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all-trans-Retinoic acid preserves viability of fibroblasts and keratinocytes in full-thickness human skin and fibroblasts in isolated dermis in organ culture

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Abstract. Human dermal fibroblast and human epidermal keratinocyte survival was examined under various conditions in organ culture. Using cell recovery from organ-cultured tissue as the criterion, it was observed that no keratinocytes and few fibroblasts survived incubation for 10–12 days in serum-free basal medium containing a low level (0.15 m*M*) of extracellular Ca^{2+}. Increasing the extracellular Ca^{2+} concentration to 1.4 m*M* or treating the tissue with 3 μ*M* retinoic acid (RA) under low Ca^{2+} conditions resulted in increased keratinocyte and fibroblast survival; the two treatments together were more effective than either treatment alone. The same treatments preserved fibroblast survival when pieces of isolated dermal tissue were incubated in organ culture and also supported fibroblast survival in monolayer culture. These findings indicate that recovery of keratinocytes and fibroblasts from skin after maintenance in organ culture provides a simple but definitive measure of the viability of the major cellular elements present in the tissue. These findings suggest that RA treatment enhances survival of both fibroblasts and keratinocytes and that these effects of RA can be seen at physiological Ca^{2+} concentrations as well as at suboptimal levels of extracellular Ca^{2+}. Finally, these results indicate that the dermis is a direct target of RA.

Key words: Skin organ culture – Retinoic acid – Cell viability

Introduction

Topical application of *all-trans*-retinoic acid (RA) alters the histological appearance of skin, and this is associated with changes in both the epidermis and dermis [15–17, 36]. A major feature of the epidermis is an overall thickening, due in part to increased keratinocyte proliferation and in part to increased synthesis of glycosaminoglycans [17, 36]. In the dermis there is evidence of fibroblast activation and new matrix synthesis [12, 15, 16]. We have recently demonstrated similar findings using an organ culture model [33, 34]. Both the topical treatment data (in vivo studies) and our own data in the organ culture model suggest that RA influences the behavior of each of the two major cellular elements of skin. However, this is difficult to "prove" on the basis of histology alone. The present study was carried out, therefore, to demonstrate that RA does indeed preserve the viability of both epidermal keratinocytes and dermal fibroblasts in organ-cultured human skin.

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Materials and methods

Retinoic acid

RA was obtained from the R.W. Johnson Pharmaceutical Research Institute (Raritan, N.J.) and prepared as a 20 mg/ml solution in dimethyl sulfoxide (DMSO). The stock solution was stored at –20° C in the dark until use. At the time of use, it was diluted directly in Keratinocyte Basal Medium (KBM) (Clonetics, San Diego, Calif.) and added to the cultures. In the cultures treated with 3 μ*M* RA (highest concentration tested), the final concentration of DMSO in the culture medium was 0.005%. This amount of DMSO had no detectable effect by itself.

Organ cultures

Organ cultures were established from healthy adult volunteers. Briefly, 2 × 2 mm full-thickness punch biopsies were obtained from the hips (normally eight biopsies per person) and immediately placed into the wells of a 24-well dish containing 0.5 ml KBM. KBM is a serum-free basal medium consisting of modified MCDB-153 medium. The Ca^{2+} concentration of the KBM was 0.15 m*M*. We routinely included duplicate biopsies per well. One group was left without further treatment while RA (3 μ*M*) was added to the KBM in other wells. In additional wells, calcium

chloride was added to bring the final Ca^{2+} concentration to 1.4 m*M*, and 3 μ*M* RA was included in some of these wells. The organ cultures were then incubated at 37° C in an atmosphere containing 5% CO_2 in the dark with fresh culture medium and treatments provided at 2-day intervals.

Additional biopsies were obtained in the normal manner and then immediately placed in a solution of 0.25% trypsin/0.05% EDTA. After 1 h of incubation, the dermis and epidermis were gently separated using forceps. The trypsin was neutralized by incubation for an additional 15 min in a solution of soybean trypsin inhibitor (100 μg/ml) in KBM. After two washes in KBM, the isolated dermal tissue pieces were incubated in KBM, treated with Ca^{2+} and/or RA and incubated for up to 12 days as described above. No attempt was made to incubate the isolated epidermal tissue pieces since histological studies indicated that they underwent rapid and complete necrosis when kept in organ culture after separation from the dermis.

Isolation of keratinocytes and fibroblasts from organ-cultured tissue

At the end of the incubation period, tissue specimens that had been incubated in either KBM alone or in KBM supplemented with extracellular Ca^{2+} and/or RA were harvested, minced using scissors and forceps and plated on tissue culture plastic in Minimal Essential Medium of Eagle with Earle's salts, non-essential amino acids and 10% fetal bovine serum (MEM-FBS). Since the total amount of tissue available from each biopsy was so small, the duplicate pieces from each well were chopped, minced and incubated together. Cultures were provided fresh medium as needed and examined daily for fibroblast and keratinocyte outgrowth. The same MEM-FBS was used for cell outgrowth from all tissues so as not to bias the chances of recovering cells from different tissues. Further, this medium has proven satisfactory in the past for isolation of both fibroblasts and keratinocytes [19, 31]. After 20 days, all tissue specimens were discarded if no keratinocytes or fibroblasts were recovered. If keratinocytes and/or fibroblasts were recovered. They were harvested with trypsin and subcultured. By first passage, fibroblasts made up virtually 100% of the remaining cells. These cells were then used to assess growth in monolayer culture. The failure to maintain keratinocytes beyond primary culture probably reflected the fact that these cells undergo differentiation in serum-containing medium and that differentiated cells fail to reattach to the substratum after exposure to proteases [19, 29].

Fibroblast growth in monolayer cluture.

Fibroblasts were harvested from culture at passage 1–3 and plated at 5×10^4 cells per well in 24-well culture dishes. MEM-FBS was used as culture medium. After the cells had had a chance to attach, duplicate wells were harvested and counted to obtain zero-time counts. The remaining cells were washed twice in KBM and treated for 2 days in KBM alone (0.15 m*M* Ca^{2+}) or in KBM supplemented with extracellular Ca^{2+} (1.4 m*M*, final concentration) and/or RA (3 μ*M*). At the end of the incubation period, cells were harvested and counted. An electronic particle counter was used to assess cell numbers after first verifying that the cells were in single-cell suspension.

Results

Recovery of fibroblasts from whole-skin organ culture and from isolated dermis

In the first series of experiments, fibroblast viability was assessed after incubation in organ culture. The results of these experiments are shown in Table 1. Fibroblasts were recovered from only 5 of 21 specimens (24%) after incubation of the tissue for 12 days in (low-Ca^{2+}) KBM alone. This compares with 8 of 23 (35%) from the group incubated in KBM supplemented with 1.4 m*M* Ca^{2+}, 8 of 20 (40%) from the group incubated in KBM supplemented with 3 μ*M* RA and 13 of 17 (76%) from the group incubated in KBM supplemented with both 1.4 m*M* Ca^{2+} and 3 μ*M* RA. By comparison, fibroblasts were recovered from 6 of 9 control tissue pieces of the same size (67%) established immediately after biopsy.

In a second experiment, isolated dermal tissue was obtained from eight additional volunteers and incubated for 12 days in KBM alone or in KBM supplemented with 1.4 m*M* Ca^{2+} and/or 3 μ*M* RA. Following incubation in organ culture, fibroblast recovery was assessed as with intact tissue. It can be seen that, just as with intact tissue, fibroblast recovery was higher in the presence of RA than

Table 1. Fibroblast isolation after incubation in organ culture

	Total number of specimens	Number of specimens from which fibroblasts recovered	%
Zero-Time control[a]	9	6	67
10–12 day organ culture			
KBM	21	5	24
KBM + Ca^{2+}	23	8	35
KBM + RA	20	8	40
KBM + Ca^{2+} + RA	17	13	76*+
10–12 day dermal organ culture			
KBM	8	2	25
KBM + Ca^{2+}	7	3	43
KBM + RA	8	7	88#
KBM + Ca^{2+} + RA	7	7	100*+

[a] Fibroblasts isolated immediately after biopsy (i.e. no time in organ culture)
* $P < 0.05$, Ca^{2+} + RA vs KBM alone; + $P < 0.05$, Ca^{2+} + RA vs Ca^{2+} alone; # $P < 0.05$, RA vs KBM alone; significance of differences determined by the Scheffe method [37]

Table 2. Keratinocyte isolation after incubation in organ culture

	Total number of specimens	Number of specimens from which keratinocytes recovered	%
Zero-Time control[a]	9	6	78
10–12 day organ culture			
KBM	13	0	0
KBM + Ca^{2+}	13	8	62!
KBM + RA	12	4	33#
KBM + Ca^{2+} + RA	11	10	91*+

[a] Keratinocytes isolated immediately after biopsy (i.e. no time in organ culture)
! $P < 0.05$, Ca^{2+} vs KBM alone, # $P < 0.05$, RA vs KBM alone; * $P < 0.05$, Ca^{2+} + RA vs KBM alone; + $P < 0.05$, Ca^{2+} + RA vs Ca^{2+} alone; significance of differences determined by the Scheffe method [37]

in its absence (Table 1). The percentage of RA-treated cultures from which fibroblasts were recovered was higher when isolated dermis was used in place of intact tissue (7 of 7 and 7 of 8 vs 8 of 20 and 13 of 17).

Recovery of keratinocytes from organ-cultured tissue

Table 2 presents the results of efforts to isolate keratinocytes from the same organ-cultured tissue pieces. In the absence of organ culture supplementation with either Ca^{2+} or RA, there was no keratinocyte recovery (zero isolates from a total of 13 different tissue). In contrast, supplementation of the organ-cultured tissue with either Ca^{2+} or RA improved keratinocyte recovery rates (Ca^{2+} being the more effective of the two) and supplementation with both Ca^{2+} and RA resulted in the highest recovery (10 of 11; 91%). By comparison, keratinocytes were recovered from 7 of 9 control specimens (78%) established immediately after biopsy.

Survival of fibroblasts in monolayer culture after isolation from organ-cultured tissue

Fibroblasts recovered from organ-cultured skin (28 separate isolates) were examined for their ability to survive for 2 days in monolayer culture under the same four conditions used with organ culture. When the data from all of 28 isolates were analyzed together, only 36% of the cells plated on day 0 were recovered after incubation for 2 days in KBM alone (Table 3). Supplementation of KBM with either 1.4 m*M* Ca^{2+} or 3 μ*M* RA resulted in significantly increased cell recovery (Table 3). Combined treatment with 1.4 m*M* Ca^{2+} and RA preserved a greater percentage of the cells than either 1.4 m*M* Ca^{2+} or 3 μ*M* RA alone (Table 3).

Although supplementation with extracellular Ca^{2+} or treatment with RA appeared to act in concert when data from all 28 of the adult fibroblast isolates were examined together (Table 3), these data do not indicate the degree of heterogeneity among the isolates. For example, 22 or the 28 isolates responded to supplementation with 1.4 m*M* Ca^{2+} (i.e. a significantly higher percentage of cells survived in this medium than in KBM alone), 15 of the 28 isolates responded to treatment with 3 μ*M* RA in low-Ca^{2+} culture medium and 28 of the 28 isolates responded to the combination of Ca^{2+} supplementation and RA treatment (Table 3). Finally, when we compared the responses of the isolates to Ca^{2+} alone and to the combination of Ca^{2+} and RA, we found that 17 of the 28 isolates responded better to the combined treatment. Interestingly, however, only one of these 17 isolates was from a tissue that had been maintained in organ culture in KBM supplemented with Ca^{2+} alone (1/7, 14%). The other 16 isolates were from tissues that had been maintained in organ culture in KBM alone, in KBM treated with RA or in KBM treated with Ca^{2+} and RA (16/21, 76%) ($P < 0.01$ by Chi-squared test).

Table 3. Fibroblast survival and growth under serum-free conditions; effects of Ca^{2+} and/or RA

Fibroblast treatment[a]	48-h cell counts[b] (no. of cells × 10^4)	Proportion of responsive isolates[c]
KBM	1.8 ± 0.6	–
KBM + Ca^{2+}	2.8 ± 1.2*	22/28
KBM + RA	2.4 ± 0.9*	15/28
KBM + Ca^{2+} + RA	3.2 ± 1.4**	28/28

[a] Treatment under which the fibroblast isolates were incubated for 2 days in monolayer culture

[b] Number of cells per well in the culture dish after incubation. Values shown are means ± standard deviations

[c] Number of isolates (out of a total of 28) that responded better to supplementation with 1.4 m*M* Ca^{2+}, RA or the combination of Ca^{2+} and RA than to low-Ca^{2+} KBM alone

* $P < 0.05$; ** $P < 0.01$ vs KBM alone; significance of differences determined by ANOVA

Discussion

Topical treatment of human skin with RA induces a number of histological changes that can be seen in both the epidermis and the dermis [15–17, 36]. The cellular and molecular events underlying the histological alterations have not been fully elucidated, and this is due, in part at least, to the difficulty in carrying out the types of interventional studies in humans that would elucidate mechanisms of action. To obviate this problem, we have recently utilized an organ culture model of human skin to evaluate the effects of RA. Using this model we found that RA preserves the histological architecture of both the epidermis and the dermis over a 12-day period in serum-free, growth factor-free basal medium under conditions of both low and high extracellular Ca^{2+} [33]. A variety of other factors that are known to support fibroblast and/or keratinocyte proliferation in monolayer culture [7, 8, 10, 11, 18, 20, 23] were incapable of duplicating this effect [33, 34]. The similarity between the histological features seen in RA-treated skin in organ culture and those observed in vivo suggest that the organ culture model provides a good approximation of intact skin and will be a useful tool for elucidating mechanisms of RA action that are applicable to in vivo use.

How retinoids work at the cellular level is not fully understood. The present study demonstrated that RA treatment of organ-cultured human skin preserved viability for both the fibroblast and the epithelial cell populations. Using cell recovery from tissue pieces after incubation in organ culture as the criterion, our data showed that there was enhanced recovery of both cellular components from RA-treated skin as compared with matched controls from the same volunteers. The previously noted improvement in histological features of organ-cultured skin following treatment with RA [33, 34] was thus correlated with preservation of cell viability in both the epidermis and the dermis. Past studies have suggested that retinoids act by reversing specific events associated with excess exposure to sunlight. These include stimulating collagen production [1, 12, 28] and inhibiting enzymes involved in collagen degradation [2, 6, 30]. While the present data do not contradict previous results, they suggest that RA also, has more basic effects as, i.e. preserving cell viability.

Although RA has growth-promoting activity for both keratinocytes and fibroblasts in monolayer culture [30, 31], it is unclear from in vivo studies as to whether the direct target of retinoid action in skin is the epidermis, the dermis or both. Induction of epithelial glands in rodent skin by retinoids is thought to results from a retinoid effect on the mesenchymal components [9, 13, 22, 35]. In contrast, results from human skin-equivalent cultures suggest that keratinocytes are the target cells and the fibroblasts act in some manner to downregulate keratinocyte responses [27]. Although the present studies do not rule out the epidermis as a direct target, our results show that the dermis clearly is. In the presence of RA, fibroblast viability in both whole-skin organ culture and isolated dermis was preserved. Indeed, RA was more effective at maintaining fibroblast viability in the isolated dermis than in the intact tissue. This may reflect epidermal metabolism of RA with a resultant decrease in the effective concentration of the retinoid reaching the dermis.

Whether the epidermis is also a direct target of RA could not be assessed as the epidermal tissue quickly degenerated after separation from the dermis. Since RA is known to promote keratinocyte growth in monolayer culture [30], it would not be unreasonable to suggest that the ability of RA to preserve epidermal structure in organ culture is a reflection of this. This may not be the case, however, since a number of different factors that stimulate keratinocyte proliferation in monolayer [7, 8, 10, 11, 18, 20, 23] are completely unable to preserve epidermal structure in organ culture [33, 34]. Furthermore, keratinocytes proliferate actively in monolayer culture at low extracellular Ca^{2+} (0.05–0.15 m*M*) levels. Raising the Ca^{2+} concentration to 1.4 m*M* slows growth and induces differentiation [3, 14, 19, 21]. The opposite is true in organ culture. Thus, observations made with isolated keratinocytes in monolayer culture are of limited value, at best, in understanding how epidermal structure is maintained in organ culture. In contrast, the histological structure of the epidermis [33, 34] and keratinocyte recovery from organ-cultured tissue (this report) are both dramatically improved under two conditions (i.e. high-Ca^{2+} KBM or low-Ca^{2+} KBM supplemented with RA) that clearly act in a positive fashion on the dermal fibroblasts [4, 5, 31, 32]. Our interpretation of these data is that, regardless of whether or not RA acts on the epidermis directly, maintenance of structure and function in the epidermis in RA-treated organ-cultured skin is primarily a reflection of a direct retinoid effect on the dermis.

Although the data presented here clearly show that fibroblasts are a target of retinoid action in both organ culture and monolayer culture, how RA acts to preserve the viability of these cells under conditions in which they would otherwise die is not fully understood. Enhanced fibroblast viability was observed in high-Ca^{2+} culture medium as well as low-Ca^{2+} culture medium. Previous studies with neonatal (foreskin) fibroblasts showed similar effects with RA under low-Ca^{2+} conditions, but in high-Ca^{2+} culture medium, neonatal fibroblasts survived without RA and the addition of RA produced no benefit [32]. It seems reasonable to suggest that RA functions in some manner to overcome the limitation imposed by insufficient Ca^{2+} on fibroblast function. While it is difficult to extrapolate from monolayer and organ culture data to what might occur in intact skin, one could postulate that a progressive decrease in responsiveness to extracellular Ca^{2+} in dermal fibroblasts as a function of aging (noted in previous studies by others [4, 5, 24–26]) provides a target of opportunity for retinoid action.

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