Wound healing in the eye may lead to undesirable sequelae such as proliferative vitreoretinopathy and scarring of glaucoma filtering fistulas. We therefore sought to manipulate in vitro two key wound healing processes—cell proliferation and contraction of extracellular matrices—using vitamin A (VA), retinoic acid (RA), and n-butyrate (BUT). These substances modulate growth and differentiation of normal and neoplastic cells. We examined the effects of these agents on cultured rabbit fibroblast proliferation and contraction of collagen matrices. Dermal fibroblast proliferation was unaffected by VA, stimulated by RA, and inhibited by BUT. Scleral fibroblast proliferation, in contrast, was stimulated by both VA and RA. All three agents mildly inhibited fibroblast contraction of collagen matrices. We conclude that 1) VA, RA, and BUT have differential effects on rabbit fibroblast proliferation; 2) retinoid effects on fibroblast growth vary with the tissue of origin; and 3) VA, RA, and BUT modestly inhibit fibroblast contraction of extracellular matrices. This study suggests that fibroblast-mediated processes in ocular wound healing and cicatricial disease may be differentially modulated by retinoids and BUT.


Materials and Methods. Cell cultures: This investigation conformed to the ARVO Resolution on the Use of Animals in Research. Rump dermal tissue and sclera (scraped free of conjunctiva and choroid with a sterile scalpel) were harvested from a New Zealand White rabbit (2.5 kg) anesthetized with ketamine and sacrificed with intravenous pentobarbital. Primary cultures were established by cutting tissues into 1-mm³ pieces with a sterile scalpel and pressing them onto the bottom of 6-cm-diameter culture dishes under sterile cover slips. Primary cultures were grown in Dulbecco’s modified Eagle’s medium (DME) containing glucose 4500 mg/l and L-glutamine 584 mg/l (Gibco, Grand Island, NY) and supplemented with 20% fetal bovine serum (Hyclone Labs, Logan, UT), 200 units/ml penicillin, 200 units/ml streptomycin, and 0.025 g/l Fungizone at 37°C in 5% carbon dioxide. DME was reported by the manufacturer to be free of all-trans retinol (VA), all-trans RA, and BUT. Fetal bovine serum was reported by the manufacturer to contain typically VA $4.5 \times 10^{-7}$ M and BUT $1.4 \times 10^{-5}$ M; RA concentrations, however, were not determined. Serum from the same bottle was used in all experiments. Cells were harvested with 0.05% trypsin with 0.02% EDTA and then maintained in DME with 10% fetal bovine serum and antimicrobials. Cells in second through sixth passages were used in these experiments.

Cell proliferation: For cell proliferation experiments, 1.5-2.0 $\times 10^4$ cells were plated into 24-multiwell plates (Falcon/Becton Dickinson, Lincoln Park, NJ). After allowing for cell attachment overnight, varying concentrations of the reagents were added to wells in quadruplicate. All-trans retinol (VA), all-trans RA, RA, and BUT were obtained from Sigma (St. Louis, MO). Fresh stock solutions of VA and RA in absolute ethanol were made weekly and diluted in absolute ethanol prior to being added to medium. The stock solutions were stored in the dark at $-20^\circ$C. VA and RA were handled only in subdued light. The final concentration of ethanol in each well was 0.1%, including corresponding control wells. BUT was dissolved directly in media. For each agent, control and treated cells were grown in a single 24-multiwell plate. After a 72-hr treatment period (without a change of media), the cells were harvested with 0.05%
trypsin/0.02% EDTA and counted with a Coulter cell
counter (Coulter Electronics, Hialeah, FL). Cell vi-
ability was assessed by the trypan blue dye exclusion
method.\textsuperscript{5}

\textbf{Contraction of collagen matrices:} Collagen matri-
ces were prepared by the method of Montesano and
Orci.\textsuperscript{6} Confluent fibroblasts were harvested with
trypsin treatment and counted, and the desired
amount was centrifuged in a plastic tube. The tube
and all solutions were placed on ice. Eight volumes of
type I rat tail collagen (Collaborative Research, Bed-
ford, MA) at a concentration of 1.25 mg/ml were
mixed with 1 volume of 10X DME and 1 volume of
sodium bicarbonate (11.76 mg/ml). The cell pellet
was resuspended with the cold collagen mixture (1
mg/ml). Aliquots (2 ml) of the collagen–cell mixture
(approximately $10^5$ cells per 2 ml collagen mixture)
were placed into each 35-mm well of six-multiwell
plates (Falcon/Becton Dickinson, Lincoln Park, NJ);
the mixtures were allowed to gel for 10 min at 37 °C
before the addition of medium. After aliquots (1 ml)
of medium containing triple-strength reagents were
added to the 2-ml gels in triplicate, the gels were de-
tached from the walls and bottoms of the wells with
bent 19-gauge needles. After 4 days (during which
time the media was not disturbed), the diameter of
each gel (mean of major and minor axes) was mea-
sured to the nearest 0.5 mm using graph paper with
1-mm divisions. To count cells, gels were dissolved in
collagenase (Advance Biofactures, Lynbrook, NY) at
a concentration of 4 mg/ml for 60–90 min at 37°C;
freed cells were counted in a hemocytometer.

\textbf{Statistical analysis:} To evaluate differences among
final cell counts or collagen gel diameters, the student
t-test was used when the effects of only two test media
were compared, and analysis of variance (ANOVA)
was used when the effects of more test media were
compared.

\textbf{Results.} The response of rabbit dermal fibroblast
proliferation to VA, RA, and BUT was different for
each of the three reagents (Fig. 1). Whereas VA did
not affect cell proliferation (ANOVA, $P = 0.5$), RA
stimulated (ANOVA, $P < 0.001$) and BUT inhibited
it (ANOVA, $P < 0.001$). The concentrations of all
three reagents tested were nontoxic as determined by
the trypan blue dye exclusion method.

Collagen matrix contraction by dermal fibroblasts
was mildly inhibited by all three reagents—VA
(ANOVA, $P = 0.001$), RA (ANOVA, $P = 0.001$), and
BUT (ANOVA, $P = 0.002$) (Fig. 2). Digestion of gels
with collagenase revealed no differences in the num-
ber of cells in gels among treatment groups (data not
shown). Therefore, differences in the degree of con-
traction of the gels were not due to differences in cell
proliferation within the gels.

Because retinoid effects on fibroblast growth have
been reported in some cases to vary with the tissue of
origin,\textsuperscript{7,8} we examined the effects of VA and RA on
rabbit scleral fibroblasts (Table 1). Proliferation was
stimulated by both VA $10^{-5}$ M and RA $10^{-5}$ M (stu-
dent t-test, $P < 0.01$ and $P < 0.001$, respectively).
Contraction of collagen matrices was inhibited by
both VA $10^{-5}$ M and RA $10^{-5}$ M (student t-test, $P$

\textbf{Discussion.} Changes in cell proliferation, migra-
tion, and interaction with the extracellular matrix are
features not only of the wound healing response but
also of oncogenesis. It is not surprising, then, that
inhibitors of DNA synthesis and mitosis evaluated
initially as cancer chemotherapeutic agents have been
reevaluated for use as potential therapeutic inhibitors
of wound healing.\textsuperscript{9}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Differential effects of VA, RA, and BUT on rabbit dermal fibroblast proliferation. Plotted values are expressed as a percent-
age of controls. Vertical bars indicate SEM ($n = 4$).}
\end{figure}
Retinoids and BUT inhibit proliferation and induce markers of differentiation in a variety of malignant cells. Retinoid effects on untransformed fibroblast proliferation are variable, however, and may vary with the tissue of origin. BUT, on the other hand, inhibits proliferation of all cell types examined. To our knowledge, the effects of these agents on fibroblast-mediated contraction of the extracellular matrix have not previously been examined.

Vitamin A, RA, and BUT all are believed to act by influencing gene expression in cells. Retinoids play an important role in morphogenesis and epithelial differentiation, probably by controlling gene transcription through a cytoplasmic receptor-mediated mechanism similar to that used by glucocorticoids. The physiologic role of BUT, which is formed naturally in the body by the hydrolysis of ethylbutyrate, and the mechanism of action of BUT are less clear, but may involve alterations in intracellular cAMP levels.

We have demonstrated that VA, RA, and BUT modulate two key processes of the wound healing response: fibroblast proliferation and fibroblast-mediated contraction of the extracellular matrix. Specifically, RA stimulated and BUT inhibited both rabbit dermal and ocular fibroblast proliferation. VA, however, demonstrated a tissue-specific effect on cell proliferation: dermal fibroblast growth was unaffected, but scleral fibroblast growth was stimulated. We cannot rule out the possibility that VA in the fetal calf serum we used may have already maximally stimulated dermal fibroblast growth, resulting in no demonstrable effect with the addition of more VA. Our observation that retinoids stimulate rabbit scleral fibroblast proliferation and a recent report that retinoids increase rabbit Tenon's capsule fibroblast migration together suggest that retinoids may stimulate fibroblast-mediated ocular wound healing. BUT, on the other hand, inhibited fibroblast proliferation and therefore may inhibit wound healing.

Vitamin A, RA, and BUT all inhibited fibroblast-mediated contraction of collagen matrices in vitro. The magnitudes of the effects were modest, however. Whether or not such effects are likely to be clinically significant is unclear.

This study suggests that BUT may be therapeutically useful in inhibiting exuberant fibroblast-mediated wound-healing that could lead to proliferative vitreoretinopathy and scarring of glaucoma filtering.

Table 1. Effects of VA and RA on rabbit scleral fibroblast proliferation and contraction of collagen matrices

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>VA 10^{-3} M</th>
<th>RA 10^{-3} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number</td>
<td>100 ± 1.6</td>
<td>115 ± 2.2</td>
<td>140 ± 1.6</td>
</tr>
<tr>
<td>(% of control)</td>
<td>(P &lt; 0.01)</td>
<td>(P &lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>Diameter of</td>
<td>10.5 ± 0.0</td>
<td>16.7 ± 1.2</td>
<td>17.5 ± 0.0</td>
</tr>
<tr>
<td>collagen</td>
<td>(P &lt; 0.01)</td>
<td>(P &lt; 0.01)</td>
<td></td>
</tr>
<tr>
<td>matrix (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM. For cell number determinations, n = 4; for diameter of collagen matrix determinations, n = 3. Results of the student t-tests are given in parentheses.
fistulas. Retinoids, on the other hand, differentially modulate fibroblast growth and contractile properties and therefore may aggravate undesirable wound-healing responses.

Key words: butyrate, proliferative vitreoretinopathy, retinoic acid, vitamin A, wound healing

From the Department of Ophthalmology, University of California, San Francisco, California. *Current affiliation: the Laboratory of Molecular and Developmental Biology, National Eye Institute, Bethesda, Maryland. Supported by grant EY-03228 from the National Eye Institute, National Institutes of Health, Bethesda, Maryland. Presented in part at the Annual ARVO Meeting, Sarasota, Florida, May 1–6, 1988. Submitted for publication: June 30, 1989; accepted October 24, 1989. Reprint requests: Dr. Robert Y. Kim, National Institutes of Health, Building 6, Room 204, Bethesda, MD 20892.

References

Separate Mechanisms for Retinal Damage by Ultraviolet-A and Mid-Visible Light

Laurence M. Rapp, Barbara L. Tolman, and Hardeep S. Dhindsa

Retinal damage by light has two distinct action spectra, one peaking in the ultraviolet-A (UVA) and the other in the mid-visible wavelengths (green light). Here we show in a single animal species, the Long Evans rat, that UVA and green light can produce histologically dissimilar types of damage. UVA light in particular produces severe retinal damage at low irradiation levels. Furthermore, the mechanism of damage by UVA light is different from that of green light as determined by their relative rhodopsin bleaching efficacies. These results provide convincing evidence that different chromophores mediate damage by UVA and green light. By producing both classes of damage in a single species, a sound model is provided for further investigation into the different forms of photic retinopathy. Invest Ophthalmol Vis Sci 31:1186–1190, 1990

Retinal phototoxicity is an incompletely understood phenomenon. One of the barriers to a better understanding of photic retinal damage has been the absence of a consistent action spectrum across the animal species that have been studied. The action spectrum for damage in the predominately rod retinas of rodents has been found to peak in the mid-visible wavelengths (green light). Ironically, green light also represents the wavelengths most efficient in bleaching the visual pigment, rhodopsin, and thus in stimulating vision.1–3 Studies on the primate retina, in contrast, have shown that the action spectrum for photic damage peaks in the ultraviolet-A (UVA) wavelengths.4 Few studies have examined the damaging capacity of UVA light in nonprimates.5–7 None of these has assayed rhodopsin bleaching efficacy of UVA light. However, vertebrate rhodopsin does have a small absorption peak in the UVA, in which rhodopsin is bleached with approximately one third the efficiency of its peak in the visible.8 In order to determine if damage by both green and UVA light is mediated by rhodopsin absorption, we sought to produce these types of damage in a single animal species, the Long Evans rat, and subsequently to characterize the rhodopsin bleaching efficacy of each spectral band.

Materials and Methods. Animals: Pregnant Long Evans (pigmented) rats were purchased from Charles