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Expression of a Releasable Form of Annexin II by Human Keratinocytes

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Abstract Annexin II is a multifunctional calcium-dependent phospholipid binding protein whose presence in epidermis has previously been reported. However, like other members of annexin family, annexin II has been regarded as either an intracellular protein or associated with the cellular membrane. Here, we report the presence of a releasable annexin II and p11, two monomers of annexin II tetramer, in keratinocyte-conditioned medium (KCM). Proteins present in KCM were fractionated on a gel filtration column and following further evaluation, a releasable protein with apparent MW of 36 kDa was identified. Further characterization identified this protein as the p36 monomer of annexin II tetramer. The phospho-tyrosine antibody did not visualize this protein as the phosphorylated form of p36. Several experiments were conducted to examine whether this protein is soluble or associated with keratinocyte cell membranes in the conditioned medium. A centrifugation of conditioned medium was not able to bring this protein down into the pellet. Surprisingly, the results of Western analysis identified p36 and p11, two monomers of the annexin II tetramer, in conditioned medium derived from either keratinocytes cultured alone or keratinocytes co-cultured with fibroblasts. In contrast to the keratinocyte-conditioned medium in which annexin II was easily detectable, both monomers were barely detectable in conditioned medium collected from dermal fibroblasts. This finding was in contrast to the cell lysates in which p36 was detectable in both keratinocytes and fibroblasts. However, the amount of this protein was markedly higher in keratinocyte lysate relative to that of dermal fibroblasts. Conditioned medium derived from keratinocyte established from adult showed a higher level of annexin II compared to that of keratinocytes established from newborn babies. The expression of p11 seems to increase with differentiation of keratinocytes derived from either adult or newborn skin samples. When the site of annexin synthesis in human skin was examined by immunohistochemical staining, the antibody for p36 localized the annexin to the keratinocyte cell members in the basal and suprabasal keratinocytes. In conclusion, Western blot detection of both p36 and p11 in conditioned medium from skin cells revealed that human keratinocytes, but not fibroblasts, express a releasable monomer form of annexin II which is regulated by differentiation status of keratinocytes. This finding is consistent with the localization of annexin II detected by immunohistochemical staining. J. Cell. Biochem. 86: 737–747, 2002.

Key words: keratinocyte; fibroblasts; differentiation; skin; annexin II; p36; p11

Dermal–epidermal interaction has been the subject of several recent studies providing evidence that keratinocytes express many growth factors (GFs) and cytokines and their receptors. It has also been demonstrated that when a cultured keratinocyte sheet is used as a temporary wound coverage, it promotes wound healing and increases wound epithelialization [Bolivar-Flores et al., 1990]. It appears that lysates of cultured keratinocytes contain mitogenic activity for keratinocytes, endothelial cells, and fibroblasts [Duinslaeger et al., 1996; Somers et al., 1996]. Further studies demonstrated that epidermal cell-derived factors regulate wound healing through stimulation of migration and proliferation of keratinocytes from sweat glands, hair follicles, and wound edges. These factors also stimulate the contrac-

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tion of collagen matrices by controlling the numbers of fibroblasts or influencing their state [Eisinger et al., 1988; Silver and Eisinger, 1988]. Proliferation of dermal fibroblasts and matrix modulation in response to keratinocyte-conditioned medium (KCM) has revealed that KCM significantly increases fibroblast replication and decreases collagen synthesis. Keratinocyte CM also stimulates the expression of type IV collagenase (72 and 92 kDa) in human keratinocytes and fibroblasts (72 kDa). This finding suggests that an autocrine/paracrine control mechanism is involved in ECM modulation in these cell types during wound healing. It has also been suggested that stimulation of wound healing seen in wounds receiving sheets of keratinocytes is partly due to expression of collagenase [Kratz et al., 1995; Garner, 1998]. Although the presence of factor(s) released from, or associated with, keratinocytes with wound healing abilities has been appreciated in these studies [Eisinger et al., 1988; Silver and Eisinger, 1988], no specific factor(s) in either cell lysate or KCM has been identified as fully responsible for accelerated wound healing and epithelialization in treated animals. Eisinger et al. [1988] suggested that epidermal cells release a factor with a molecular weight of ~1.0 kDa that stimulates their own proliferation and that its biological effect is different from that of EGF. In a keratinocyte/fibroblast co-culture system, Goulet et al. [1996] reported a cellular cooperation between human keratinocytes and dermal fibroblasts resulted in an increase in DNA synthesis by keratinocytes through secretion of some soluble factors in the culture medium. These authors proposed that an unidentified 35–40 kDa protein released into conditioned medium might be responsible [Goulet et al., 1996]. These findings collectively suggest that keratinocyte-derived factors are likely to be a mixture of several unrecognized factors with overlapping biological activities to previously identified cytokines or GFs [Sauder et al., 1990]. The purpose of this study was, therefore, to examine the presence of keratinocyte releasable factors with molecular weight between 30 and 45 kDa in KCM using gel filtration column fractionation. Here, we observed a major releasable protein in this range, which was later identified to be annexin II.

Annexins are a family of calcium-dependent, phospholipid-binding proteins, which have been found in many multicellular organisms [Raynal and Pollard, 1994; Morgan and Fernandez, 1997]. They are characterized by the highly conserved primary and tertiary structures of their core-repeat region [Burger et al., 1996]. Annexin II has been identified and re-discovered under a variety of different names such as lipocortin II, calpactin I heavy chain, protein I, chromobindin 8, p34, p36, p39, and placental protein IV [Raynal and Pollard, 1994]. However, a vast number of in vitro experiments carried out for the last two decades revealed that they play major roles in various cellular processes in which their effects on membrane dynamics are particularly important. The physiological roles of annexins in the maintenance of calcium homeostasis, signal transduction, regulation of synthesis of lipid second messengers, secretion of neurotransmitters and hormones, receptor mediated endocytosis, cell proliferation and growth regulation have already been established [reviewed in Bandorowicz-Pikula et al., 2001].

In this study, we used a gel filtration column and Western blot analysis to detect a keratinocyte releasable protein with MW of 36 kDa in KCM. Further characterization identified this protein as being the p36 monomer of annexin II tetramer. As our previous report [Khorramizadeh et al., 1999] demonstrated a significant difference in expression of ECM mRNA between adult and newborn skin fibroblasts, in this study, we also examined the expression of p11 and p36 in both keratinocytes and fibroblasts established from either adult or newborn skin. Further, the localization of annexin II synthesis in human skin tissue was evaluated.

MATERIALS AND METHODS

Cell Cultures

To establish fibroblast cultures, either fetal foreskin or normal skin punch biopsies, obtained from adult patients undergoing elective reconstructive surgery, were collected individually and washed three times in sterile Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with an antibiotic–antimycotic preparation (100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (Gibco). Specimens were dissected free of fat and minced into small pieces of less than 0.5 mm in diameter, washed six times with DMEM, and distributed into 25 cm

flask, 3 pieces per flask. Four milliliters of DMEM, supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotic–antimycotic, was added to each dish and these were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air at 95% relative humidity. Medium was replaced weekly for 4 weeks, by which time fibroblasts covered more than 50% of the growth surface. At this time the fibroblasts were released from the dishes by brief (less than 5 min) incubation with 0.25% trypsin and subsequently seeded into 75-cm^2 culture flasks (Corning, NY) in DMEM-10% FBS and then further incubated. Upon reaching confluence, the cells were released by trypsinization, split for subculture at a ratio of 1:6, and reseeded into 75-cm^2 flasks [Ghahary et al., 1994]. Strains of dermal fibroblasts at passages 3–7 were used in this study. The procedure of Rheinwald and Green [1975] was used for cultivation of human foreskin keratinocytes using serum-free keratinocyte medium (KSFM), (Gibco) supplemented with bovine pituitary extract (50 μg/ml) and EGF (5 μg/ml). Primary cultured keratinocytes at passages 3–5 were used. Keratinocytes were then grown in either medium with additives or test medium consisting of 50% DMEM and 50% KSFM without any additives. Test medium contained a higher calcium concentration than KSFM alone [Ghahary et al., 2001] which induced differentiation of keratinocytes. In a coculture system, keratinocytes and fibroblasts were grown in the upper and lower chambers of a double chamber well, respectively. These chambers were separated by a 0.4 μm porous membrane. Conditioned medium or corresponding harvested cell lysates were collected and used to detect either p36 or p11 by Western blotting.

Fractionation of Protein in Keratinocyte-Conditioned Media

The media collected from KCM every 48 h over a 24-day period was subjected to a 65% ammonium sulfate precipitation followed by centrifugation at 10,000*g* for 15 min. The pellet was resuspended in a minimum volume of buffer A (10 mM sodium phosphate, pH 7.3, 150 mM NaCl, 4 mM 2-mercaptoethanol, protease inhibitor cocktail (Sigma)), and dialyzed overnight at 4°C in the same buffer. Approximately 200 μg of protein in 50 μl was loaded onto a Superdex-75 PC 3.2/30 gel filtration column attached to a SMART micropurification system (Amersham Pharmacia BioTech). Protein was eluted with buffer A and twenty-five 80-μl fractions were collected. The fractions were examined by electrophoresis on a 10% SDS–polyacrylamide gel and staining with Coomassie Blue.

Mass Spectrometry

Proteins in bands excised from SDS/polyacrylamide gels were subjected to trypsin digestion according to a published procedure [Wilm et al., 1996] and MS analysis [Dai et al., 1996]. Matrix assisted laser desorption mass spectrometry (MALDI MS) and matrix assisted laser desorption/ionization post source decay mass spectrometry (MALDI PSD MS) were performed on a Voyager Elite MALDI MS instrument (Voyager Elite, PerSeptive Biosystem, Inc., Framingham, MA) equipped with a delayed extraction (DE) device. A two-layer method was used for MALDI MS analysis in which 1–2 μl of first layer solution [10 mg/ml of 4-hydroxy-alpha-cyanocinnamic acid (HCCA) per milliliter of 20% methanol/acetone (v/v)] was deposited onto a probe tip, and evaporated to form a thin matrix layer, and then 0.5–1 μL of gel extract from 50% acetonitrile or 40% methanol saturated by HCCA was deposited onto the first layer, allowed to air dry, and washed three times with water. The PSD spectra were recorded in the PSD mode of the Voyager Elite instrument. Nanoelectrospray (NanoES) ion trap MS was performed on an Esquire-LC ion trap spectrometer (Hewlett-Packard, Reno, NV) with NanoES interface. Spectra were acquired over the mass range 200–2,200 Da.

Western Blot Analysis

For Western blot analysis, fractions obtained from a gel filtration column were run on a 10% SDS–PAGE and transferred onto a nitrocellulose membrane. For evaluation of p36 and p11 in conditioned medium, keratinocytes and fibroblasts were grown in either 6-well plates at 1×10^6 cells/well, or 24-well plates at 1×10^5 cells/well. Medium was collected 48 h later unless otherwise indicated. Two milliliters of the conditioned medium was concentrated in Centricon® YM-3, 3,000 kDa MWCO filter devices at 4,000*g*. All samples were adjusted to the same final volume with water. At each collection, one well was trypsinized and the cells were counted. A separate well was harvested in

100 μl with a cell scraper using 1 × SDS–PAGE sample buffer. A proportion of the conditioned medium or cell lysate that was equivalent to the cell number was loaded onto the corresponding lane of each sample on an SDS–PAGE gel. Following electrophoresis, the gel was transferred to nitrocellulose, then subjected to Western blotting. The binding of non-specific proteins on the membrane were blocked by incubating each blot in 5% skim milk powder in 0.1% Tween-20 in PBS solution at room temperature overnight. For immunodetection, the blots were incubated with either anti-annexin II or p11 monoclonal antibodies (initially obtained from ICN Biomedicals, Inc., Aurora, OH and subsequently as gifts of Dr. D. M. Waisman, Bio/Can Scientific, Calgary, Canada) at final concentration of 1:5,000 dilution. The blots were then washed and incubated with goat anti-mouse horseradish peroxidase-coupled secondary antibodies (Jackson Immunology Res) at final concentration of 1:10,000 dilution. The membranes were washed again and the immunoreactive proteins were detected by using Western blotting luminal reagents (Santa Cruz, Biotechnology, Inc.). Annexin II isolated from cow lung (Biodesign International, 60 Industrial Park Road, Saco, Maine) was run in parallel as a control.

Determination of Lactate Dehydrogenase

To address the question of whether annexin II found in KCM is a releasable form or released from dead (floater) cells, conditioned media from test and control cells were collected and subjected to lactate dehydrogenase (LDH) measurement. The procedure is the same as that described in the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). This assay, which is a colorimetric alternative to radioactive cytotoxicity assays, measures LDH, a stable cytosolic enzyme that is released upon cell lysis, in much the same way as [^{51}Cr] is released in radioactive assays. Thus, to address this question, keratinocytes were cultured in either keratinocyte serum free medium (KSFM) or test medium consisting of 50% KSFM plus 50% DMEM with 2% serum for a duration of either 48 and 96 h. Media were then collected, filtered and subjected to LDH measurement and Western blot analysis for p36 and p11 of annexin II detection. Both blank KSFM and test medium were run and evaluated for LDH and annexin II detection as negative controls. The corresponding cells were also stained with propidium iodide (PI) for cell viability prior to media collection.

Localization of Annexin II in Human Skin Sections

Immunoreactive annexin II protein in sections of normal human tissue was detected by immunoperoxidase staining. Briefly, punch biopsies were immediately fixed at room temperature in 4% paraformaldehyde and processed for paraffin embedding. Tissue sectioning was performed by conventional procedures. To block the endogenous peroxidase, sections of 5 μm thickness were deparaffinized, re-hydrated, and incubated in 10% hydrogen peroxide in methanol for 10 min. Non-specific binding was blocked by incubating the sections in 10% rabbit serum in BSA for 30 min. Mouse anti-human anti-annexin II monoclonal antibody (ICN Biomedicals, Inc., Aurora, OH) at a concentration of 1.0 mg/ml was diluted 1:2,000 in PBS and used as the primary antibody. Sections were incubated over night at 4°C. After three washes (5 min each) in PBS, slides were incubated with biotinylated rabbit anti-mouse immunoglobulin (DAKO Diagnostics Canada, Inc., Mississauga, Ontario, Canada) at a final concentration of 1:200 dilution in PBS for 40 min. The procedure of immunostaining was then performed according to the instructions provided by the ABC kit manufacturer. The immunoperoxidase staining was performed by incubating the tissue sections in ABC solutions consisting of streptavidin and biotinylated horseradish peroxidase (DAKO Diagnostics Canada, Inc.) for 1 h. The immunoreactivity was visualized by incubating the sections in DAB solution for 5 min. Slides were then counterstained with hematoxylin and dehydrated through serial dilutions of ethanol and xylene. Non-immuno mouse IgG at the same concentration was used as a negative control.

RESULTS

Detection of Releasable Annexin II in KCM

KCM was collected every 48 h over a 24-day period. Protein in the medium was then precipitated, redissolved, and fractionated, according to size, on a gel filtration column. Based on the observation of Goulet et al. [1996] of an unidentified ECM-modulating factor with an apparent molecular weight of 35–40 kDa, we focussed on proteins in the range of 30–45 kDa,

which, based on size markers, were expected to elute from the column in fractions 7–9. To confirm this and to examine the protein content of each fraction, an aliquot of each fraction was analyzed by SDS–PAGE. Figure 1 shows the proteins eluted in fractions 4–10. It is clear that fractions 7–9 contained proteins in the appropriate size range. The three most abundant proteins in fraction 8 were excised and digested in-gel with trypsin. The peptide extracts were analyzed by mass spectrometry using MALDI-TOF and NanoES ion trap instruments. We were able to identify the protein in the two bands marked with arrows as annexin II. In total 46 MS peaks matched with theoretical tryptic digestion data of annexin II. The identification was confirmed by sequencing one of the peptides by MALDI PSD MS, which yielded the sequence GVDEVTIVNILTNR. There was insufficient material to establish the identity of any protein in the third major band.

To further confirm this finding, a Western blot analysis was conducted and, as shown in Figure 1, panel B, the antibody raised against p36 identified a monomer of annexin II with apparent size of 36 kDa in fractions 7, 8, and 9. However, the intensity of annexin II monomer which seems to be a doublet band in fraction 8 was markedly higher relative to that of either fraction 7 or 9.

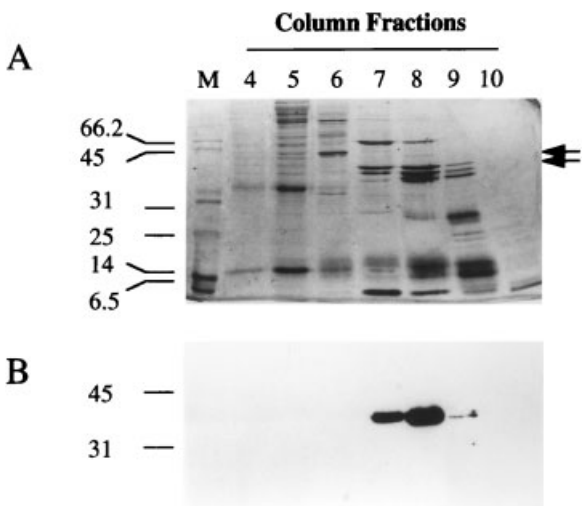

Fig. 1. Partial purification of annexin II in a gel filtration column. Ammonium sulfate precipitated proteins prepared from 1 L of keratinocyte conditioned medium (KCM) were fractionated on a gel filtration column and an aliquot of each fraction was visualized by polyacrylamide gel electrophoresis. Fractions 4–10 are shown in **panel A**. Three candidate bands (two arrows and the third lower band) in fractions 7–9 were separately excised and analyzed by mass spectrometry. The result of peptide mapping identified the two upper bands (two arrows on the right) with MW of 36 and 38 as monomers of annexin II. As shown in **panel B**, in a Western blot analysis, anti-annexin II antibody (p36) reacted with a protein with MW of 36 kDa in fraction 7, 8, and 9.

Annexin II is Not Phosphorylated

To determine whether the p36 has a phosphorylated form, two sets of samples of recombinant annexin II and KCM were run on a polyacrylamide gel in parallel. The first set was blotted with an anti-p36 antibody; while the other was blotted with anti-phospho-tyrosine antibody. The result, shown in Figure 2, indicates that neither of the two bands identified by p36 antibody is phosphorylated. However, an unknown protein with slightly smaller size was visualized by phospho-tyrosine antibody in KCM and the identity of this protein is under our current investigation.

Presence of Annexin II in Keratinocytes/ Fibroblasts Conditioned Medium

As dermal–epidermal interaction seems to be important in pathophysiology of skin, here, we examine whether the level of annexin II is influenced by keratinocyte–fibroblast interaction. To achieve this, the presence of annexin II was further explored in conditioned medium derived from cultured fibroblasts alone, keratinocytes alone, or fibroblast/keratinocyte coculture system. Antibody specific for p36 visua-

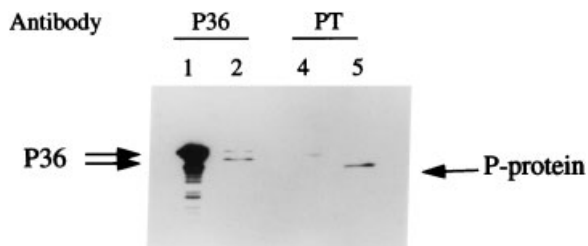

Fig. 2. Detection of annexin II in KCM. To determine the presence of annexin II and possibly its phosphorylated form in KCM, keratinocytes were grown in test medium consisting of 50% of KSFM and 50% DMEM containing 2% FBS for 5 days. Aliquots of conditioned medium were concentrated by microfiltration and subjected to Western blot analysis. To evaluate the presence of phosphorylated annexin II in KCM, two blots with the same samples were prepared. The blot corresponding to the samples of the recombinant human annexin II (**lane 1**) and KCM (**lane 2**) was reacted with human annexin II antibody; while the other blot with the same samples (**lanes 3** and **4**) was reacted with phospho-tyrosine antibody. Note, p36 antibody identified a doublet of bands related to annexin II, while the phospho-tyrosine antibody reacted with a protein which is smaller than either of the two protein bands seen by annexin II antibody.

lized two bands in conditioned medium-derived from either keratinocytes alone or those cultured with fibroblasts, collected for either 48 or 96 h (Fig. 3, panels A and B). However, a faint band with molecular size comparable to the upper band of that seen in keratinocytes was also detectable in 48 h conditioned medium obtained from fibroblasts cultured alone (panel A). Neither of these bands were detectable in 96 h conditioned medium collected from fibroblasts (Fig. 3, panel B). Interestingly, the intensity of p36 was markedly higher in conditioned medium collected from the keratinocyte/fibroblast co-cultured system.

Detection of Intra- and Extra-Cellular Annexin II and p11 Monomers

To date, annexin II has been regarded as either an intracellular protein or associated with the cellular membrane. We, therefore, examined the releasable form of annexin II in conditioned medium derived from either neonatal or adult keratinocytes and fibroblasts and compared it to the intracellular form (Fig. 4). The result of this experiment showed a high level of intracellular p36 in both neonatal and adult keratinocytes. Similarly, intracellular p36 protein was easily detectable by Western blotting in fibroblasts established from skin of a newborn baby or an adult. However, p11 was only detectable in cell lysate prepared from keratinocytes, but not in fibroblasts. On the other hand, the levels of releasable p36 were high only in conditioned medium derived from keratinocytes established from adult, but not from newborn babies. This time, the p36 antibody identified three bands related to annexin II monomer in conditioned medium from adult KCM. The presence of three bands might be due to degradation of releasable p36 in conditioned medium. In contrast to keratinocytes regardless of their origin, conditioned medium derived from fibroblasts showed little or no detectable p36 (Fig. 4, panel B).

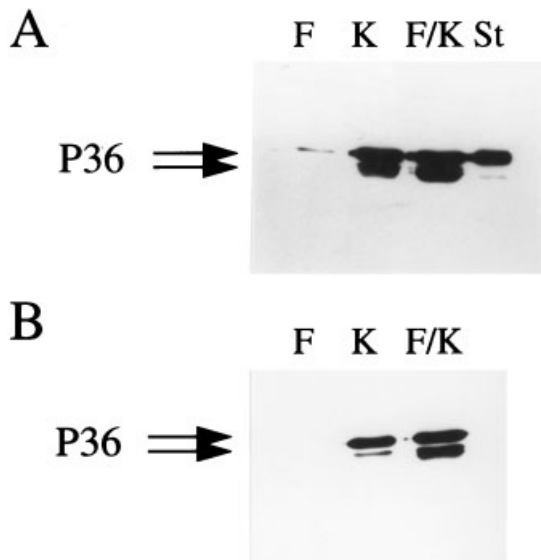

Fig. 3. Detection of human annexin II in fibroblast and KCM. To further detect and compare the levels of annexin II in keratinocyte and fibroblast conditioned medium, conditioned medium from either cultured fibroblasts alone (F), keratinocytes alone (K) or co-culture of fibroblast/keratinocyte (F/K) was collected and subjected to Western blot analysis. Conditioned medium from each experimental condition was either collected after 48 (**panel A**) or 96 h (**panel B**). Human annexin II antibody detects a doublet related to annexin II in keratinocytes cultured either alone or with fibroblasts. A faint band related to annexin II was also identified in fibroblast culture medium. This antibody also positively reacted with human recombinant annexin II (St) which was used as control.

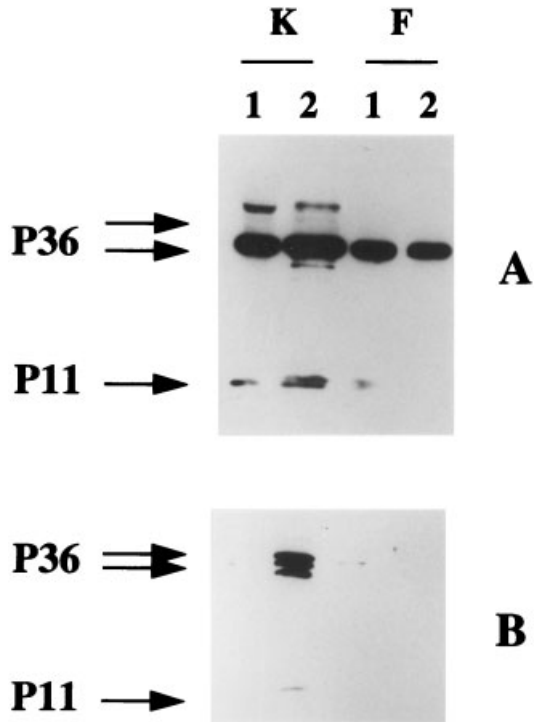

Fig. 4. Detection of annexin II and p11 in neonatal and adult fibroblast and keratinocyte cell lysates and conditioned medium. To demonstrate the releasable annexin II and p11 by keratinocytes and fibroblasts, either neonatal (**lane K1**) and adult (**lane K2**) keratinocytes or neonatal (**lane F1**) and adult (**lane F2**) fibroblast cell lysates (**panel A**) and corresponding conditioned medium (**panel B**) were collected and evaluated for the presence of annexin II and p11 by Western analysis. Note, the releasable annexin II and p11 was mainly detected in conditioned medium derived from adult keratinocytes (panel B).

Determination of Annexin II and LDH in KCM

To clarify the issue of whether the presence of annexin II in KCM is not due to lysis of dead (floater) cells, the same conditioned medium derived from keratinocytes cultured in either KSFM or test medium for a duration of either 48 or 96 h was used to determine the levels of LDH and the quantity of p36 and p11, two monomers of annexin II. The corresponding cells were also stained with PI viability dye. Both blank KSFM and test medium were run and evaluated for LDH and annexin II detection as negative controls. The results of these experiments revealed no detectable level of LDH above those detected in negative controls. In contrast, both p36 and p11 were detectable only in conditioned medium derived from cells grown in either KSFM and test medium for 48 and 96 h (Fig. 5). Interestingly, the intensity of p36 and p11 was higher in conditioned medium derived from 48 h compared to that of 96 h, a time at which one expects to observe more annexin II possibly due to more dead or lysed cells. This was further confirmed by microscopic evaluation of keratinocytes grown in test medium for 96 h. The result of PI staining confirmed that keratinoyctes in both KSFM and test medium at 96 h remained attached and there was no sign of dead cells (data not shown). These findings are consistent with the previous results indicating that the presence of soluble annexin II in KCM is not due to dead or dislodged cells.

Detection of Annexin II in Human Dermal Section

To examine whether keratinocytes in human skin also express annexin II protein, immunohistochemical staining was used to detect p36 in sections of human skin. The result showed a high expression of p36 mainly in suprabasal keratinocytes (Fig. 6). The pattern of staining indicates that p36 was mainly cell membrane associated. The expression of this protein was less pronounced in basal keratinocytes relative to that found in suprabasal keratinocytes. In contrast, the presence of cell associated p36 in fibroblasts embedded in dermis was not apparent. However, some staining was also found in fibroblast cytoplasm which was difficult to be distinguished from that found associated with ECM in dermis (Fig. 6).

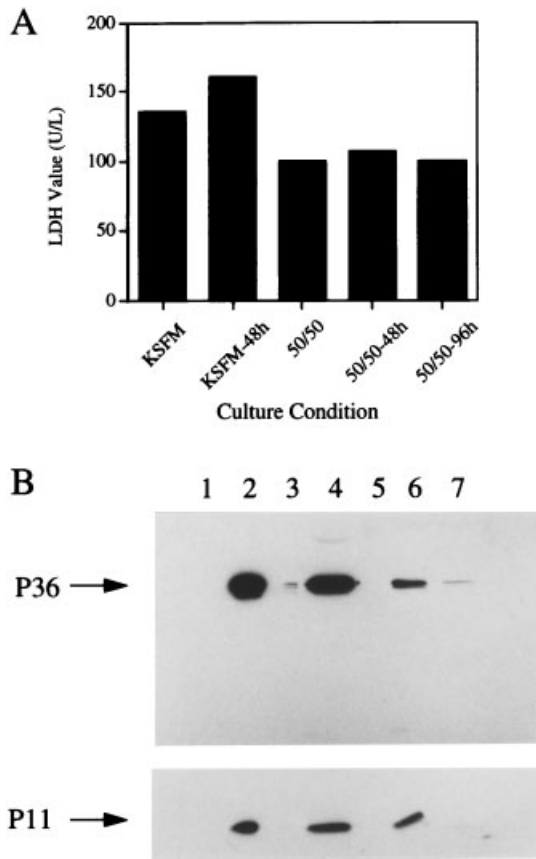

Fig. 5. Detection of lactate dehydrogenase and annexin II in KCM. To determine the levels of LDH and annexin II in the same KCM, cells were cultured in either keratinocyte serum free medium (KSFM) or test medium consist of 50% KSFM plus 50% DMEM with 2% serum for a duration of either 48 and 96 h. Media were then collected, filtered, and subjected to LDH measurement (**panel A**) and Western blot analysis for p36 and p11 of annexin II detection (**panel B, lanes 3–7**) using procedures described in the Materials and Methods. Both blank KSFM and test medium (50/50) were run and evaluated for LDH and annexin II detection as negative controls. **Panel B, lane 1 and 2** are markers and recombinant annexin II (positive control).

DISCUSSION

Annexins belong to a multigenic family of multifunctional proteins. They are calcium-dependent phospholipid binding proteins encoded by some 20 different genes [Siever and Erickson, 1997]. One of these annexins, annexin II, is a 36 kDa calcium-binding protein that has been localized to the apical plasma membrane and to the extracellular surface of many different cell types [Raynal and Pollard, 1994]. Annexin II was originally discovered as an intracellular calcium and phospholipid-binding protein and later findings showed its potential role in the regulation of membrane trafficking such as endocytosis and exocytosis

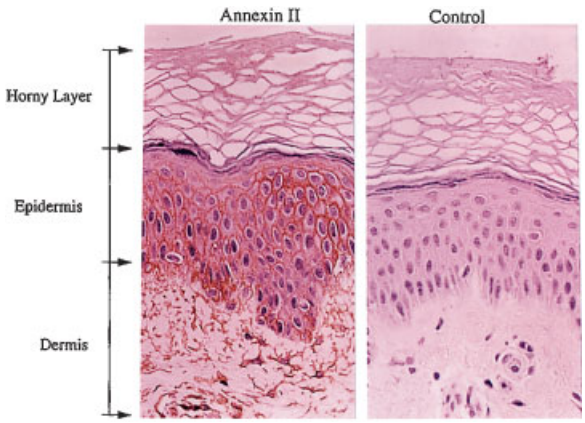

Fig. 6. Immunohistochemical staining of annexin II in human tissue section. Paraffin embedded normal human skin biopsies were sectioned in 6 μm thickness and stained with human annexin II antibody using the procedure of immunohistochemical staining described in the Materials and Methods. Note, annexin II staining is shown as a granular pattern mainly associated with keratinocyte cell membrane (**left panel**), while this staining is missing in negative control (**right panel**).

and t-PA-mediated plasminogen activation [Waisman, 1995]. Annexin II is present in cells as both a monomer and a heterotetramer, which are called annexin II (AII) and annexin II tetramer (AIIt), respectively. Annexin II tetramer consists of two annexin II molecules and two molecules of an 11-kDa regulatory subunit called p11 light chain whose regulatory role in annexin II activities is important [Drust and Creutz, 1988]. Several lines of study showed the presence of different annexins in human skin and isolated keratinocytes. Ma and Ozers [1996] investigated the topography and possible functions of annexin I, II, IV, and VI in skin sections in cultured epidermal keratinocytes by immunostaining. These investigators found the presence of annexin I and II staining in a granular pattern in the monolayer epithelial cells. However, in tissue sections, annexin I was detected as an envelop pattern in the stratified keratinocytes, while, annexin II was identified in all nucleated epidermal cells. The pattern of staining suggests that these annexins may interact with membrane cytoskeleton in other biological functions. Annexins IV and VI were predominantly found in dermal cells but not in cultured keratinocytes [Ma and Ozers, 1996]. In another study, Munz et al. [1997] used RT-PCR and immunohistochemical staining and showed the expression of p36 and p11 mRNA and proteins in cultured keratinocytes and sections of human normal and injured skin, respectively. The expression of annexin II was shown in cytoplasmic membrane of human keratinocytes in the basal layer without significant staining in cellular nuclei. The presence of annexin II in both the cytoplasmic membrane and the nucleus of the keratinocytes in basal and spinous layers of human cholesteatoma was also demonstrated [Kim et al., 1998].

Although, the presence of annexin I and II has been demonstrated in keratinocytes, none of these studies demonstrated a releasable form of annexin II or p11 from human cultured keratinocytes. The results of this study consistently demonstrated the presence of a keratinocyte-releasable form of annexin II and p11 monomers of the annexin II tetramer in conditioned medium. The presence of annexin II and p11 is unlikely to be cell membrane associated as the KCM was always centrifuged prior to its use for different experimental procedures such as ammonium sulfate precipitation and protein fractionation. The size of annexin II on gel filtration column was 36 kDa and that supports the idea that annexin II may in fact be released from keratinocyte in its monomeric form. This finding becomes particularly important when annexin II and P 11 were consistently found in conditioned medium derived from cultured keratinocytes but not fibroblasts. As both keratinocyte serum free medium and 50/50% test medium utilized in this study lack EGTA, which can be used to strip the annexin II out from cell surface, the presence of annexin II in KCM can not be due to EGTA induced dissociation of annexin II and p11 from cell membrane. This was further confirmed by the fact that conditioned medium loaded onto our gel filtration columns was collected from keratinocytes grown in 50/50% test medium, which contains relatively high concentration of calcium. As calcium-dependent binding to phospholipid membranes is a hallmark of most annexins [Siever and Erickson, 1997], the presence of annexin II in KCM is not due to low calcium induced dissociation of annexin II from cell membrane. Lack of p36 and p11 in conditioned medium from newborn keratinocytes and adult fibroblasts further confirm that releasable annexin II is not a common phenomenon for all cell strains, rather it seems to be cell strain specific.

Finally, we used a non-radioactive LDH cytotoxicity assay to determine whether the releasable form of annexin found in KCM is due to cell lysis. This quantitative assay determines a

stable cytosolic LDH that is released upon cell lysis, in much the same way as [^{51}Cr] is released in radioactive assays. The amount of color formed is proportional to the number of lysed cells [Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988]. The finding of these experiments demonstrated no detectable level of LDH above those detected in negative controls. A background in LDH levels seen in both blank and test medium is more likely to be due to phenol red of the culture medium. Thus, the lack of LDH in KCM which has also been used to evaluate the level of annexin II suggests that the presence of p36 and p11 in this medium is not due to leakage of cytosolic proteins. This can be further confirmed by the fact that the intensity of p36 and p11 was higher in conditioned medium derived from 48 h compared to that of 96 h.

Annexins lack known signal peptides and can not pass through the classic endoplasmic reticulum secretory pathway [Siever and Erickson, 1997]. As for other proteins that lack signal peptide, there must be another mechanism by which annexin II and p11 are released into KCM. There is now supporting evidence to indicate that there are several well known releasable proteins such as interleukin-1 [Andree et al., 1992; Corradi et al., 1995], fibroblast growth factor-2 [Albuquerque et al., 1998], and endothelial cell growth factor [Jaye et al., 1986] which lack signal peptides. Thus, this is not unique to annexin II and as suggested by these investigators, the release of annexin II may occur by an externalization mechanism through which the protein of interest is released into conditioned medium by shedding and peeling off with and without phospholipids from the membrane. We speculate that when high annexin II expressing cells, such as keratinocytes, synthesize an excessive amount of annexin II and p11, which exceeds the number of molecules needed to saturate the membrane associated phospholipids, the unbound annexin molecules may be released into conditioned medium. This speculation is based on the fact that only 4% of total annexins produced by cells are cell membrane bound [Siever and Erickson, 1997]. If true, the amount of annexin II found in conditioned medium derived from any cell type should be less than 4% of total cellular annexin II. Our results clearly showed that this is not the case for keratinocytes. This is because the amount of annexin II found in adult KCM was markedly greater than 4% of total intracellular annexin II (data not shown).

Keratinocyte differentiation induced by 50/50% test medium [Ghahary et al., 2001] appears to upregulate the expression of intracellular p11 in comparison to that obtained from keratinocytes grown in serum free medium in which cells are in proliferating status. Further, the expression of p11, but not p36, is markedly increased in conditioned medium derived from adult keratinocytes relative to that obtained from cells derived from newborn infant. This may indicate that cell aging is another regulatory element in expression of p11, but not p36.

In all Western blot experiments conducted, the annexin II antibody identified only a single band related to intracellular annexin II. However, in the same experimental condition, frequently two and sometimes three bands related to annexin II were found in conditioned medium derived from keratinocytes. The multiple annexin II reactive bands ranging from 31 to 37 kDa have been previously reported. The additional bands may be generated by proteolytic cleavage of annexin II molecules at two different sites located near the NH_2 terminus [Chung and Erickson, 1994; Matsuda et al., 1999].

As shown in this study, under similar experimental conditions, dermal primary fibroblasts synthesized considerably less annexin II and p11 than those seen in keratinocytes. In fact, little or no detectable level of p11 monomer was observed in fibroblast cell lysates. If formation of annexin II tetramer is required for the release of this protein, the low expression of p11 might be another reason why only keratinocytes but not fibroblasts synthesize and release a relatively large amount of annexin II and p11 into conditioned medium. The releasable form of annexin II is unlikely to be phosphorylated as the band identified by the phospho-tyrosine antibody was even smaller than the shorter degradative product of annexin II. The size of tyrosine phosphorylated form of intracellular annexin II induced by PDGF reported to be 39 kDa [Brambilla et al., 1991] which did not match the band found in our study.

Although, annexin II is known as a multifunctional protein, the mechanism of its function remains largely unknown. For example, tenascin is an extracellular matrix protein whose binding to cell membrane associated annexin II is well established. It is also known

that binding of tenascin-C to annexin II receptors induces a loss of local adhesion, increases cell migration, and enhances cell division. However, it is not clear how extracellular annexin II signals cross the cellular membrane and lead to such cellular responses [reviewed in Siever and Erickson, 1997]. Plasminogen and plasminogen activator are two plasma proteins involved in a fibrinolytic system which specifically bind to annexin II and through which plasminogen is activated to plasmin. It is, therefore, possible that both proteins bind simultaneously to a single annexin molecule that activates catalysis of plasminogen to plasmin. Further, membrane bound annexin II is reported to be important in metastatic lymphoma cells and cell–cell adherence of non-metastatic cells. From this study, it is not clear whether a releasable form of annexin II plays any similar roles as annexin II associated with cellular membrane. For example, it would be particularly interesting to know whether the releasable form of annexin II binds to a soluble form of tenascin or activates plasminogen to plasmin. It is also important to explore why only adult keratinocytes, but not newborn keratinocytes or even adult fibroblasts, release annexin II. Finally, it is critical to understand the mechanism through which annexin II is released in KCM. Thus, this new insight on the presence of a releasable form of annexin II in KCM merits further studies through which some of these questions could be addressed in the future.

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