Abstract

Annexin I plays an important role in the process of keratinization as a component of the cornified envelope. To elucidate the function of annexin I in keratinization, we investigated the effects of calcium, epinephrine, hydrocortisone, and 12-O-tetradecanoyl phorbol 13-acetate (TPA) on the expression and localization of annexin I in cultured human keratinocytes. Normal human keratinocytes were cultured in serum-free culture medium (0.15 mM calcium) until 70% confluence. After incubation with a higher concentration of calcium (1.8 mM), TPA (100 nM), epinephrine (50 µM), or hydrocortisone (10 µM) for 24 h, the expression of annexin I protein and mRNA was examined using immunofluorescence, Western blot, and Northern blot techniques. Immunofluorescence microscopy showed increased membrane staining of annexin I by calcium, which was inhibited by the addition of epinephrine or hydrocortisone. Western blotting confirmed elevated annexin I on the cell membrane. It was increased in the cell membrane fraction, but not in the cytosol fraction. Interestingly, the mRNA level of annexin I was slightly reduced after incubation with calcium, whereas TPA upregulated both membrane expression and the mRNA level. Secretion of annexin I was increased by TPA but inhibited by calcium. Because calcium and TPA are known to promote keratinization, our data suggest that annexin I expression on the cell membrane is involved in the process of keratinization.

Key words Keratinization · Cornified envelope · 12-O-Tetradecanoyl phorbol 13-acetate (TPA) · Epinephrine

Introduction

Annexin I was initially described as a corticosteroid-inducible protein (Flower et al. 1984). Annexin I has recently been recognized as a component of the cornified envelope (Robinson et al. 1997) and it has been suggested that it plays an important role in keratinization. Studies examining the expression of annexin I have shown that the annexin I located in the cell membrane plays an important role in keratinocyte differentiation (Kitajima et al. 1991; Ma and Ozers 1996; Serres et al. 1994). Recently, we have demonstrated strong expression of annexin I mRNA in vivo in keratinizing foci such as the borders of psoriatic lesions and horn pearls of well-differentiated squamous cell carcinoma (Sato-Matsumura et al. 1996). To elucidate the function of annexin I in keratinization, we examined the effects of calcium, epinephrine, hydrocortisone, and 12-O-tetradecanoyl phorbol 13-acetate (TPA) on the expression and localization of annexin I in cultured human keratinocytes.

Materials and methods

Cell culture

Normal human neonatal foreskin keratinocytes and serum-free medium and supplements for cell culture were purchased from Clonetics Co. (San Diego, Calif.). The keratinocytes were cultured in serum-free medium following the method of Boyce and Ham (1983). Cells were cultured in keratinocyte basal medium (Clonetics) supplemented with 5 ng/ml human recombinant epidermal growth factor, 5 µg/ml insulin, 50 µg/ml gentamicin, 50 ng/ml amphotericin-B, and 0.5% bovine pituitary extract, in an atmosphere of 95% air and 5% CO₂ at 37°C. The Ca²⁺ concentration of the medium was 0.15 mM. Cells in the third passage cultured up to 70% confluence were used for the experiments. For Western and Northern blotting, cells were rinsed with RNase-free ice-cold phosphate-buffered saline three times following incubation for 24 h in the medium sup-
plemented with calcium (1.8 mM), epinephrine (50 µM), hydro-
cortisone (10 µM), or TPA (100 nM). In some experiments, the
cells were stored at −70 °C until use. To detect annexin I released
into the medium, 2 ml of culture medium was concentrated with
Centricon-30 (Amicon, Beverly, Mass.) to a final volume of 100 µl
and stored at −70 °C. For immunohistochemistry, cells were cultured
in LabTek Chamber Slides (Nalge Nunc International, Naperville,
Ill.) and fixed with ice-cold acetone following the 24 h of culture
described above.

Immunohistochemistry

Specimens were incubated with a mouse monoclonal antibody
against annexin I (MSP-04; Nippon Shin-yaku Company, Kyoto,
Japan) for 30 min at room temperature followed by three washes
with phosphate-buffered saline for 5 min. The specimens were then
incubated with goat anti-mouse IgG conjugated with fluorescein
isothiocyanate (FITC) (Jackson Immuno Research Laboratories,
West Grove, Pa.) or goat anti-mouse IgG conjugated with tetra-
methyl rhodamine isothiocyanate (TRITC) (EY Laboratories, San
Mateo, Calif.) for 30 min at room temperature followed by three
washes with phosphate-buffered saline for 5 min. Some specimens
were double-stained with FITC-labeled phalloidin (Sigma, St.
Louis, Mo.) and examined with a confocal laser scanning micro-
scope (Laser Scanning Confocal Imaging System MRC1024; Bio-Rad,
Richmond, Calif.).

Western blot analysis

First, 1 × 10⁶ keratinocytes were homogenized and divided into
membrane and cytosol fractions as described previously (Kotzumi
et al. 1997). The pellet of the membrane fraction was solubilized in
a conical glass homogenizer with 20 mM Tris HCl, pH 7.5, con-
taining 0.25% Triton-X100, 2 mM EDTA, 2 mM EGTA, and 2 mM
phenylmethylsulfonylfluoride (PMSF). The samples of the cytosol
fraction, membrane fraction, and concentrated culture supernatant
(Serres et al. 1994) were separated by 12.5% SDS-PAGE accord-
ing to the method described by Laemmli (1970). Each lane contained
50 µg protein, which was evaluated by the method of Bradford
(1976). Coomassie staining of one of the duplicated SDS-PAGE
gels confirmed that the same amount of protein was applied to
each lane. Proteins were transferred onto polyvinyldene difluoride
membranes (Bio-Rad, Richmond, Calif.), blocked with 0.1% bovine
serum albumin/Tris-buffered saline (TBS; 10 mM Tris buffer,
pH 7.4, 140 mM NaCl) containing 0.1% Tween 20 (TBS-T), incu-
bated with the mouse monoclonal antibody against annexin I (Z013;
Zymed Laboratories, San Francisco, Calif.) followed by the addi-
tion of the anti-mouse antibody conjugated with horseradish per-
oxidase (Bio-Rad, Richmond, Calif.). Chemiluminescence was vi-
sualized using an ECL system (Amersham, Little Chalfont, UK)
by exposing immunoblots to Kodak X-Omat film (Eastman Kod-
dak, Rochester, N.Y.) for up to 10 min.

Northern blot analysis

Northern blotting was performed as described previously (Church
and Gilbert 1984). Human annexin I cDNA was kindly supplied by
Dr. Hiroshi Teraoka of the Shionogi Research Laboratory. A Hind
III-Xba I 548-bp fragment of human glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) cDNA (GenBank J04038) was subcloned
into plasmid pBR322 and purified using a Qiagen plasmid kit
(Qiagen, Santa Clarita, Calif.). The sequence of the inserted frag-
ment of GAPDH was verified using an ABI prism 310 genetic an-
alyzer (PE Biosystems, Foster City, Calif.). mRNA was prepared
from 1 × 10⁶ keratinocytes using a Micro-Fast Track mRNA Isola-
tion Kit (Invitrogen, Carlsbad, Calif.) following the recommenda-
tions of the manufacturer. Extracted mRNA (1 µg) was loaded into
each lane. DNA probes for annexin I were hybridized after radio-
labeling by random priming using [γ-32P]dCTP (NE Life Science
Products, Wilmington, Del.). Washings of the highest stringency
were done at 65 °C in 0.1 × standard saline citrate and 0.1%
sodium dodecyl sulfate for 20 min. For autoradiography, Kodak
BioMax film was exposed at −70°C for up to 3 days with an in-
tensifying screen (Amersham Pharmacia Biotech, Uppsala, Swe-
den).

Results

Immunohistochemistry

In proliferating monolayer keratinocytes, annexin I was
detected diffusely in the cytoplasm. In contrast, stratified
keratinocytes expressed annexin I intensively on the mem-
brane (Fig. 1). Membrane staining was also increased by
elevating the calcium concentration, and inhibited by the
addition of epinephrine or hydrocortisone (Fig. 2). TPA also
increased membrane staining of annexin I, which was
slightly augmented by the addition of calcium.

Western blotting

Annexin I was increased in the membrane fraction after in-
cubation with calcium or TPA, and was decreased after in-
cubation with epinephrine (Fig. 3a). Hydrocortisone also
reduced annexin I in the membrane fraction, although the
effect was less obvious than that of epinephrine. Annexin I
in the cytosol fraction was almost unchanged under these
conditions (Fig. 3b). To detect annexin I released from ke-
rinocytes, culture supernatants were also analyzed. An-
nexin I was undetectable in the supernatant after incubation
with calcium, whereas TPA markedly increased annexin I
released from keratinocytes (Fig. 3c). Epinephrine and hy-
drocortisone had almost no effect.