

Inhibition of retinoic acid-induced skin irritation in calorie-restricted mice

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Received: 4 June 2007 / Revised: 13 September 2007 / Accepted: 28 September 2007 / Published online: 30 October 2007
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Abstract Mice on a calorie-restricted (CR) diet (total calories restricted to 70% of ad libitum; AL) for periods of time ranging from 3 to 18 months were examined for response to topical treatment with all-trans retinoic acid (RA). Daily application of a 0.1% solution of RA to the shaved skin of UM-HET3 mice on an AL diet produced a severe irritation that was evident by day 4, maximal at day 7–8 and still detectable at day 14. Skin irritation was characterized by redness, dryness, flaking and failure of the hair to grow at the treated site. In CR mice, the same treatment produced little detectable irritation. Animals were sacrificed at the end of the retinoid-treatment period (day 7 or day 14) and skin from these animals was examined histologically. In both AL and CR mice, a similar degree of epidermal hyperplasia was observed. Numerous inflammatory cells (mononuclear cells and granulocytes) were present in the skin of both groups. Occasional S100-positive cells (presumably Langerhans cells) were also observed in the epidermis of skin from both groups. S100-positive cells were also observed in the dermis. When skin from CR and AL mice was incubated in organ culture for 3 days (on day 7 after initiation of RA treatment), similar levels of four different pro-inflammatory cytokines were found in the

conditioned medium. Soluble type I collagen levels were also similar. In contrast, the level of matrix metalloproteinase-9 was lower in the conditioned medium of skin from CR mice than in conditioned medium from skin cultures of AL mice. Taken together, these studies suggest that CR may provide a way to mitigate the irritation that normally accompanies RA treatment without compromising the beneficial effects of retinoid use. CR appears to exert a protective effect at the target tissue level rather than by a reduction in pro-inflammatory events, per se.

Keywords Retinoic acid · Skin irritation · Calorie restriction · Epidermal hyperplasia · Inflammation · Cytokines · Matrix metalloproteinases

Introduction

Topical application of all-trans retinoic acid (RA) improves the appearance of skin that has been damaged as a consequence of the natural (chronological) aging process [21, 26] or as a result of chronic exposure to ultraviolet radiation from the sun (photoaging) [29, 54]. In both photoaging and natural aging, there is a loss of intact collagen and an increase in fragmented collagen in the skin [12, 45, 49]. RA treatment reverses the increased expression of connective tissue-degrading matrix metalloproteinases (MMPs) [10, 11] and concomitantly stimulates new collagen synthesis [15, 27, 28, 49]. Given the ability of RA to reduce collagen damage and induce new collagen synthesis, it is not surprising that topical retinoid use not only improves the appearance of damaged skin but also results in better function. Specifically, a number of past studies have demonstrated that RA-pretreatment of skin that has been damaged as a result of aging/photoaging or as a consequence of either diabetes or chronic corticosteroid

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use improves the healing of superficial wounds that occur subsequent to treatment [1, 33, 42, 55, 57].

Topical retinoid use typically causes skin irritation. Irritated skin is characterized by redness, dryness and flaking of the skin at the treated site. At the histological level, one sees a perivascular accumulation of mononuclear cells, with neutrophils and monocytes scattered throughout the dermis and an occasional foci of neutrophils in the dermis or epidermis [3, 14, 23]. Epidermal thickening along with features of abnormal differentiation are also seen. Irritation is a major cause of non-compliance among retinoid users. In addition, excessive irritation may counteract the beneficial effects of topical retinoid use. While the molecular events that underlie retinoid-irritation have not been completely delineated, the retinoid irritation response is, fundamentally, a form of inflammation.

Calorie restriction (CR) in rodents has been known for years to extend life and reduce age-related diseases in aged animals (for reviews, see [34, 36]). A number of hypotheses have been put forward to explain the beneficial effects of calorie restriction. While the mechanisms by which CR might exert its effects have not been fully elucidated, a reduction in pro-inflammatory events seems to be an important component of the overall response to CR diets. Numerous observations specifically linking CR to a reduction in oxidant-mediated, pro-inflammatory processes have been made [7, 34, 37]. Given this background information, the present studies were carried out to assess retinoid irritation in CR mice as compared to their ad libitum (AL)-fed counterparts. Our results indicate that CR dramatically blunts RA-induced skin irritation under conditions in which beneficial effects of retinoid use (i.e., RA-induced epidermal proliferation and dermal collagen production) are largely unaffected.

Materials and methods

Mice

Animals used in this study were bred as the progeny of CB6F1 females and C3D2F1 males, a cross referred to in other publications as UM-HET3 [38, 40]. Because the parents are heterozygous at many loci, each mouse in the UM-HET3 population is genetically unique, and can be considered a full sibling of every other mouse tested. Mice were weaned into same sex cages at the age of 4 weeks (3–4 mice/cage) and provided free access to laboratory chow and fresh water. To document the specific pathogen-free status of the colony, sentinel animals (not part of the test population) were exposed to pooled spent bedding and then examined for pinworms and for serological evidence of infection with Sendai virus, mycoplasma, or mouse coronavirus;

such testing was conducted quarterly and proved negative throughout the course of the experiment.

CR protocol and AL controls

The CR protocol used in these studies was a modification of a previously described procedure [39]. Briefly, beginning at the age of 1 month, half of the animals were subjected to CR, reducing their daily food intake to 90% of AL food intake of animals during the same week. Two weeks later, food was further restricted to 80% of the AL consumption, and 2 weeks later diminished further to 70%, which was then maintained for the remainder of the study. Food consumption of AL animals was monitored throughout the study and the CR animals were fed daily, at approx. 0900 hours.

RA-induced epidermal hyperplasia and skin irritation

A 0.1% solution of RA was prepared in DMSO. At the start of the experiment, the skin over the back and flanks of the animals was shaved. One day later, treatment with RA was initiated. Once daily for 14 days, each animal was topically treated over the shaved area with 100 μ l of the retinoid solution. In each experiment, some mice (AL and CR) were kept as non-retinoid-treated controls. These animals received 100 μ l of DMSO alone. Previous studies have demonstrated that treatment of hairless mice with RA in this manner leads to an intense irritation response that can be seen as early as 3–4 days after initiation of treatment and is maximal by day 7 or 8. Irritation is associated with increased keratinocyte proliferation and epidermal thickening [51]. The animals were examined for signs of skin irritation each day after RA treatment. Irritation was characterized by redness, dryness (with small cracks developing in the skin), epidermal flaking and failure of hair to grow at the treated site. The animals were carefully evaluated for these features and given an overall “irritation score” of 1+ to 4+. A 1+ score indicated no difference between the RA-treated animals and controls and a score of 4+ indicated maximal irritation. All measurements were made in an unblinded fashion. On day 7 and day 14, cohorts of CR and AL animals were sacrificed by cervical dislocation after exposure to CO₂. Two biopsies (2 mm full-thickness) from the treated area were fixed in 10% buffered formalin and used for histology. Epidermal thickness (i.e., distance from the dermal–epidermal juncture to the upper level of viable cells) was assessed randomly at 4 or 5 sites in each tissue section. Two additional 2 mm full-thickness biopsies were put into organ culture using Keratinocyte Basal Medium (KBM) (Lonza, Walkersville, MD) supplemented with Ca²⁺ to a final concentration of 1.4 mM as culture medium. Incubation was at 37°C in an atmosphere of 95% air and 5% CO₂. Three days later, the conditioned

medium was obtained and used for the various analyses indicated below.

MMP production

Substrate embedded enzymography (zymography) was used to assess levels of latent and active MMP-2 and MMP-9 in organ culture fluids. As described previously [10, 49], SDS-PAGE gels were prepared with the incorporation of gelatin (1 mg/ml) at the time of casting. After electrophoresis under non-reducing conditions to separate proteins and overnight incubation to allow for substrate digestion, zones of hydrolysis were identified as “holes” in the stained gels and quantified. Values for latent and active MMP-2 and MMP-9 bands were obtained following digitization.

Soluble type I collagen

Culture fluids were assayed for soluble type I collagen by western blotting [52]. Briefly, culture fluids representing equal quantities of protein were resolved by SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat milk solution in Tris-buffered saline with 0.1% Tween (TTBS) for 1 h at room temperature and then incubated with an anti-mouse collagen type I antibody (Abcam Inc., Cambridge, MA) (1:10,000) in the same buffer overnight. The membranes were washed with TTBS and bound antibody was detected using the Phototope-HRP Western detection kit (Cell Signaling Technologies Inc.). Images were digitized and quantified.

Cytokines

Levels of four cytokines IL-1 β , IL-6, KC/IL-8 and MCP-1 were quantified using individual ELISA kits from R&D Systems (Minneapolis, MN). Briefly, the ELISA plates (Immulon 4, Dynex Technologies, Inc., Chantilly, VA) were coated with 10 μ g/ml of capture antibody in carbonate buffer (pH 9.6) and allowed to bind overnight at 4°C. The plates were then washed with PBS containing 0.02% Tween 20 (BioRad, Hercules, CA) and then 50 μ l of either sample or standard was added to appropriate wells. The plates were then incubated for 2 h at room temperature, washed twice and 50 μ l of biotinylated secondary antibody was added. The plates were then incubated for 1 h at room temperature, washed twice, and 50 μ l of streptavidin conjugated HRP (Zymed, San Francisco, CA) diluted 1:3,000 added to each well. The plates were then incubated at room temperature for 30 min, washed twice and 200 μ l of OPD substrate (Sigma Chemical Co., St Louis, MO) was added and color allowed to develop. The reaction was stopped with 50 μ l of 3 M sulfuric acid and the optical density

(490 nm) determined using an ELISA Plate reader (Bio-Tek Instruments).

S100 staining

A rabbit polyclonal antibody (Dako North America, Carpinteria, CA) was used to stain tissue sections from AL and CR mice. Briefly, formalin-fixed tissue was stained with antibody at a 1:400 dilution. The sections were stained by the immunoperoxidase method and the reaction product was visualized using diaminobenzadine as the chromogenic substrate. Immunostained sections were examined by light microscopy. In the skin, S100 reactivity is thought to be restricted to Langerhans and to some cells of the melanocytic series. Dermal staining may reflect migration of Langerhans cells out of the epidermis in response to inflammatory stimuli. Alternatively, S100 may have reactivity with some of the dendritic cells normally present in the dermis [16, 22].

Results

Skin irritation induced by RA in AL and CR mice after 3, 8 and 18 months on diet

Groups of AL and CR mice were maintained in the Core Facility for Aged Rodents at the University of Michigan for periods of time ranging from 3 to 18 months. At the selected ages, mice were treated topically with a 0.1% solution of RA. Animals were examined daily for overall health and for signs of skin irritation including redness, dryness, flaking of excess epidermis and failure of hair to grow at the treated site. Consistent with past findings in hairless mice [51], AL mice at all three ages developed an intense skin irritation as a result of the retinoid treatment. Irritation in these mice was evident 4 days after initiation of treatment and was maximal by day 7–8. By day 14, irritation had subsided, but the RA-treated AL mice were still distinguishable from AL controls. In contrast to these findings with AL mice, mice on the CR diet for 3, 8 or 18 months demonstrated much less irritation in response to topical RA treatment than their AL controls. Figure 1 presents irritation scores over the 14 day retinoid treatment period in AL and CR animals on diet for 18 months. The insert in Fig. 1 demonstrates the typical appearance of RA-treated mice from the AL and CR groups on day 7 after initiation of retinoid treatment.

In a final set of experiments, mice that had been maintained on the CR diet for 18 months were maintained for a further 2 week period with access to AL chow. When allowed unlimited access to food, the amount consumed by animals previously on the CR diet immediately re-equilibrated at the level consumed by age-matched animals that had not been subjected to the CR diet. When these mice

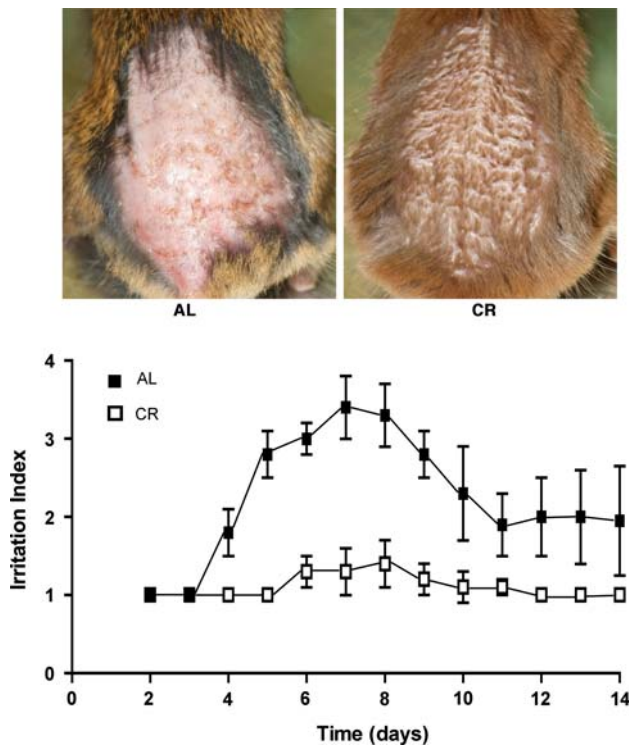


Fig. 1 Retinoid-induced skin irritation in CR and AL mice. Mice on AL and CR diets for 18 months were treated daily for 14 days with RA. Irritation was assessed on days 2–14 as indicated in the “Materials and Methods” section. Each animal was given a score of 1+ to 4+ based on four criteria with 1+ indicating no difference in appearance from non-RA treated controls and 4+ indicating maximal skin irritation. Values are averages and standard deviations based on 9 animals per group from days 2–7 and 4 animals per group from days 8–14. (Insert: Gross appearance of the skin from CR and AL mice. The mice were on their respective diets for 8 months and examined on day 8 of RA treatment. The AL animal demonstrates skin irritation. The skin is red and flaky and small cracks can be observed. Hair has failed to grow on the treated site. The animal on the CR diet shows little or no inflammatory changes in the skin)

were treated for 6 days with RA and examined on day 7, they still demonstrated protection from skin irritation (irritation score = 1.9 ± 0.7 in CR mice as compared to age matched AL control irritation score of 3.3 ± 0.5 ; mean + SD; $n = 8$; $P < 0.01$).

Histological features of RA-treated skin in AL and CR mice after 3, 8 and 18 months on diet

Figure 2 presents a quantitative assessment of epidermal thickness in RA-treated AL and CR mice on day 7 after initiation of treatment. Both groups of RA-treated mice demonstrated significant epidermal thickness as compared to non-RA-treated controls. No significant difference between CR and AL mice was evident at any of the three ages tested (3, 8 or 18 months on diet). Figure 3 shows histological features of skin from AL and CR mice (8 months on diet). The increase in epidermal thickness is apparent in RA-

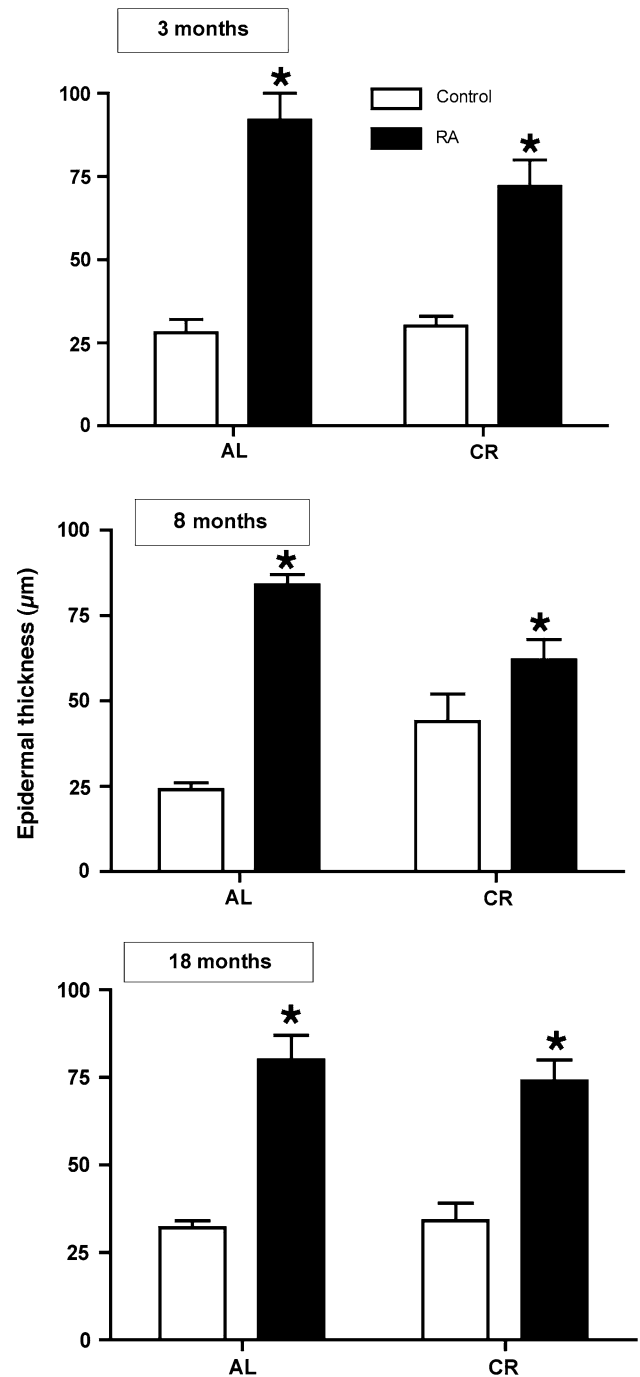
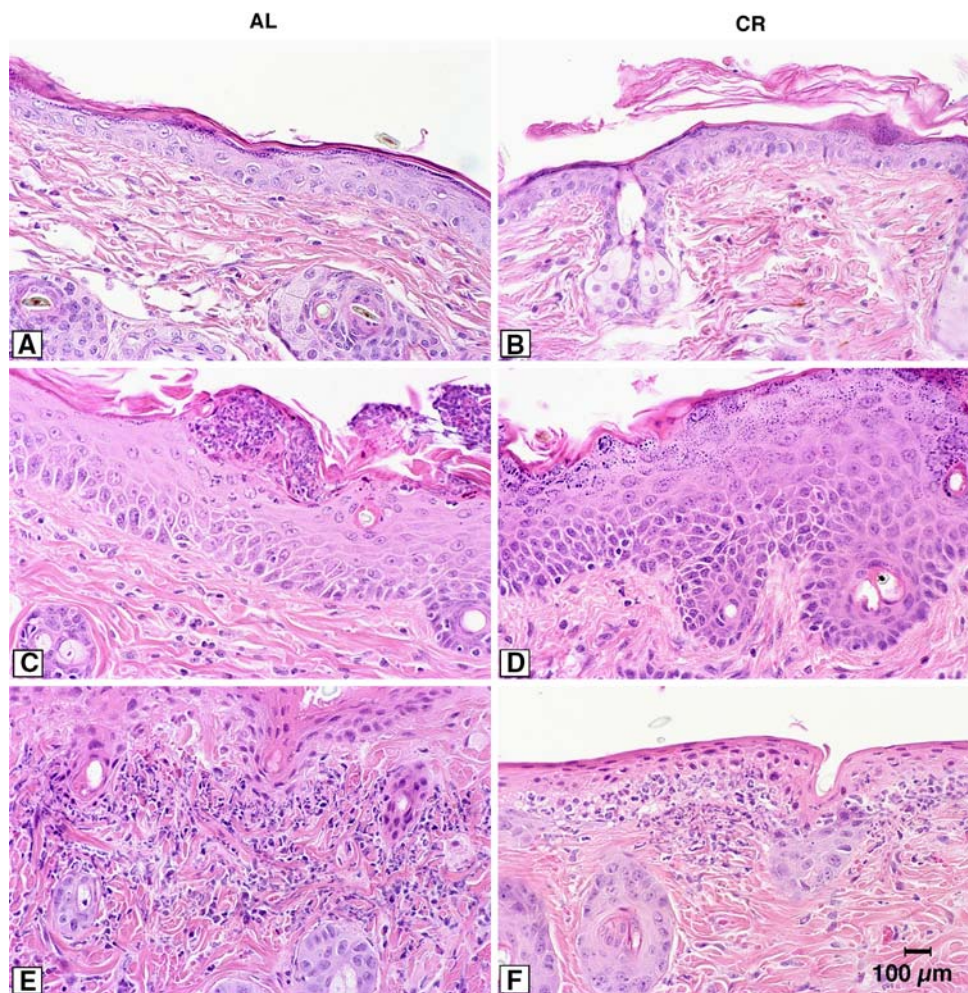


Fig. 2 Epidermal thickness of skin from CR and AL mice. Values are means and standard errors based on 4–7 mice per control group and 7–14 mice per RA group. Statistical significance of the difference among samples at each age was determined by ANOVA followed by paired-group comparisons. *Indicates $P < 0.05$ relative to the appropriate control group

treated mice regardless of CR status (compare panels c and d with panels a and b).

In addition to assessing epidermal thickness, we also examined the same histological sections for evidence of inflammation. With mice on the AL diet for 3, 8 and

Fig. 3 Histological features of the skin from 8 month CR and AL mice. **a, b** Skin sections from AL (left) and CR (right) mice without RA exposure. **c, d** Skin sections from AL (left) and CR mice (right) after treatment with RA for 6 days and sacrifice on day 7. Epidermal thickening is observed in both animals. The AL animal also demonstrates a cluster of neutrophils at the epidermal surface. **e, f** Skin sections from animals of the same AL (left) and CR groups (right). Numerous inflammatory cells (mostly granulocytes) are present in the dermis of both animals (All panels are hematoxylin and eosin $\times 166$)



18 months, treatment with RA produced an intense inflammatory infiltrate that was evident on day 7 of treatment. Perivascular accumulation of granulocytes and mononuclear cells was observed throughout the sections in virtually every animal. In addition, granulocytes were scattered throughout the dermis and granulocyte-rich foci could be seen in the dermis and at the surface of the epidermis (see Fig. 3, panels c and e). In some places, the inflammation was so intense that the epidermis was almost completely eroded. In these areas, keratinocyte necrosis was evident (Fig. 3e). A similar inflammatory infiltrate was seen in the skin of retinoid-treated CR mice of all three ages. Just as observed in AL mice, there were mononuclear cells and granulocytes scattered throughout the dermis and occasional foci within the dermis (Fig. 3f). There appeared to be fewer epidermal neutrophils in CR mice than in AL mice (Fig. 3f), but variability from animal to animal was high and efforts to quantify this difference produced inconsistent and not statistically significant results.

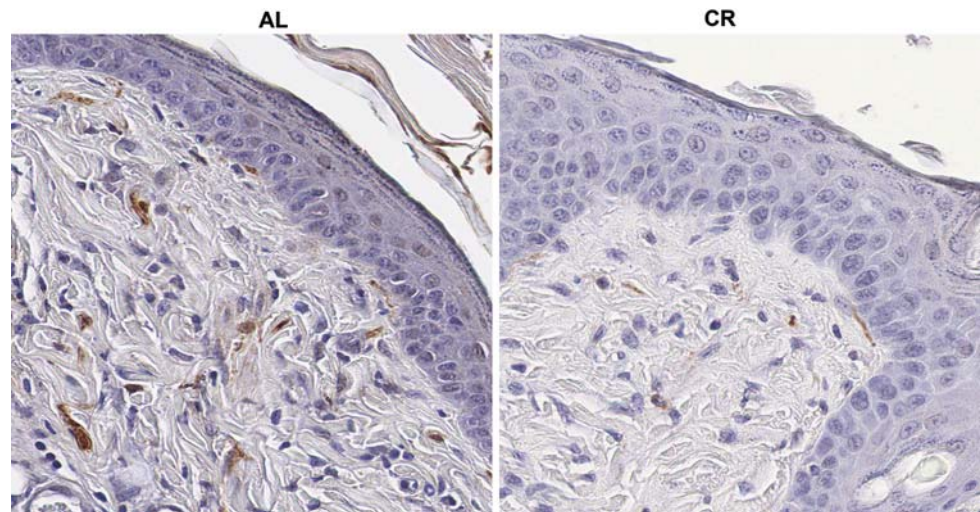
Although neutrophil-rich areas could be readily identified in the hematoxylin and eosin-stained sections, this was not sufficient to detect Langerhans cells. To aid in this, we

stained skin sections of AL and CR mice (18 month on diet) with antibody to the S100 protein. Occasional S100-positive cells were observed in the epidermis, but there was no difference between the two groups (Fig. 4). There were also occasional cells that stained in the interstitium. Again, there were no significant differences between AL and CR mice (Fig. 4). The ratio of S100-positive cells in AL to CR mice was 1:1.19 in the epidermis and 1:0.86 in the dermis ($n = 8$).

Cytokines, soluble type I collagen and MMPs induced by RA in AL and CR mice after 18 months on diet

At the time of sacrifice, skin from the RA-treated site (18 month AL and CR mice; 6–9 per group) was harvested into organ culture and incubated for 3 days. At the end of the incubation period, the serum-free culture medium was collected and assayed for IL-1 β , TNF- α , KC/IL-8 and MCP-1. With IL-1 β and TNF- α , there was a slight but statistically significant increase in the levels in organ culture fluid from CR mice as compared to AL controls (IL-1 β : 0.30 + 0.04 versus 0.17 + 0.01 and TNF- α :

Fig. 4 S100 staining of tissue section from 18 month *CR* and *AL* mice. Skin sections from an *AL* and *CR* animal. Sections were stained by the immunoperoxidase method and the reaction product visualized using diaminobenzadine as the chromogenic substrate. Occasional S100-positive cells are apparent in the epidermis and in the interstitium in sections from both *AL* and *CR* mice



0.31 + 0.02 versus 0.14 + 0.02; $P < 0.001$ for both). These values were only slightly above background. In contrast, there were much higher levels of KC/IL-8 and MCP-1. However, there were no differences in levels of these cytokines between the two groups (Fig. 5).

The same organ culture fluids from *AL* and *CR* mice were analyzed for soluble type I collagen by Western blotting. Newly synthesized collagen is secreted as a propeptide, and then cleaved and cross-linked as it is incorporated into the extracellular matrix. The material in solution (not yet cross-linked) is a measure of newly synthesized collagen. Results from these studies are shown in the upper panel of Fig. 6. Soluble type I collagen levels were similar in the culture fluids from both groups of mice.

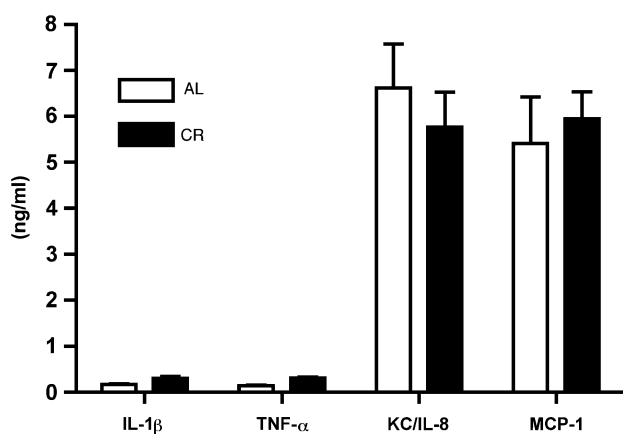


Fig. 5 Cytokines in culture fluid from 18 month *CR* and *AL* mice. Mice on *AL* and *CR* diets for 18 months were treated daily for 6 days with RA and sacrificed on day 7. Skin from *AL* and *CR* mice was maintained in organ culture for 3 days. At the end of the incubation period, organ culture fluid was collected and assessed by ELISA for the four cytokines. Values shown are means and standard deviations, based on organ cultures from 8 *AL* mice and 9 *CR* mice

The lower panel of Fig. 6 demonstrates levels of MMP-2 and MMP-9 in the same organ culture fluids based on gelatin zymography. While similar levels of MMP-2 were detected in the skin organ culture media of both *AL* and *CR* mice, levels of MMP-9 were reduced in the culture fluid from *CR* mice relative to that seen with *AL* controls.

Discussion

We report here that *CR* can prevent skin irritation following topical retinoid treatment of mice without significantly inhibiting the beneficial effects of RA use, which include increased epidermal thickening and increased collagen production. This observation is of interest from a number of standpoints. First, the data clearly support the idea that retinoid beneficial effects can be attained under conditions in which irritation is reduced. Second, these findings provide insight into possible mechanisms by which *CR* functions (and possible approaches for mitigating retinoid irritation). Finally, our results suggest a possible biological test for the beneficial effects of *CR* that could be useful for assessment of *CR* and related diets in humans.

Retinoids have multiple effects on the skin. Many of these occur via activation of nuclear retinoic acid receptors (RARs) [4, 5]. Additionally, retinoids have detergent-like properties [35] and influence such fundamental properties as membrane fluidity [48] and cation transport [46, 47]. Through one mechanism or another, topical treatment of skin with RA results in a substantial keratinocyte proliferative response, leading to increased epidermal thickness [21, 23, 26, 29, 50, 54]. Altered differentiation [9, 13, 20] and a reduction in cell–cell cohesion [45] accompany increased proliferation. Our recent studies have shown that several pro-inflammatory cytokines are elaborated in the rapidly proliferating epidermis [52, 53]. Cytokine production may

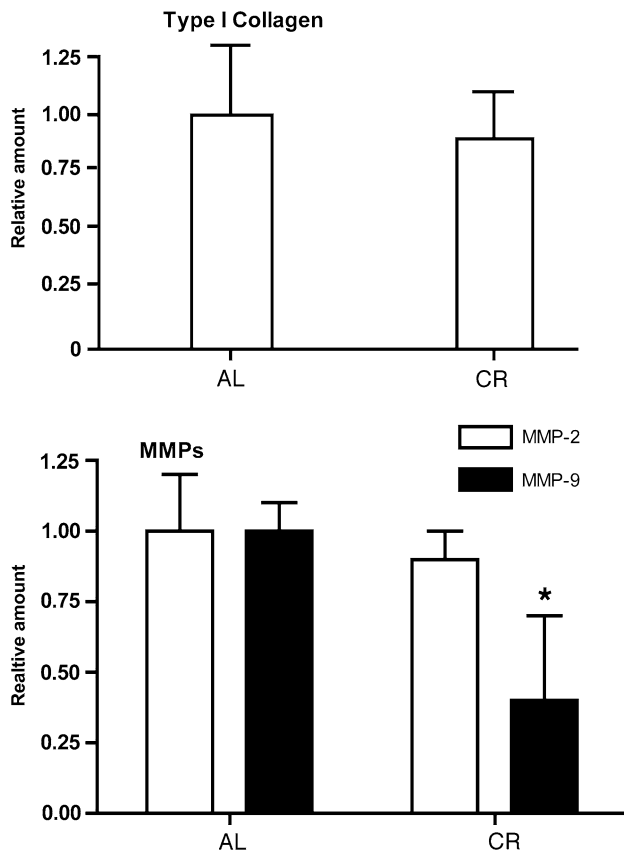


Fig. 6 Type I collagen and MMPs in culture fluid from *CR* and *AL* mice. *Upper panel*: Type I collagen. Mice on *AL* and *CR* diets for 18 months were treated daily for 6 days with RA and sacrificed on day 7. Skin from *AL* and *CR* mice was maintained in organ culture for 3 days. At the end of the incubation period, organ culture fluid was collected and assessed for soluble type I collagen by Western blotting followed by digitization of the collagen band. Values shown (normalized to 1.0 in the *AL* group) are means and standard errors, based on organ cultures from 8 *AL* mice and 6 *CR* mice. *Lower panel*: MMPs. The same organ culture fluids were assessed for MMP-2 and MMP-9 by gelatin zymography followed by digitization of the latent and active enzyme forms. Values shown (normalized to 1.0 in the *AL* group) are means and standard deviations, based on organ cultures from 8 *AL* mice and 6 *CR* mice (duplicate organ cultures per mouse). Statistical significance of the difference between paired groups was determined using the student *t*-test. * $P < 0.05$ relative to *AL* control skin

reflect altered differentiation/cohesion rather than proliferation per se, since disruption of epidermal barrier function (without significant hyperplasia) also leads to cytokine production [56].

With the plethora of cellular responses to RA, it may be difficult to determine how CR reduces retinoid irritation. Given the similar inflammatory cell infiltrate found in both *AL* and *CR* mice and given that there were no apparent differences in elaboration of pro-inflammatory cytokines between the two groups, it seems reasonable to suggest that CR acts somehow to protect the tissue from inflammatory injury rather than by blunting pro-inflammatory events, per

se. Consistent with this, previous studies have demonstrated changes in cellular membrane composition resulting (directly or indirectly) from CR [19, 30, 31]. A recent study [19] showed that cells maintained in culture with serum from CR animals had a significant increase in the plasma membrane redox system antioxidants (α -tocopherol and coenzyme Q10). Since oxidants are a major contributor to cell injury in inflammation [17], increased cellular anti-oxidant levels, especially in cellular membranes, could be expected to provide protection.

Alternatively, CR has been shown to interfere with signaling events that influence AP-1 and NF- κ B-mediated gene transcription [24, 25, 41]. Since both of these transcription complexes regulate synthesis of tissue-destructive MMPs, [2, 10, 11], interference with either or both pathways could be expected to reduce MMP levels. There was a decrease in MMP-9 in skin organ culture fluid from CR mice (RA-treated) as compared to *AL* controls. Although we used MMP-9 as our “readout” in these studies, it should be noted that several other MMPs are also regulated at the transcriptional level in parallel to MMP-9 [10, 11]. While there is no direct evidence that MMPs are primarily responsible for tissue damage in retinoid irritation, these enzymes are important mediators of inflammatory tissue injury in other contexts [6, 8, 44]. The lack of decrease with MMP-2 is consistent with this model, because MMP-2, unlike most other MMPs, is not regulated at the transcriptional level [10]. In contrast to these results, we did not see a decrease in the level of various pro-inflammatory cytokines in the CR mice. KC/IL-8 and MCP-1 were both well-represented in organ culture fluid from both *AL* and *CR* mice, but there was no significant difference between the two groups. In contrast, IL-1 β and TNF- α were barely detectable, but unexpectedly, the levels of these two were actually higher in organ culture fluid from CR mice as compared to *AL* controls. We have no explanation for this.

In addition to helping elucidate mechanisms by which CR interferes with tissue injury, the data presented here suggest that the retinoid irritation response might provide a useful tool for evaluating the physiological effects of CR diets in humans. Retinoid patch-testing is a sensitive, reliable and easily quantified way to assess retinoid irritation in human skin [14, 23]. As studies on CR in humans move forward [18, 32, 43], it will be important to have sensitive measures for functional outcomes as well as for metabolic changes that result from dietary modification. One could envision using retinoid irritation as a predictor of responsiveness to treatment on an individual basis. Such a marker could also be useful for assessing how rapidly beneficial effects are observed and how long they persist once CR is stopped. Finally, this could be used to help elucidate mechanisms of CR action in humans.

In summary, the present studies indicate that mice maintained on a diet that restricts their calorie intake to approximately 70% of the calories consumed by AL animals demonstrate significant resistance to the irritating effects of topical retinoid application. The beneficial retinoid activities are, however, maintained. These data support the notion that retinoid beneficial effects and retinoid irritation can be separated. These findings also provide insight into possible mechanisms by which both RA and CR mediate their effects. Finally, these results suggest a possible biological test for the beneficial effects of CR that can be adapted to humans.

Acknowledgments This study was supported in part by an Older Americans Independence Center Grant (NIH AG024824) from the USPHS.

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