Effects of Biscoclaurine Alkaloid Cepharanthine on Newborn Rat Skin Epidermal and Dermal Cells

Kazuto OHKURA,*^a Kenji URAKAMI,^a Shio MAKINO,^a and Hiroshi TERADA^b

Department of Applied Biological Sciences, School of Agricultural Sciences, Nagoya University,^a Furo-cho, Chikusa-ku, Nagoya 464-01, Japan and Faculty of Pharmaceutical Sciences, University of Tokushima,^b Shomachi-1, Tokushima 770, Japan. Received August 30, 1995; accepted October 26, 1995

Effects of biscoclaurine alkaloid cepharanthine on proliferation of skin cells were examined *in vitro*. The alkaloid was found to enhance attachment and cell layer formation of newborn rat skin epidermal cells in culture on type I collagen-coated Millipore filter, and to potentiate production of keratins in these cells. Spreading and growth of skin dermal fibroblasts were inhibited by cepharanthine in a dose-dependent manner, but the alkaloid-treated cells still retained the ability to synthesize type IV collagen. In culture of skin cells containing both epidermal and dermal cells, no growth of epidermal cells or over-growth of dermal fibroblasts was observed in medium without cepharanthine. In contrast, the presence of the alkaloid suppressed the growth of fibroblasts and promoted the formation of epidermal cell layers accompanied by production of keratins. Thus, in comparison with the cells untreated with the alkaloid, cepharanthine-treated skin epidermal and dermal cells *in vitro* expressed *in vivo*-like characters.

Key words skin; epidermal cell; dermal cell; cepharanthine

Cellular metabolic changes induced by hormones, antibodies and lectins have been indicated to be accompanied by modifications of cell surface membranes.^{1–4)} Cepharanthine, an alkaloid from *Stephania cepharantha*,⁵⁾ is known as an agent that stabilizes membranes by affecting the fluidity of the lipid bilayer in the membrane and that causes metabolic changes in several types of cells.^{6–8)} For instance, it exerts a potent inhibitory action on snake venom-induced hemolysis.^{9,10)} The alkaloid also enhances the activity of anthracycline type anti-cancer agents in leukemia and lymphoma cell lines.^{11–15)} In skin, it has been suggested that cepharanthine shows therapeutic effects on a burn.¹⁶⁾

The objective of this study was to describe the possible mode of action of cepharanthine on skin cells, by studying the effects of the agent on cultivation and protein synthesis of newborn rat skin epidermal and dermal cells. Skin is constructed from the epidermal layer, the dermal layer and the basement membrane which intervenes both layers.¹⁷⁾ Each layer is essentially formed by epidermal cells and dermal fibroblasts, respectively.¹⁷⁾ Epidermis founded on the basement membrane is composed of several types of epidermal cells which are classified in their level of differentiation and produce differentiation-specific keratins.^{18–21)} We prepared epidermal cells from the basal layer of newborn rat epidermis and cultured them on a type I collagen-coated Millipore filter, an artificial-basement membrane. We also cultured dermal fibroblasts, which support epidermal cells in intact skin, on a plastic plate. Both the cepharanthine-treated epidermal and the dermal cells showed behaviors resembling intact cells in the *in vivo* state when compared to those of the untreated cells. The effects of the alkaloid on the proliferation of epidermal cells containing both epidermal and dermal cells are described.

MATERIALS AND METHODS

Reagents The reagents used were as follows: Eagle's

minimum essential medium (MEM), Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; fetal bovine serum (FBS), Whittaker M.A. Bioproducts, Walkersville, MD, U.S.A.; TPCCK-trypsin, Sigma, St. Louis, MO, U.S.A.; dispase, Godo-Shusei Co., Tokyo, Japan; type I collagen (bovine skin dermis), Koken, Tokyo, Japan; Millicell-CM (12-mm diameter), Millipore Products, Bedford, MA, U.S.A.; anti-type IV collagen serum (rabbit), Collaborative Biomedical Products, Bedford, MA, U.S.A.; horse-radish peroxidase conjugated anti-rabbit IgG (goat), Organon Tekunika, West Chester, PA, U.S.A.; cepharanthine, Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Preparation and Culture of Epidermal Cells Skin epidermal cells were prepared from Wistar rats of 3 d old as previously described.²²⁾ Epidermal cells were suspended with several amounts of cepharanthine in Eagle's MEM containing 10% FBS, 23.8 mM sodium bicarbonate, 20 mM Hepes (MEM-FBS). The suspension (0.5 ml) was seeded on a type I collagen-coated Millipore filter at an initial density of 5.0×10^6 cells/ml,²³⁾ and the cells were incubated at 37 °C in a CO₂ incubator.

Preparation and Culture of Dermal Fibroblasts Whole skin was removed from newborn rat and cut into pieces (5 × 5 mm), which were placed on a 100-mm culture dish. After the pieces adhered to the dish (about 5 min), they were incubated in 10 ml of MEM-FBS at 37 °C in a CO₂ incubator. Dermal fibroblasts spread from the pieces during 7 d in culture. The pieces of skin were then removed from the dish, and the fibroblasts were recovered by trypsin (0.25%)–EDTA (0.02%) digestion at 37 °C for 1 min. The fibroblasts were cultured on new culture dishes for several days until use.

Preparation and Culture of Skin Cells Epidermal sheet was stripped from newborn rat skin as previously described.²²⁾ The residual skin sheet was digested by collagenase (0.05%) in 10 mM phosphate buffer (pH 7.4) containing 5 mM CaCl₂ at 37 °C for 2 h. Then the skin cells were recovered by centrifugation at 1200 rpm for 1 min in Ca²⁺- and Mg²⁺-free phosphate buffered saline

* To whom correspondence should be addressed.

(PBS). The skin cells were suspended in MEM-FBS and were cultured on a 24-well multiplate at 37 °C in a CO₂ incubator.

Measurements of Adhesion and Viability of Epidermal Cells After culture for an appropriate period, the culture medium was discarded with the nonadherent cells, 0.5 ml of PBS was added and adherent cells were gently dissociated from the collagen layer by a rubber policeman. The number of adherent cells was counted with a hemocytometer, and their viability was examined by trypan blue exclusion test.

Immunoblotting Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5% gel as described by Laemmli.²⁴⁾ Suspensions of epidermal or dermal cells in an appropriate volume of PBS without or with 8 M urea were sonicated in a Branson Sonifier Cell Disrupter (model 185) at 0 °C for 10 s. The sonicates (10 μ l) of epidermal (5.0×10^5) or dermal cells (4.0×10^3) were mixed with an equal volume of 0.5 M Tris-HCl (pH 6.8) containing 1% SDS, 650 mM 2-mercaptoethanol and 25% glycerol, boiled for 1 min, and then loaded on the gel. After electrophoresis,

Fig. 1. Effects of Cepharanthine on Attachment (A) and Viability (B) of Epidermal Cells

Epidermal cells (2.5×10^6) were placed on a type I collagen-coated filter and incubated at 37 °C in a CO₂ incubator in MEM-FBS without (○) or with 0.1 μ M (●), 1 μ M (△) and 5 μ M (▲) cepharanthine. After culture for the indicated period, the cells attached to the filter were recovered, their number counted and their viability measured. Results are means ($\pm$ S.E.) of three determinations.

Fig. 2. Micrographs of Epidermal Cells in Culture

Epidermal cells (2.5×10^6) were cultured in medium without (A and B) and with 5 μ M cepharanthine (C and D), as described in the legend of Fig. 1, and then micrographed. Bar, 10 μ m. A and C, cultured for 2 d; B and D, cultured for 6 d. Arrowheads show detached areas of epidermal cell layer (A) and areas of naked collagen-coated filter (B).

Fig. 3. Effect of Cepharanthine on Production of Keratins from Epidermal Cells

Epidermal cells were cultured for 2 d in medium without or with cepharanthine, and the proteins were extracted from 5×10^4 of the cells. The extracts were electrophoresed and immunoblotted with antiserum raised against keratins from newborn rat skin, as described in Materials and Methods. Concentrations of cepharanthine in the culture medium are: lane 1, $5 \mu\text{M}$; lane 2, $1 \mu\text{M}$; lane 3, $0.1 \mu\text{M}$; lane 4, none. Arrowheads indicate the positions of major bands detected.

Fig. 4. Effects of Cepharanthine on Growth (A) and Viability (B) of Dermal Fibroblasts

Fibroblasts were cultured on a 24-well multiplate in MEM-FBS without or with cepharanthine. After culture for the indicated period, the cell number was counted and viability was examined. Concentrations of cepharanthine in the culture medium are: ○, none; ●, $0.1 \mu\text{M}$; △, $1 \mu\text{M}$; ▲, $5 \mu\text{M}$. Results are means ($\pm$ S.E.) of three determinations.

Fig. 5. Micrographs of Dermal Fibroblasts Cultured in the Medium without or with Cepharanthine

Fibroblasts (1.7×10^4) were cultured for 4 d on a 24-well plate in MEM-FBS without (A) or with $0.1 \mu\text{M}$ (B), $1 \mu\text{M}$ (C), $5 \mu\text{M}$ (D) cepharanthine, and then micrographed. Bar, $10 \mu\text{m}$.

Western-blotting was performed as described by Towbin *et al.*²⁵⁾ Marker proteins used were: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa).

Morphological Observation and Immuno-Fluorescent Staining of Cultured Cells Following an appropriate period of culture, cells were observed by a microscope (Olympus IMT-2). Keratins produced from epidermal cells were detected by an Olympus fluorescent system (model IMT2-RFC). Antiserum against keratin from newborn rat skin was raised in rabbit as previously described.²³⁾

RESULTS

Effects of Cepharanthine on Adhesion and Keratin Accumulation of Epidermal Cells The density of epidermal cells placed on a type I collagen-coated Millipore filter, 2.5×10^6 cells/well, corresponded to >95% confluence. After cultured for the indicated period, the effects of cepharanthine on cell attachment to the collagen layer (Fig. 1A) and viability of the attached cells (Fig. 1B) were determined. In the absence of cepharanthine, half of the inoculated cells became detached from the collagen layer after 2 d. Cepharanthine enhanced both cell attachment and viability of the attached cells in a dose-dependent manner. There was a nearly parallel relationship between the cell number attached to the collagen layer and the cell viability.

The effect of cepharanthine on the adhesion of epidermal cells was further examined microscopically (Fig. 2). In the absence of cepharanthine, detached areas in the cultured epidermal cell layer were seen after 2 d of culture, and after 6 d a naked-collagen layer was visible. Such a notable detachment of the cells was not detected when culture was done in medium containing $5 \mu\text{M}$ of cepharanthine.

Skin epidermal cells *in vivo* synthesize a variety of types of keratins, which are characteristic of cell differentiation, and the ability to produce the proteins is one of the indexes to judge activity of the cells. We examined whether cepharanthine affects the keratin synthesis of the cells or not, using the cells cultured for 2 d. As shown in Fig. 3, the amounts of keratin accumulated in epidermal cells markedly increased with the concentration of cepharanthine added to the culture medium, and three major bands of 55 kDa, 60 kDa and 68 kDa were detected as previously reported.²³⁾

Effects of Cepharanthine on Growth and Type IV Collagen Synthesis of Dermal Fibroblasts Dermal fibroblasts (1.7×10^4 cells) seeded on a plastic plate grew in MEM-FBS without cepharanthine and cell number reached 1.4×10^5 after 6 d in culture (Fig. 4A). Cepharanthine suppressed the cell growth in a dose-dependent manner, and no growth occurred in the presence of $5 \mu\text{M}$ cepharanthine. However, the alkaloid did not affect cell viability (Fig. 4B). Figure 5 shows micrographs of fibroblasts cultured for 4 d on a 24-well plate in the absence or presence of cepharanthine. It is apparent that spreading of the cells is impaired by the alkaloid, while well-spread fibroblasts are observed in the absence of cepharanthine. These observations are not incompatible with the results

Fig. 6. Effect of Cepharanthine on Production of Type IV Collagen from Dermal Fibroblasts

Fibroblasts (1.7×10^4) were cultured for 6 d in MEM-FBS without or with cepharanthine, as described in the legend of Fig. 4A. A: Proteins extracted from 4×10^3 of fibroblasts were electrophoresed and immunoblotted with anti-type IV collagen antiserum. B: Proteins from the culture supernatant ($0.7 \mu\text{g}$) were also analyzed. Concentrations of cepharanthine in the culture medium are: lane 1, $5 \mu\text{M}$; lane 2, $1 \mu\text{M}$; lane 3, $0.1 \mu\text{M}$; lane 4, none. Arrowheads indicate the positions of major bands detected. C: Control experiment; the same samples as used in Fig. 6A (lane 1-4) and 6B (lane 5-8) were loaded on a gel and immunoblotted without anti-type IV collagen serum (concentrations of cepharanthine are: lane 1 and 5, $5 \mu\text{M}$; lane 2 and 6, $1 \mu\text{M}$; lane 3 and 7, $0.1 \mu\text{M}$; lane 4 and 8, none).

of Fig. 4A.

One function of dermal fibroblasts *in vivo* is to synthesize type IV collagen, a major constituent of the basement membrane.¹⁷⁾ As seen in Fig. 4A, dermal fibroblasts grew in MEM-FBS without cepharanthine, but the cells produced only detectable amounts of type IV collagen, as indicated by faint 170 and 180 kDa bands recognized with anti-type IV collagen antiserum (Fig. 6A). Although the addition of cepharanthine to the medium markedly inhibited growth of the cells, it rather potentiated the production of type IV collagen. It was further indicated that the produced type IV collagen is secreted from the cells (Fig. 6B). Figure 6C shows the control experiment

Fig. 7. Effect of Cepharanthine on Proliferation of Epidermal Cells

Skin cells (2.5×10^6) were cultured for 2 d on a 24-well plate in MEM-FBS without (C and D) or with $5 \mu\text{M}$ cepharanthine (A and B), then micrographed. Micrographs A and C are phase-contrast of the cells, and micrographs B and D show distribution of keratins visualized by the immunofluorescence method. Bar, $10 \mu\text{m}$.

of Fig. 6A (lane 1—4) and 6B (lane 5—8), and no bands were detected.

Effects of Cepharanthine on Differentiation of Epidermal Cells Skin cells (2.5×10^6 cells) containing both epidermal and dermal cells were cultured in MEM-FBS on a plastic plate. After 2 d of culture in medium without cepharanthine, only dermal fibroblasts grew (Fig. 7C) and merely background levels of keratins were detected as shown by immunofluorescence staining (Fig. 7D). On the contrary, in the presence of cepharanthine epidermal cells attached tightly to the culture plate and the cell layers developed during 2 d of culture (Fig. 7A), keratins were produced over the whole surface of the cell layer (Fig. 7B), while dermal fibroblasts were hardly detected at all.

DISCUSSION

Cepharanthine has been indicated to cause a variety of metabolic changes in cells,^{3,4,13)} and to be effective for skin damage such as burns.¹⁶⁾ In the present study, we examined the effects of cepharanthine on proliferation of skin epidermal and dermal cells *in vitro*. It has been reported that an artificial basement membrane mimicking the *in vivo* membrane is required for culture of skin epidermal cells.¹⁷⁾ Consistent with the fact that type IV collagen is a major component of the basement membrane,¹⁷⁾ we have previously shown that type IV collagen layer provides a better basement layer for attachment of epidermal cells than type I collagen layer.²²⁾ In the present experiment examining the effects of cepharanthine on attachment and viability of epidermal

cells, however, type I collagen layer was rather pertinent for the purpose. The alkaloid enhanced attachment of epidermal cells to type I collagen layer as well as cell viability, while it suppressed the spread and growth of dermal fibroblasts on a plastic plate without affecting the viability; the cepharanthine-treated cells, however, did not lose the ability to produce type IV collagen. Skin fibroblasts *in vivo* are known to be in a quiescent state and to produce some types of collagen.^{17,26)} In these respects, it appears that dermal cells treated with cepharanthine behave like intact cells in skin.

The effects of cepharanthine on skin cells used in this study has thus been shown to be a synergism of the actions on epidermal cells and dermal fibroblasts observed in culture of each type of cell. Skin epidermal cells, in general, require feeder fibroblasts to grow and differentiate in culture on a plastic plate.²⁷⁾ Mouse fibroblast 3T3 cells, which were γ ray-irradiated and lost growth potential, have validity as feeder cells to grow epidermal cells in culture.²⁷⁾ As shown in Fig. 7, proliferation of epidermal cells in skin cells treated with cepharanthine was markedly stimulated by the coexistence of dermal fibroblasts. In the culture system in which fibroblasts did not grow, they probably act as feeder cells for epidermal cells. In addition, production of type IV collagen from dermal fibroblasts, which was enhanced in the presence of cepharanthine, might promote attachment and growth of epidermal cells in such a culture system. Thus, the action of cepharanthine on skin cells as revealed in the *in vitro* system may account for the therapeutic effect on damaged skin cells *in vivo*. It is not obvious, however, whether epidermal cells grown

in this culture system are subject to differentiation or not; studies are in progress to answer this question.

The underlying mechanism of the actions of cepharanthine on developments of epidermal and dermal cells is not clear. As in the cases of other cationic amphipathic drugs, it seems that cepharanthine modifies membrane functions in cells through interaction with polar lipids, especially with phosphatidylserine.²⁸⁻³¹⁾ Although more detailed studies are needed, it is likely that such interactions may perturb the response of skin cells to some of the growth factors, affecting cell proliferation.

REFERENCES

- 1) Han T., *Clin. Exp. Immunol.*, **13**, 165—170 (1973).
- 2) Inbar M., Shinitzky M., *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2128—2133 (1974).
- 3) Novogrodsky A., *Nature* (London), **250**, 788—790 (1974).
- 4) Ruishauser U., Sachs L., *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2456—2461 (1974).
- 5) Tomita M., Fujitani K., Aoyagi Y., *Tetrahedron Lett.*, **13**, 1201—1206 (1967).
- 6) Nagatsuka S., Nakazawa T., *Biochim. Biophys. Acta*, **691**, 171—177 (1982).
- 7) Shiraishi N., Arima T., Aono K., Inoue B., Morimoto Y., Utsumi K., *Physiol. Chem. Phys.*, **12**, 299—305 (1980).
- 8) Utsumi K., Miyahara M., Sugiyama K., *Acta Histochem. Cytochem.*, **9**, 59—68 (1976).
- 9) Utsumi K., Miyahara M., Inoue M., Mori M., Sugiyama K., Yamashita S., *Cell Struct. Funct.*, **1**, 133—136 (1978).
- 10) Miyahara M., Aono K., Quesada J. S., Shimano K., Baba Y., Yamashita S., *Cell Struct. Funct.*, **3**, 61—65 (1978).
- 11) Shiraishi N., Akiyama S., Nakagawa M., Kobayashi M., Kuwano M., *Cancer Res.*, **47**, 2413—2416 (1987).
- 12) Nagaoka S., Kawasaki S., Karino Y., Sakai K., Nakanishi, T., *Eur. J. Cancer Clin. Oncol.*, **23**, 1297—1302 (1987).
- 13) Wheeler C., Rader R., Kessel D., *Biochem. Pharmacol.*, **31**, 2691—2693 (1982).
- 14) Sigfried J. A., Kennedy K. A., Sartorelli A. C., Tritton T. R., *J. Biol. Chem.*, **258**, 339—343 (1983).
- 15) Rintoul D. A., Center M. C., *Cancer Res.*, **44**, 4978—4980 (1984).
- 16) Tanimura J., Sadanobu K., Sato K., *Acta Urol. Jpn.*, **11**, 1316—1323 (1965).
- 17) Fusenig N. E., "Biology of the Integument," Vol. 2, ed. by Hahn J. B., Natolsky A. G., Richards K.S., Springer-Verlag, New York, 1986, pp. 409—442.
- 18) Timpl R., Paulsson M., Dziadek M., Fujiwara S., *Methods Enzymol.*, **145**, 363—391 (1987).
- 19) Dlugosz A. A., Yuspa S.H., *J. Cell Biol.*, **120**, 217—225 (1993).
- 20) Schweizer J., Winter H., *J. Biol. Chem.*, **258**, 13268—13272 (1983).
- 21) Eichner R., Sun T. T., Aebi U., *J. Cell Biol.*, **102**, 1767—1777 (1986).
- 22) Ohkura K., Fujii T., Konishi R., Terada H., *Cell Struct. Funct.*, **15**, 143—150 (1990).
- 23) Ohkura K., Terada H., *Biochim. Biophys. Acta*, **1133**, 153—159 (1992).
- 24) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
- 25) Towbin H., Staehelin T., Gordon J., *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350—4354 (1979).
- 26) Lapiere C. M., Nusgens B. V. "Pharmacology of the Skin," ed. by Greaves M. W., Shuster S., Springer-Verlag, New York, 1989, pp. 69—88.
- 27) Rheinwald J.G., Green H., *Cell*, **6**, 331—344 (1975).
- 28) Lullmann H., Lullman-Rauch R., Wassermann O., *Biochem. Pharmacol.*, **27**, 1103—1108 (1978).
- 29) Lullmann H., Wehling M., *Biochem. Pharmacol.*, **28**, 3409—3451 (1979).
- 30) Sheetz M. P., Singer S. J., *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4457—4461 (1974).
- 31) Sato T., Kanaho Y., Fujii T., *Cell Struct. Funct.*, **5**, 155—163 (1980).