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EXTRACELLULAR CALCIUM ALTERS THE EFFECTS OF RETINOIC ACID ON DNA SYNTHESIS IN CULTURED MURINE KERATINOCYTES

Philip Tong, Douglas Mayes, and Larry Wheeler

Discovery Research, Allergan Eye and Skin Care Group, Herbert Laboratories
Irvine, California 92715

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The rate of proliferation of epidermal keratinocytes was manipulated by growing the cells in medium containing high or low concentrations of calcium. Keratinocytes cultured in high extracellular Ca^{++} (1.4 mM and 2.8 mM) proliferated twice as fast as those grown in low Ca^{++} medium (0.09 mM) as measured by incorporation of [^{3}H] thymidine into DNA. Exposure of high calcium keratinocytes to all-trans retinoic acid for 4 days caused a dose-related inhibition of DNA synthesis with an IC_{50} of about 10 μM. In contrast, incubating low calcium keratinocytes with all-trans retinoic acid caused a dose-related stimulation of DNA synthesis with maximum increase of 278% over control at 10 μM. This increase was accompanied by increases in culture confluency with maximum increase of 109% in cell number over control at 10 μM. These results are of importance since they suggest Ca^{++} may influence the effect of retinoids on keratinocytes.

The mode of action of retinoids on cellular proliferation remains unclear from the paradoxical responses exhibited in different biological systems. On the one hand, retinoids have been demonstrated to inhibit hyperproliferative disorders, epithelial cancers, carcinogenesis (1, 2), and the proliferation of non-transformed and transformed cell lines (3, 4). On the other hand, they have been shown to cause hyperproliferation in *in vivo* (5, 6) as well as in *in vitro* systems (3, 4, 5). Since the ability to modulate cellular proliferation may form the basis by which retinoids control hyperproliferation and neoplasm, a knowledge of how these compounds exert the dual effects is important toward understanding their mechanism of action.

In vivo data suggest that normal and hyperproliferative keratinocytes respond differently to retinoids (1, 2, 5, 6). Since extracellular Ca^{++} has been reported to dramatically affect proliferation and differentiation in murine keratinocytes we examined the effects of all-trans retinoic acid

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(RA) in cultures maintained in low Ca^{++} and high Ca^{++} media. In contrast to previously published studies (Hennings et al., 7), keratinocytes maintained in low Ca^{++} had a lower rate of DNA synthesis than high Ca^{++} keratinocytes. In addition, RA had paradoxical effects on DNA synthesis - inhibiting in high Ca^{++} but stimulating in low Ca^{++} medium.

MATERIALS AND METHODS

Epidermal keratinocyte cultures

The procedure previously described for the isolation of basal keratinocytes from the skins of neonatal Balb/c mice (8) was followed with some modifications. The trypsin solution and Ficoll gradient were prepared in Ca^{++}-free medium 199 (Gibco) without and with 10% (v/v) chelexed fetal bovine serum (FBS) respectively. FBS was treated with chelex 100 as described by (9). Ca^{++}-free medium 199 plus 10% chelexed FBS had 0.02 mM Ca^{++} as determined by atomic absorption spectroscopy (Beckman Product Service Lab, CA). The concentrations of viable basal keratinocytes were determined by fluorescein diacetate (FDA, Sigma) staining and counting in a hemacytometer. The isolated basal keratinocytes were resuspended in Ca^{++}-free M199 containing 10% chelexed FBS and 1% Fungibact (Irvine Scientific Co., Ca.) which were made to final Ca^{++} concentrations of 0.09 mM (LC medium), 1.4 mM (HC medium) or 2.8 mM by adding $CaCl_2$ from a 0.3 M $CaCl_2$ solution. 2 x 10^6 viable cells were plated per 35-mm plastic culture dish in either LC or HC medium. The cultures were incubated in 5% CO_2 in air gassing at 34°C, and the medium was changed every 2 days. Cells plated in LC and HC medium had plating efficiencies of 39% and 53%, respectively. LC cultures grew as monolayers and took more than 2 weeks to reach confluency. HC cultures formed multilayers and became confluent within 5-7 days.

Addition of all-trans retinoic acid (RA)

RA (Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and was used to supplement the media to the desired RA concentrations. DMSO, 0.2% (v/v), which did not affect keratinocyte DNA synthesis, was added to parallel cultures as vehicle control. All handling of RA was done in dimmed light. Quadruplicate LC and HC cultures or 2.8 mM Ca^{++} cultures were incubated in media containing various concentrations of retinoids on day 4 after plating, and with each medium change for a total of 4 days.

Measurement of DNA synthesis

DMSO- and RA-treated keratinocyte cultures were pulse-labeled for 6 hours with 1 μCi [^{3}H] thymidine. DNA was extracted and assayed for quantity and radioactivity as described by Marcelo and Madison (10). The data are presented as percent of the DMSO vehicle control (retinoid-treated culture cpm/μg DNA divided by 0.2% DMSO control cpm/μg DNA x 100) ± S.E. in the retinoid studies.

Viable cell counts

Octuplicate LC cultures treated with 0.2% DMSO or various concentrations of RA were trypsinized in 0.05% trypsin/0.02% EDTA. The number of viable cells was determined by FDA staining.

RESULTS

The rate of proliferation of mouse epidermal keratinocytes cultured in medium containing 0.09 mM Ca^{++} (LC) and 1.4 mM Ca^{++} (HC) medium was

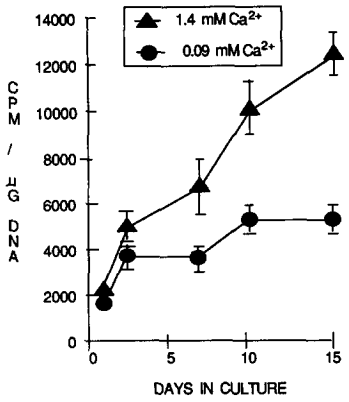

Figure 1. DNA synthesis in epidermal keratinocytes cultured in LC and HC medium.

Mouse epidermal keratinocytes were prepared in LC medium (0.09 mM Ca^{++}) or HC medium (1.4 mM Ca^{++}). At 1, 4, 7, 10 and 14 days after plating, quadruplicate LC and HC cultures were pulse-labeled with [^{3}H] thymidine. The DNA was extracted and assayed for quantity and radioactivity (10). The data are CPM/µg DNA ± SEM.

studied by determining the incorporation of [^{3}H] thymidine into DNA after pulse-labeling LC and HC cultures at the time indicated in Figure 1.

Both LC and HC cells had a low rate of DNA synthesis after 1 day in culture. DNA synthesis in LC cells increased by 2-3 fold and that in HC cells by 4-6 fold after 9 more days of incubation, at which time the HC cultures incorporated 73% more [^{3}H] thymidine per microgram DNA than the LC cultures. DNA synthesis in HC cultures further increased in the HC cultures but remained at the day 10 level in the LC cultures on 14 days of incubation.

These results show that DNA synthesis in epidermal keratinocytes is inducible by extracellular calcium and that the induction is time dependent.

The effects of all-trans retinoic acid (RA) on the proliferation of LC and HC cultures were studied by incubating 4-day-old cultures in media containing various concentrations of the retinoid. The cultures were pulse-labeled with [^{3}H] thymidine on the fourth day of treatment and the incorporation of [^{3}H] thymidine into DNA is presented as percent of incorporation found in the vehicle (DMSO)- treated cultures in Figure 2.

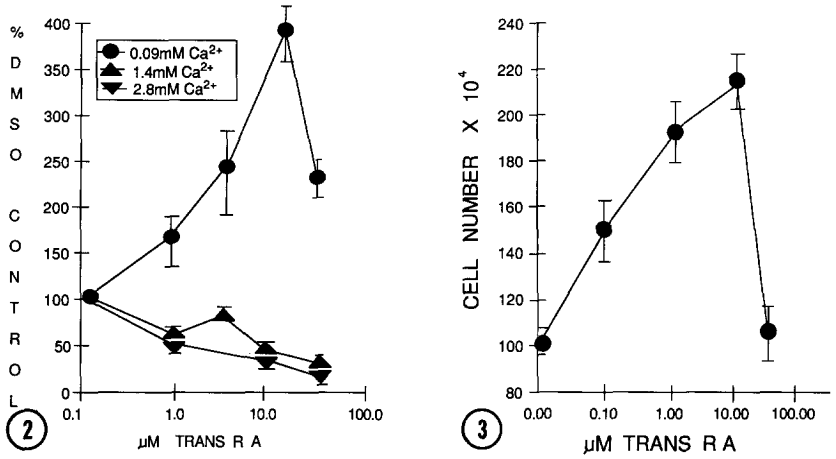

Figure 2. Effects of all-trans retinoid acid (RA) on DNA synthesis in epidermal keratinocytes cultured in LC and HC medium.

Four-day-old LC or HC keratinocyte cultures were incubated in medium containing various concentrations of RA, or the vehicle solvent DMSO. After 4 more days of incubation, the cultures were pulse-labeled with [^{3}H] thymidine and the DNA was extracted and assayed for quantity and radioactivity. The data from 2 separate experiments were presented: (1) effect of RA on LC (-●-)and 1.4 mM Ca^{++} cultures (-▲-), and (2) effect of RA on 2.8 mM Ca^{++} cultures (-▼-). The data are presented as percent of vehicle (DMSO) control ± SEM.

Figure 3. Effect of all-trans retinoic acid (RA) on cell production in keratinocytes cultured in LC medium.

The culture conditions and treatment with RA was similar to those described in Figure 2. After 4 days of incubation in DMSO (0.0 μM) or in various RA concentrations, the number of viable cells from 8 cultures per treatment was determined by the fluorescein diacetate staining technique. Data represent viable cell number ± SEM per 35-mm culture.

RA at concentrations below 0.1 μM had no appreciable effects on LC or HC keratinocyte proliferation (data not shown), but stimulated LC keratinocyte DNA synthesis in a dose-related manner between 1 μM and 10 μM. A 278% ± 20 ($p<0.005$ t-test) stimulation was found in LC cultures treated with 10μM RA. At 33 μM RA, however, the stimulation declined to 125% ± 35. At this and higher RA concentrations, some LC keratinocytes began to round up and detach from the culture dish, indicating cytotoxicity of RA.

Epidermal keratinocytes cultured in medium containing 1.4 mM or 2.8 mM Ca^{++}, on the other hand, were both inhibited by RA with an approximate IC_{50} of 10 μM. These results demonstrate the opposite effects of retinoids on the proliferation of LC and HC keratinocytes.

To see if the RA stimulation of DNA synthesis actually reflects stimulation of cell replication, a similar experiment was conducted in LC cultures where the viable cell numbers in control and RA-treated cultures were determined (Figure 3). LC cultures produced 47% ± 13, 87% ± 13 and 109% ± 10 more cells over the control cultures when treated with 0.1 μM, 1 μM and 10 μM RA, respectively. The number of cells in the 33 μM RA-treated cultures was similar to those of the control cultures. These cultures again began to show signs of toxicity. Autoradiographic studies also showed many more [^{3}H] thymidine-labeled cells in the RA-treated than the DMSO-treated LC cultures (data not shown). These results demonstrate that RA is mitogenic to LC keratinocytes, and that [^{3}H] thymidine incorporation into DNA is indicative of keratinocyte proliferation.

DISCUSSION

Our results indicate that epidermal keratinocytes cultured in HC medium proliferate faster than those grown in LC medium. This finding is in agreement with the observation that non- neoplastic cells require extracellular calcium for proliferation (11) and that high calcium concentrations cause cellular hyperproliferation (12).

Our observations, however, are at variance with those reported by Hennings, et al. (7), who found that mouse epidermal keratinocytes cultured in medium containing 0.05 to 0.1 mM calcium synthesized 2 to 4 times the highest amounts of DNA found in keratinocytes grown in medium containing 1.2 mM to 3.0 mM calcium. We do not know what causes these contradictory observations in two similar culture systems, although differences in culture techniques, serum composition, and initial culture confluency could affect the proliferation and differentiation status of the keratinocytes so that subsequent responses to extracellular calcium are altered.

Our findings that retinoids stimulate DNA synthesis in LC keratinocytes, but inhibit that process in HC keratinocytes, reflects the response of normal and hyperproliferative epidermis to retinoids, in harmony with the results of other investigators (5, 6). These similarities further support

that the LC keratinocytes have a slower rate of proliferation than the HC keratinocytes.

These dual effects of retinoids are important to the question of how these compounds control epidermal hyperproliferation and how they restore normal structure and function in disease epidermis. The LC and HC keratinocyte cultures should be useful for studying the mechanism underlying these dual effects.

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