Evidence suggests that some of the effects of retinoids on sensitive target tissues are mediated by specific cellular binding proteins. This study demonstrated the presence of cellular retinoic acid-binding proteins (cRABP) in human skin. The approximate concentration and affinity of cRABP was determined independently in epidermal and sebaceous follicle cytosol, using both tritiated retinoic acid (RA) and tritiated 13-cis-retinoic acid (13-cis-RA) as radiolabeled ligands. Scatchard analysis suggested that on the basis of binding per mg of cytosol protein, the binding capacities by epidermal and sebaceous follicle cytosol were similar for RA and 13-cis-RA. However, the dissociation constants of binding of 13-cis-RA by sebaceous follicle cRABP was more than 9-fold the K_d for RA (1096 nM compared to 117 nM). The K_d for binding 13-cis-RA by epidermal cRABP was 31-fold the K_d for binding RA (3582 nM compared to 114 nM). Thus the affinity for 13-cis-RA by epidermal and sebaceous follicle cRABP is significantly less than for RA. It appears that the specificity of response of human sebaceous glands to 13-cis-RA is dependent on factors other than the concentration or affinity of cRABP in these structures.

Vitamin A (retinol) is recognized as an important regulator of normal epithelial cell growth and differentiation. This has become particularly significant in recent years since the discovery that synthetic analogs of vitamin A, many of which are less toxic to mammalian systems than is retinol, are effective clinically in the treatment of a variety of disorders that involve abnormal epithelial cell differentiation. In dermatology, retinoids (natural and synthetic forms of vitamin A) have been used successfully in the experimental treatment of a number of skin diseases, including nodulocystic acne, psoriasis, pityriasis rubra pilaris, lamellar ichthyosis, and Darier’s disease [1–6]. Evidence suggests that small differences in the chemical structure of retinoids may have striking effects on their clinical efficacy [3].

13-cis-Retinoic acid (13-cis-RA, isotretinoin, Accutane) has been found to induce dramatic clinical improvement and long-lasting remission from disease in patients with nodulocystic acne [4–10]. A very specific effect of this retinoid in human skin is the suppression of sebaceous gland lipogenesis [10–11].

Suppression of sebum production by 13-cis-RA is more effective than has been induced by any other agent. Oral therapy at doses of 1 mg/kg body weight per day have induced up to 90% reduction in sebum secretion by the 3rd to 4th weeks of therapy [6]. Other retinoids, notably the aromatic retinoid RO 10-9559 (etretinate) have also been observed to reduce sebaceous gland activity in human skin, but this effect is minimal in comparison to 13-cis-RA [12].

Our understanding of the mechanisms of retinoid action remains unclear. Some research suggests that their effects are mediated by specific cellular binding proteins which act as receptors in transporting the retinoids to cell nuclei where they may induce and/or repress gene activity [13–15]. Two such proteins have been identified: cellular retinol-binding protein (cRBP) which is specific for retinoids with a free alcohol function [16–18], and cellular retinoic acid-binding protein (cRABP) [19–22] which is specific for retinoids with a free carboxylic acid function. Generally it appears that although both kinds of binding proteins are present in all fetal tissues, adult tissues have lower concentrations of these proteins. Increased levels of cRABP have been reported in cancerous tissues compared to normal tissues of the same organs [23–27]. Despite the fact that there have been instances where the biologic effects of retinoids have failed to correlate with either the affinity or the concentration of cRABP in the target tissues, it has also been suggested that in other instances differences in concentration and in affinity of cRABP may explain the responsiveness of tissues to specific retinoids [28].

In the present study we have assayed cRABP levels in specific compartments of human skin. Using microdissection of facial skin, we collected large quantities of isolated sebaceous follicles and sebaceous gland-free epidermis. Cytosol from both these cutaneous compartments was then compared for proteins that specifically bound [3H]retinoic acid (RA) and [3H]13-cis-RA to find out whether the effect of 13-cis-RA on sebaceous gland differentiation could be explained by the presence of increased concentrations and/or increased affinity of binding proteins for this compound.

MATERIALS AND METHODS

Bovine serum albumin, whale skeletal muscle myoglobin (Type II), collagenase (Type IA), all-trans-retinoic acid and retinol were obtained from Sigma Chemicals, St. Louis, Missouri; 13-cis-retinoic acid was a gift from Hoffmann-La Roche, Nutley, New Jersey; [11-3H]all-trans-retinoic acid (sp act 1.64 Ci/mmol) and [11-3H]13-cis-retinoic acid (sp act 1.75 Ci/mmol) were gifts from the Chemoprevention Program, National Cancer Institute; trypsin, Hanks’ balanced salt solution (HBSS), Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum, and antibiotic and antimycotic mixture were obtained from Gibco (Grand Island, New York). The stability of the tritiated 13-cis-RA was evaluated by thin-layer chromatography before and after incubation for 16 h at 4°C using hexane/ethylic acid (90:10) as the solvent system. Tritiated chemicals were stored at –60°C in dimethyl sulfoxide containing 0.025% butylated hydroxytoluene.

Tissues

Human facial skin was obtained from cosmetic surgery procedures and immediately placed into HBSS at 4°C. Within an hour after excision specimens were transported to the laboratory on ice, the subcutaneous fat and lower dermis were trimmed off, the skin cut into 2 x 3 mm pieces, and placed in 0.04% collagenase solution in DMEM.
containing 5% fetal bovine serum as protease neutralizer, and antibiotics, at 37°C for 18 h.

The use of this digestion procedure was based on results of preliminary experiments in which, in addition to the collagenase digestion described above, identical pooled samples of skin were also incubated in 0.25% trypsin at 4°C overnight and in 0.06% collagenase (without the addition of 5% fetal calf serum) at 4°C overnight. Each digestion procedure was evaluated for ease of separating sebaceous follicles and epidermis from the dermis and also for preservation of CRABP activity (compared to the activity in epidermis obtained by scalp scraping of fresh skin).

Following removal from collagenase, tissues were rinsed by gentle shaking in 4 changes of 100 ml of HBSS. Microdissection of skin was carried out at 5°C using Dumont stainless steel tweezers ST 3-5 (Ted Pella Inc., Tustin, California) and Vannas straight microscissors (Circon Corporation, Galeta, California). Each piece of skin was micro-dissected as follows. The epidermis, with the sebaceous follicles attached, was carefully peeled from the dermis and placed dermal side up in HBSS. The ease with which this could be accomplished varied greatly with the tissues. Generally, squeezing the dermis gently before peeling off the epidermis forced the glands upward and facilitated successful dissection. Follicles were cut off as close to the epidermis as possible and pooled on one corner of the slide. After all ducts and glands were removed, the epidermis was transferred to 0.5 ml of Tris-HCl homogenizing solution (see below) in a 5-ml beaker, and the pooled follicles were transferred to 0.5 ml of homogenizing fluid in a 1-ml beaker. Only glabrous skin was used in these studies. Skin from 4-6 face-lift surgeries provided 8-10 mg of sebaceous follicle cytosol protein.

Preparation of Tissue Cytosol

Immediately after dissection, tissues were homogenized in 0.05 M Tris-HCl, pH 7.5 buffer, containing 1 mM EDTA and 1 mM dithiothreitol, with a Polytron tissue homogenizer set at 6 for 20 s. Cytosol was separated by centrifugation of the homogenate at 31,000 g for 20 min followed by centrifugation of the supernatant at 100,000 g for 60 min at 5°C. Cytosol from samples not immediately used was stored at −60°C for up to 6 weeks in delayed experiments.

Before using cytosol for retinoid-binding protein assays, small-molecular-weight lipid and lipoprotein material was removed by acid precipitation with 1 N acetic acid as previously described by Ong and Chyi [29]. Protein content of the preparations was determined using the Bradford Coomassie Blue assay [30].

Single Concentration Assay for CRABP

The binding proteins were quantitated by incubation of samples with [3H]RA and [3H]13-cis RA followed by sucrose density gradient centrifugation as previously described [21]. Briefly, cytosol solutions were adjusted to contain 5-4 mg of protein/ml. Half-milliliter volumes were incubated with 0.1 µCi of [3H]RA or [3H]13-cis RA in a total of 200 µmol for 4°C.

Following incubation the mixtures were absorbed with charcoal-coated dextran to remove unbound radioactive ligand. Suspensions were clarified by low-speed centrifugation and the clarified supernatants (0.5 ml) layered onto 5-20% sucrose density gradients in 10 mM Tris-HCl and centrifuged in a Beckman L2-75Ti ultracentrifuge with an SW 50.1 rotor for 18 h at 180,000 g. Whole myoglobin and bovine serum albumin standards were run in separate gradients as external markers. After centrifugation, the gradients were fractionated from the bottom in 0.22-ml fractions and radioactivity of the fractions counted with a Beckman L57500 liquid scintillation counter. The radioactivity profile was established and the radioactivity in the 28 peak (specific binding) was used to quantitate the pmol of radioactive ligand bound per mg of tissue cytosol protein.

Specificity Determinations

Competitive inhibition assays were run to establish specificity of the binding. Each radioligand was incubated with a 200-fold excess of unlabeled RA, 13-cis-RA, and retinol in the presence of cytosol from the cutaneous compartments investigated.

Scatchard Analyses

To compare the approximate total binding capacity and the dissociation constants (Kd) of cytosol from sebaceous follicles and from epidermis, Scatchard analyses were carried out by running binding assays with increasing amounts of unlabeled RA and 13-cis-RA in the presence of constant amounts of the corresponding tritiated material [31].

RESULTS

Comparison of 3 methods for separation of epidermis and sebaceous glands from the dermis indicated that incubation of skin at 37°C for 18 h in 0.04% collagenase and 5% fetal bovine serum resulted in optimal preservation of intact sebaceous follicles (Fig 1) which could be dissected from the epidermis leaving appendage-free epidermal samples (Fig 2). This method also appeared to give greatest preservation of CRABP activity when compared to other digestion procedures, and to binding values obtained by using homogenates of untreated scraped epidermis as a source for cytosol (Table I).

![Fig 1. Sample of facial epidermis demonstrating presence of intact sebaceous follicle following collagenase digestion as described in Materials and Methods.](image1)

![Fig 2. Epidermis following microdissection and removal of sebaceous follicles.](image2)

<table>
<thead>
<tr>
<th>Digestion procedure</th>
<th>Total dpm in 25 peak/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25% trypsin for 18 h at 4°C</td>
<td>1,733</td>
</tr>
<tr>
<td>0.06% collagenase for 18 h at 4°C</td>
<td>5,791</td>
</tr>
<tr>
<td>0.04% collagenase + 5% fetal bovine serum for 18 h at 37°C</td>
<td>8,943</td>
</tr>
<tr>
<td>Untreated skin, epidermis obtained by scraping</td>
<td>6,607</td>
</tr>
</tbody>
</table>

Results based on single determinations of CRABP activity using [3H]RA incubated with 2 mg of epidermal cytosol obtained from identical pooled samples of skin (see Materials and Methods).
Stability of cRABP in Cytosol Preparations

Less than 10% of binding activity was lost in cytosol stored frozen at -60°C for up to 6 weeks.

Single Concentration Curves

Radioactivity profiles of sucrose density gradients of epidermal and sebaceous follicle cytosol incubated with 0.1 μCi of [3H]RA are shown in Figs 3 and 4. The 2S peak of binding of [3H]RA was higher, approximately 10.0 ± 1.1 pmol/mg protein, with epidermal cytosol compared to 8.0 ± 1.6 pmol/mg protein in cytosol from sebaceous follicles. This difference in binding by the two tissues was not statistically significant when results from 4 different pools of tissues were compared (Student’s t-test, p > 0.05).

Figs 5 and 6 demonstrate the radioactivity profiles of the cytosol from epidermis and sebaceous follicles incubated with [3H]13-cis-RA. With this retinoid the 2S peak was higher with sebaceous follicle cytosol than with epidermal cytosol but this difference also was not statistically significant (p > 0.10) when results of several experiments were compared. The approximate binding of [3H]13-cis-RA was 2.1 ± 0.3 pmol/mg protein in cytosol from epidermis compared to 3.1 ± 0.7 pmol/mg protein in sebaceous follicles.

Specificity of Binding

Competitive inhibition studies indicated that a 200-fold excess of unlabeled RA or unlabeled 13-cis-RA completely eliminated the radioactivity peaks in the 2S fraction of both sebaceous follicle and epidermal cytosol incubated with either [3H]RA or [3H]13-cis-RA (Figs 3–6). On this basis it was assumed that the 2S peak represented specific binding by cRABP. A 200-fold excess of unlabeled retinol did not affect the binding of [3H]RA but had a partial inhibitory effect on the binding of [3H]13-cis-RA (Figs 5, 6).

In assays with [3H]13-cis-RA a second radioactivity peak present in the 4S marker region was not inhibited by addition of excess unlabeled RA, 13-cis-RA, or retinol, and was assumed to represent nonspecific binding of [3H]13-cis-RA by albumin contaminating the cytosol preparations.

Scatchard Analysis

Summaries of results of the Scatchard analysis are presented in Table II. These figures were derived from standard Scatchard plot analysis using computer plotting of nonlinear regression curves with correction for nonspecific binding.

Results show estimated dissociation constants and the relative binding capacity of epidermal and sebaceous follicle cytosol using [3H]RA and [3H]13-cis-RA.

Using [3H]RA: The estimated K_d for cRABP were 114.7 and
117.6 nm for epidermis and sebaceous follicles, respectively. These values are very similar and fall well within the range that has been reported for other retinoid-sensitive tissues [32]. Binding in terms of pmol/mg of cytosol protein was somewhat higher with epidermal than with sebaceous follicle cytosol, but these results cannot be regarded as reflecting significant differences.

Using $[^3H]13$-cis-RA: The dissociation constants for both epidermal and sebaceous follicle cRABP were 31- to 9-fold higher than what was estimated with $[^3H]$RA. In other words, affinity of both epidermal and sebaceous follicle cRABP is 31- to 9-fold lower for 13-cis-RA than for RA. Binding capacity of $[^3H]13$-cis-RA in terms of pmol/mg cytosol protein was similar for epidermal and sebaceous follicle cytosol.

![Graph](image)

**Fig. 6.** Sucrose density gradient analysis of human sebaceous follicle cytosol (1 mg protein) incubated with 0.1 μCi of 13-cis-RA (●●●●●). As with epidermal cRABP, the 2S (↑) binding peak is lost in the presence of a 200-fold excess of RA (○○○○○) and 13-cis-RA (ΔΔΔΔ), but is only partially affected by a 200-fold excess of retinol (■■■■). The 4S (↑) peak is not affected by the addition of excess retinoids, suggesting that this peak represents non-specific binding by tissue albumin.

**Table II.** Summary of $K_d$ and binding capacities (± SE) of epidermal and sebaceous follicle cRABP calculated from nonlinear regression curves of Scatchard analysis data, using $[^3H]$RA as well as $[^3H]13$-cis-RA as radiolabeled ligands.

<table>
<thead>
<tr>
<th></th>
<th>With $[^3H]$ RA</th>
<th>With $[^3H]13$-cis-RA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$</td>
<td>Specific binding</td>
</tr>
<tr>
<td>Epidermal</td>
<td>114.7 ± 8.6</td>
<td>18.3 ± 0.8</td>
</tr>
<tr>
<td>cytosol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sebaceous</td>
<td>117.6 ± 16.4</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>follicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytosol</td>
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</table>

**DISCUSSION**

Sebaceous follicles from human facial skin are difficult to obtain in pure form in meaningful quantities. Even though the digestion procedure described in this paper gave excellent preservation of intact sebaceous follicles, the average net weight of a single follicle was 15–25 μg, and only a fraction of that was soluble protein. Thus a lot of tedious microdissection had to be performed in order to carry out the work described.

To our knowledge this digestion procedure is the only available method for isolating “functioning” sebaceous follicles in which at least some of the metabolic parameters are seemingly intact. Protease digestion procedures such as trypsin treatment, as well as chemical digests are clearly unsatisfactory since they denature metabolically active proteins. Unfortunately, because there are no alternative methods for isolating sebaceous follicles, any variables in effect of collagenase treatment on retinoid binding by epidermis compared to sebaceous follicles remain unresolved. In epidermis, cRABP activity appears to be higher in samples obtained following collagenase pretreatment of skin compared to samples obtained by scraping skin (Table I). The explanation for this is not clear. In the present study, histologic examination of the separated epidermis indicated that there was a higher proportion of basal cells in epidermal samples obtained following collagenase treatment of skin in contrast to direct scraping. However, the exact location of cRABP in the epidermis remains unresolved and the relative proportion of basal cells may be unrelated to cRABP activity. Gates and King [33] have observed in a preliminary report that collagenase treatment of chick embryo skin increased cRABP activity, suggesting that collagenase may have an activating or liberating effect on cutaneous cRABP. Clearly future studies to localize cRABP in specific compartments of epidermis, as well as in sebaceous follicles, are warranted. In the meantime it must be noted that the effects of collagenase on cRABP activity in sebaceous follicles may differ from the effects on cRABP activity in the epidermis, and that this uncontrolled variable may have affected the results of the present study.

The single concentration assay used in the present paper has been the basis of most studies which have approximated cRABP levels in different tissues, particularly studies on cRABP in cancerous tissue compared to normal tissues of the same organs [24–27,32,34–39]. This methodology is acceptable for demonstrating presence and specificity of binding proteins, but is less accurate than the Scatchard analysis, for approximating numbers of binding sites and dissociation constants for specific ligands [40].

In the present study it was found that both human epidermis and sebaceous follicles contained cellular binding proteins specific for RA. A 200-fold excess of unlabeled RA or unlabeled 13-cis-RA added to the assay mixture completely eliminated binding by the prelabeled ligand, but a 200-fold excess of unlabeled retinol did not compete for binding with the cRABP. This was as expected since cRABP is known not to bind with retinoids having a free alcohol function. The fact that excess unlabeled retinol competed for binding when $[^3H]13$-cis-RA was used as the radiolabeled ligand was contrary to expectations and suggests the presence of some cross-reacting contaminants in the retinol preparation.
The magnitude of the standard errors obtained in the Scatchard analysis using [3H]13-cis-RA as radiolabeled ligand was a reflection of the technical limitations of our assay. The relatively low specific activity of the available tritiated 13-cis-RA combined with the low affinity for 13-cis-RA by the cRABP made it impossible to establish more definitive Kd values in the present system.

To our knowledge the present study is the first quantitative analysis of cRABP in human epidermis and sebaceous follicles. Since these tissues are retinoid-sensitive target organs, it was not surprising to find dissociation constants and binding capacities in the same range as has been reported by others in retinoid-sensitive malignant cell lines [37]. What was more surprising was the finding that despite the high biologic activity of 13-cis-RA on human sebaceous gland differentiation, the affinity of sebaceous cRABP for this retinoid was more than 9-fold lower than for RA. This confirms what has been suggested by others, namely that for retinoids, a high biologic activity does not always correlate with a high degree of binding [41]. Responsiveness of tissues to retinoids appears to be dependent to some degree on the presence of cellular binding proteins, but numerous other factors, such as for example, cellular metabolism of the retinoid by the tissues, are clearly more important in determining the final biologic effect. Whether retinoids can have biologic activity in tissues without the presence of binding proteins, as has been suggested by Libby and Bertram [42], is another matter, which probably will not be determined until more sensitive assays, such as radioimmunoassays can establish with more certainty the absolute presence or absence of binding proteins.

The primary focus of this work was to look for cRABP in sebaceous follicles and epidermis. However, in preliminary assays we also tested dermal cytosol and found binding of RA to be minimal.

On the basis of cytosol protein content, sebaceous follicles and epidermis appeared to have similar capacities for binding RA. However, if one were to describe the primary site of cRABP in skin, it would clearly be in the epidermis. In terms of area, almost twice as much skin was required to provide 1 mg of sebaceous follicle cytosol, than was required to provide the same amount of epidermal cytosol. This despite the fact that in the present studies only facial skin was used, which probably has more sebaceous follicles than any other area of the body, with the possible exceptions of scalp and scrotal skin.

As the present studies have shown, skin is a good source of cRABP. Since it is also an easily manipulable tissue it may provide a good model in which to study the factors which regulate cRABP synthesis.

The excellent technical help of Gloria Carruth is gratefully acknowledged. The calculations of the Scatchard analysis data were performed by Elliot M. Landau, M.D., Ph.D., of the Department of Biomathematics at UCLA Center for Health Science.

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