronectin synthesis. In regard to fibronectin secretion, there is a report that the pathway for collagen secretion differs from that of fibronectin secretion [Hedman, 1980]. Oleamide-treated chick embryo calvaria explants show that the accumulation of collagen at the

periphery of cells is blocked. The optimal release of newly synthesized collagen appears to require GJIC. In contrast, the secretion of another connective tissue protein, fibronectin, is unaffected by the inclusion of GJIC uncouplers. It suggests that blocking GJIC does not inhibit the secretion of all proteins.

What is the possible mechanism for the selective inhibition of Type I collagen secretion? One option is disruption of intercellular calcium signaling inhibits collagen secretion. Endosulfan is an intracellular calcium signaling inhibitor as well an uncoupler of GJIC. Oleamide is a more specific uncoupler of GJIC and has no intercellular calcium signaling inhibiting activity [Boger et al., 1998]. Since oleamide is as effective as endosulfan at blocking collagen secretion, disruption of calcium signaling is unlikely. The level of GAPGH mRNA is not altered in endosulfan-treated dermal fibroblasts. Added uncouplers to cultured human dermal fibroblasts had minimal affect on the expression of Type I procollagen mRNA. It shows that uncoupler obstruction of Type I collagen synthesis is not by blocking Type I procollagen mRNA expression. Endosulfan does not appear to be an inhibitor of mRNA transcription. In contrast knocking out the expression of Cx-43 in transformed rat bone cells, blocks the expression of Type I procollagen mRNA [Stains et al., 2003]. Here, we demonstrate that blocking GJIC with uncouplers in human dermal fibroblasts does not inhibit the transcription of pro α1 (I) mRNA. One possible explanation for these differences is that Cx-43 expression is linked to the transcription of pro α1 (I) mRNA. On the other hand, blocking GJIC with an uncoupler does not alter procollagen mRNA expression. Another possibility is collagen synthesis in transformed osteoblasts is linked to GJIC, but collagen synthesis is independent of GJIC in human dermal fibroblasts. Another observation is blocking GJIC by added uncouplers is reversible in 4 h, which is consistent with no change in the expression of procollagen mRNA.

To optimize the secretion of collagen, intact microtubules are required. Is there a link between microtubules, gap junctions, and collagen secretion? A relationship between Cx and microtubules is reported, where the assembly of Cx-43 into connexons within the plasma membrane at the cell surface involves microtubules [Lauf et al., 2002]. Newly synthesized Cx-43 transport to the plasma membrane involves microtubules [Rassat et al., 1982; Guo et al., 2003]. The placement and pattern of connexons on the cell surface is influenced by microtubules [Meller, 1981; Zylberberg et al., 1988]. There is a link showing a relationship between microtubules and gap junction involvement with the orientation of collagen fibers in development of fish scales. At the base of fish scales the fibroblasts and collagen fibers run in an orthogonal pattern as flat sheets organized at 90° angles to one another. The disruption of microtubules and/or GJIC prevents that pattern of connective tissue orientation [Zylberberg et al., 1988]. What links collagen secretion to microtubules and GJIC? It is proposed that microtubules involvement in protein exocytosis is influenced by GJIC. One possibility is that the anchorage of microtubules at the plasma membrane requires GJIC [Lauf et al., 2002]. The disruption of GJIC alters that anchorage of microtubules at the cell surface, which disrupts the translocation of procollagen secretory granules along microtubules.

The initial observation in oleamide-treated rat PVA sponge implants of less connective tissue deposited between fibroblasts appears related to decreased synthesis and deposition of collagen. There may be some role for increased fibroblast proliferation also. The inclusion of GJIC uncouplers endosulfan or oleamide with cultured dermal fibroblasts, as well as, with chick embryo calvaria explants inhibits the secretion and synthesis of collagen. These findings generate questions about GJIC and fibrosis. Does that enhancement of GJIC increase the secretion of collagen? Do GJIC uncouplers offer an approach for controlling the secretion of collagen and connective tissue deposition? Excess collagen deposition is the problem with pathological fibrotic lesions, such as hypertrophic scar and keloid. Will the local applications of GJIC uncouplers prevent or reduce the severity of excessive scarring? The cell density of early granulation tissue is high, but as granulation tissue matures into scar the cell density declines. There is the possibility that the early exposure of wounds to uncouplers will prolong the proliferative phase of repair. On the other hand, promoting GJIC in young granulation tissue may advance the more rapid maturation of granulation tissue into scar.

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