Int. J. Dev. Biol. 34: 433-439 (1990)

Original Article

Effect of vitamin A on wound epidermis during forelimb regeneration in adult newts

STAUROS KOUSSOULAKOS*, KUMAR KRISHAN SHARMA and HERMANN JOSEF ANTON

Zoological Institute, University of Cologne, Germany

ABSTRACT The effects of vitamin A on blastemal epidermis were studied during the early postamputational period of forelimb regeneration in *Triturus alpestris*. Vitamin A was administered through oral intubation at a dose of 250 IU per gram of body weight per day. The results were evaluated by morphometry, histology, and autoradiography. After 7, 11 and 14 days of treatment, several alterations were observed in the wound epidermis: a) reversal of keratinization; fewer keratinized cells were counted in sections from vitamin A-treated limbs; b) decrease in the incorporation of tritiated thymidine, as judged by estimation of labeling indices; c) increased mitotic activity in the cells of the *stratum germinativum*, and in the middle layer of the epithelial cells, as well. The significance of these cellular effects is discussed against the relevant literature.

KEY WORDS: *vitamin A, epidermis, regeneration, urodeles*

Introduction

Soon after amputation of a urodele amphibian limb, cells from the basal layer of the adjacent epidermis extend pseudopodia and migrate over the cut surface and cover the trauma by a wound epidermis (Repesh and Oberpriller, 1980). Piling up of the migrating cells at the center of the amputation surface results in a thickening of the wound epidermis to form the apical epidermal cap (Tank *et al.*, 1976). Wound epidermis is composed of several cell types, such as keratinocytes, phagocytes, mucous-excreting cells, Leydig cells, ovoid cells (Stocum, 1985), and various epithelial cells active in RNA (Bodemer, 1963) and protein (Anton, 1965) synthesis. There is substantial evidence that wound epidermis synthesizes several hydrolytic enzymes associated with the dedifferentiated state of the stump mesodermal cells (Eisen and Gross, 1965; Grillo *et al.*, 1968), and various growth factors as well (Globus *et al.*, 1980). All these products in combination provide an environment which retards blastema cell redifferentiation allowing sufficient time for cell proliferation, so that regenerate outgrowth can occur.

One of the major differences between skin epidermis and wound epidermis is that the latter remains dermis-free through all stages of limb regeneration (Singer and Salpeter, 1961). The absence of the dermis underneath the wound epidermis brings into intimate contact ectodermal and mesodermal cells, whose interactions play important roles in several morphogenetic procedures, particularly in determining the proximal-distal organization during amphibian and avian limb development (Saunders, 1948; Zwilling, 1956; Tschumi, 1957; Faber, 1971), and amphibian limb regeneration as well (Stocum and Dearlove, 1972).

Apart from the above cited vital functions and properties of the wound epidermis, there is some evidence that it might also have a role in pattern specification. Elegant experiments performed by Carlson (1975) revealed that no asymmetry in morphogenetic quality can be detected in the axes of the epidermis. In this respect, additional experiments (Maden, 1977; Stocum, 1978, 1980) have shown that actually epidermis does not possess graded positional values, but a single one, uniformly distributed over the entire limb surface, instructing the underlying mesodermal cells where the outer boundary of the regenerate is. Thus, wound epidermis seems to be endowed with a number of qualities, most of which can be influenced by the action of vitamin A and its analogues, the retinoids.

Retinoids exhibit a wide range of different and frequently opposite effects when administered to various biological systems *in vivo* and *in vitro*. Excess of retinoids change keratinizing epithelia to mucous secreting ones (Fell and Mellanby, 1953), modify glycosaminoglycan and glycoprotein synthesis (Levin *et al.*, 1983), inhibit cell division in a wide variety of cell types (Lotan, 1980) and in axolotl blastema mesenchymal cells (Maden, 1983; Sharma and Anton, 1986), intensify blastema cell proliferation in anurans (Jangir and Niazi, 1978; Alam, 1983) and terrestrial urodeles (Koussoulakos and Anton, 1988a), alter the pattern of synthesis of many proteins and enzymes, such as fibronectin, laminin, collagen, plasminogen, glycosaminoglycan, protein kinases, transglutaminase, etc. (Roberts and Sporn, 1984), and finally they respecify cell

Abbreviations used in this paper: CRABP, cellular retinoic acid binding protein; RAR, retinoic acid receptor.

***Address for reprints**: Zoological Laboratory, University of Athens, Panepistimiopolis, GR-157 84 Athens, Greece. FAX: 724.31.63

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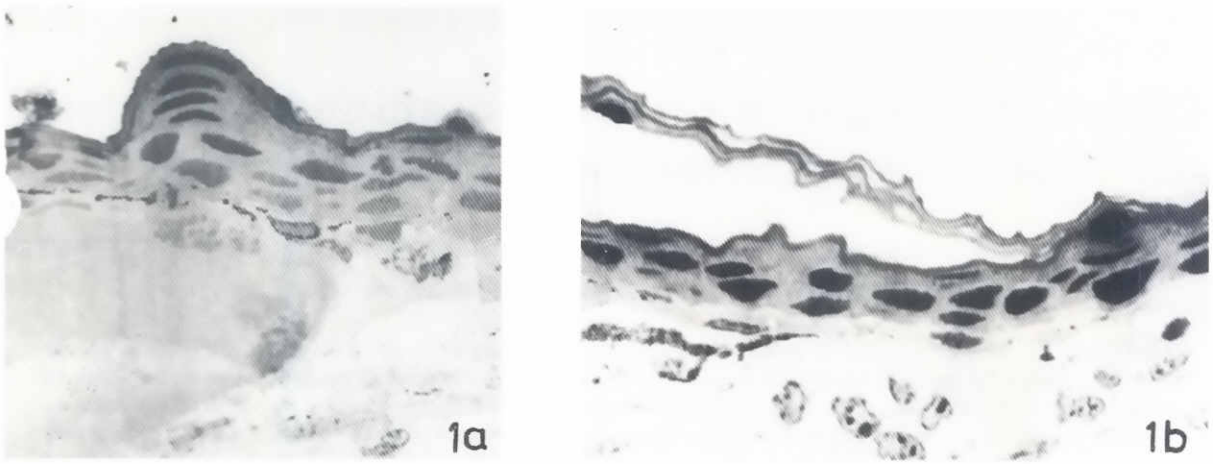

Fig. 1. Longitudinal, vertical sections at the middle of the zeugopod of the adult newt *Triturus alpestris*. **(a)** *Structure of the physiological skin with dermal glands. As is usual in amphibians, epidermis exhibits considerable variation in thickness, even in neighboring areas. The horny layer either was shed immediately before, or it was lost during histological procedure. The more flattened epidermal cells at the outermost layer are in a transitional stage of cornification with evident nuclei.* **(b)** *Shedding of the outer keratinized layer. Ostensibly, after molting, the epidermis displays a uniform thickness.*

positional memory during avian limb development (Tickle *et al.*, 1982; Summerbell, 1983) and amphibian limb regeneration (Maden 1983; Kim and Stocum, 1986; Koussoulakos *et al.*, 1988).

Taking into consideration the possibilities of putative influences of retinoids on wound epidermis, it seems unfortunate that most of the studies of the effects of vitamin A during limb development and regeneration are confined to their effects on blastema mesenchyme, whereas, only two works have been hitherto published dealing with vitamin A-treated wound epidermis (Maden, 1983, 1984). Our interest in studying further the effects of retinoids on the wound epidermis motivated the present investigation.

We report here that systemic administration of vitamin A to young, postmetamorphic *Triturus alpestris* influences considerably both developmental parameters of the wound epidermal cells, namely differentiated state and proliferation. This is the first time that DNA synthesis and mitotic activity have been studied in wound epidermal cells of regenerating urodele limbs after treatment with retinoids. Our findings are evaluated within the framework of background knowledge in the field of ectodermal-mesodermal interactions.

Results

Morphological observations

As expected, untreated animals regenerated only the distal limb elements removed by amputation. In the treated group, the degree of proximalization depended on the time-window of exposure to the drug. The most consistent results were obtained after 11 days of

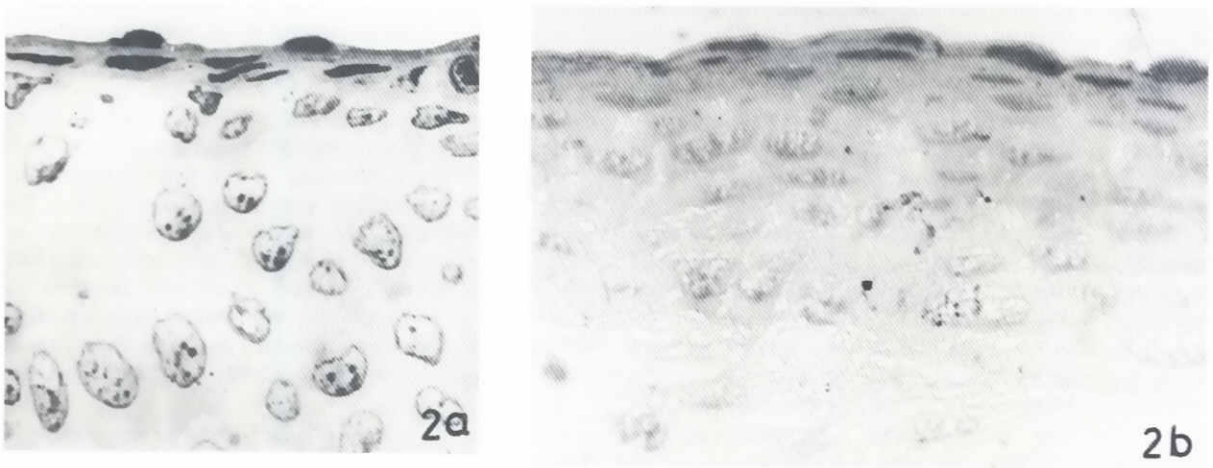

Fig. 2. Vertical sections along the anterior-posterior axis through the wound epithelium of a regenerate at the 4th day post amputation. **(a)** *Control epidermis,* **(b)** *treated epidermis. No obvious difference is encountered concerning the keratinization of the outer layer of this peculiar epithelium. Only 2 layers of dark, flattened cells, with evident nuclei are shown. No mitosis was observed, since this epithelium originates solely by migration and piling up of stump mesodermal cells.*

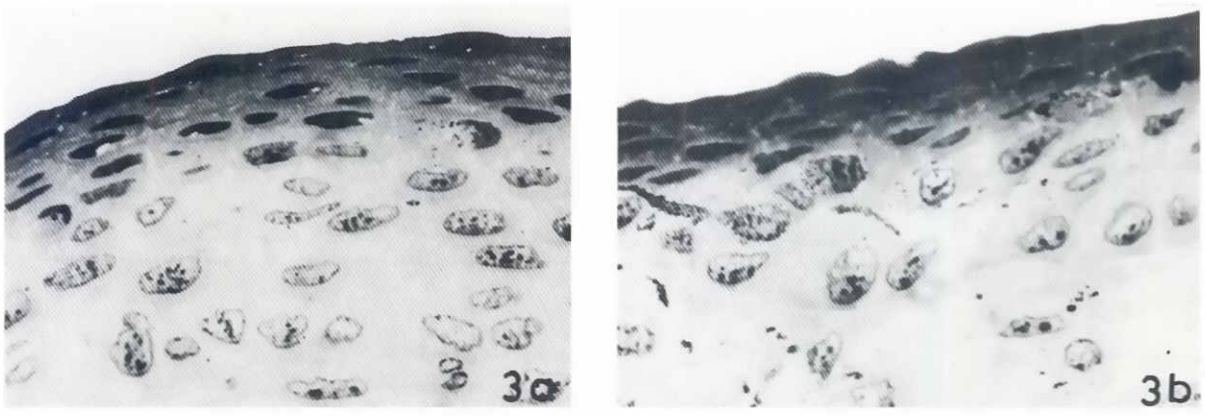

Fig. 3. Vertical sections at the distal part of forelimb blastemata growing in the absence (a), and in the presence (b) of vitamin A palmitate. *At this stage (7 days post amputation), cell counting of compressed, blackened cells revealed fewer keratinized cells in the treated epithelium.*

vitamin A treatment. Therefore our studies on DNA synthesis and mitosis concern mainly the 11-day regenerates (for details on limb morphology, see Koussoulakos *et al.*, 1988).

The skin of the adult *Triturus alpestris* is made up of two components, the epidermis and the dermis. These two embryologically different tissues are physically separated by a distinct basement membrane. The epidermis rests on the basement membrane by a layer of columnar to cuboidal cells, which, due to their high proliferative capacity, constitute the *stratum germinativum*. The cells of the *stratum germinativum* divide, and one of the daughter cells migrates upwards changing its morphology to polyhedral. As they move towards the surface they still continue dividing and start producing keratohyalin granules. Still further out lie cells in which nearly all the intracellular organelles have disappeared, the nucleus, if still present, is flattened and darkly stained, and finally such cells are reduced to flattened scales (Fig. 1a). These cells contain practically nothing but keratin, thus justifying their characterization as the *stratum corneum*. This keratinized layer of epidermal cells is shed regularly (Fig. 1b), and the lost cells are continually replaced by proliferation in the *stratum germinativum* and in the middle layer, as well.

On the 4th day after amputation, both vitamin A-treated and control animals displayed stumps covered by a 3- to 4-cell-thick layer of wound epidermis. Most of the epidermal cells were polyhedral, whereas the outermost layer consisted of keratinized cells (Fig. 2a, b). At this early developmental stage it was difficult to reveal any quantitative differences in the percentages of keratinized cells between treated and control animals.

Seven days after surgery the number of epidermal cells becoming keratinized increased in the control animals, as compared to their treated counterparts (Fig. 3a, b).

Examination of untreated and vitamin A-treated 11-day regenerates revealed great differences in the organization of the epidermis covering the underlying mesodermal components. In the vitamin A-treated regenerates, wound epidermis appeared to be still in a juvenile state, consisting mainly of healthy, polyhedral epithelial

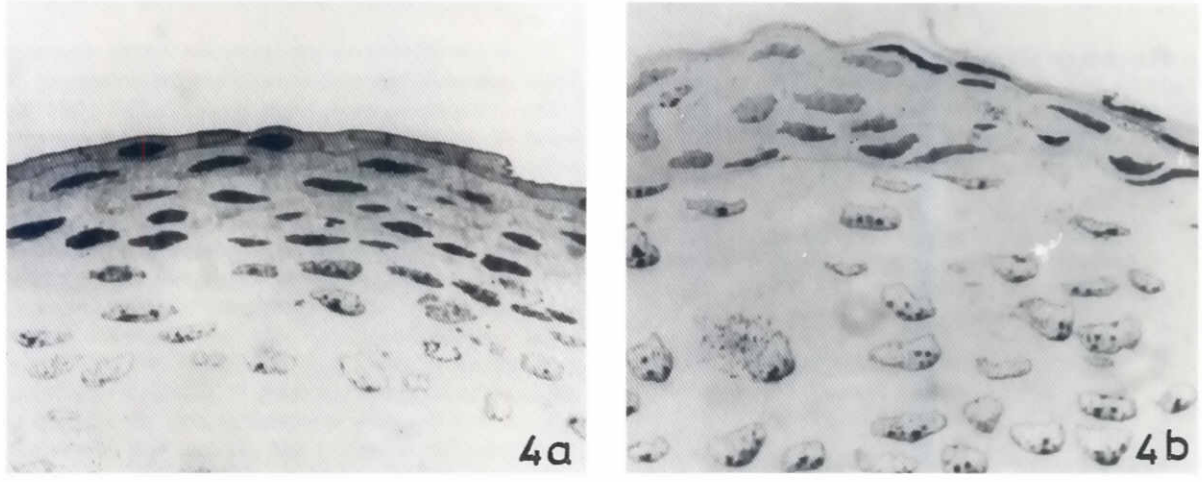

Fig. 4. Vertical sections at the wound epithelium 11 days post amputation. *At this stage, the difference in the keratinized state is obvious. The transitional layer of cells, which will be shed in due time, is clearly shown only in control wound epithelium* **(a)**, *where also the number of darkly stained cells is increasing. In contrast, the well known phenomenon of hypokeratinization after vitamin A treatment is expressed in treated animals* **(b)**.

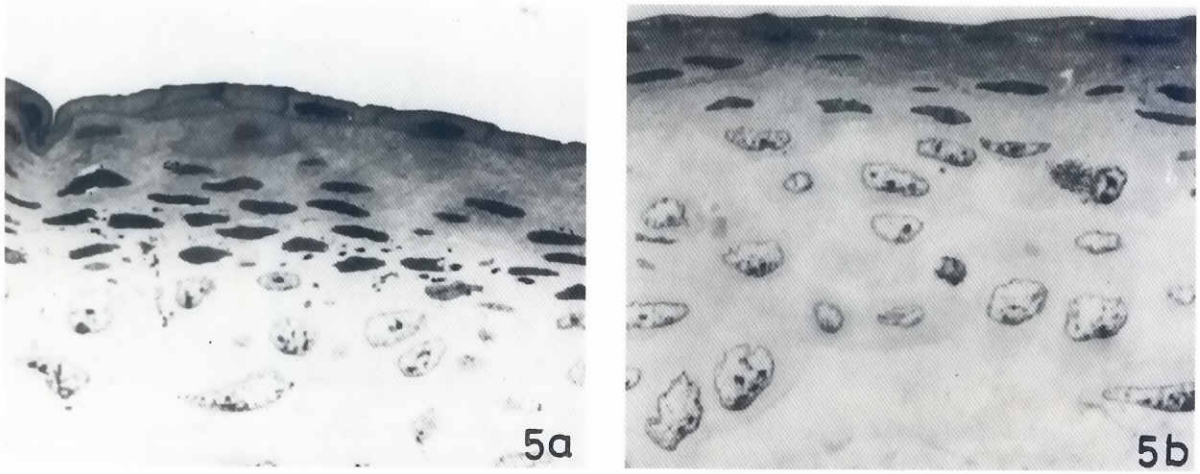

Fig. 5. Vertical sections at the wound epithelium 14 days after amputation. *At this time-point the keratinization in the control limbs,* **(a)**, *is advanced, whereas, experimental limbs,* **(b)**, *display a figure of considerably delayed differentiation of keratinizing cells. The fusion of the transitional cell layer is quite evident, as these cells prepare for the periodic hormone-dependent sloughing.*

cells. The outermost layer, the *stratum corneum,* had a limited number of darkly stained, flattened cells (Fig. 4b). On the other hand, in the group of untreated animals, the transition of cells from the polyhedral shape to the flattened, keratinized state seemed to be more advanced, as judged by the increased number of such cells. It is worth noticing that even at this advanced developmental stage, a basement membrane is still lacking (Fig. 4a). Similar results were obtained from the 14th-day regenerates, displayed in Fig. 5a, b.

In 11-day blastemata from control and experimental animals, various sections from several specimens were subjected to counts of keratinized, radiolabeled, and mitotic cells. The results are depicted in Table 1, and indicate that, vitamin A obviously affects the process of keratinization of epithelial cells, resulting in a decrease in the percentage of such cells (40%) in comparison with control figures (60%). At first glance, it seems that the well known phenomenon of mitotic inhibition of several cell types by retinoids is expressed in the decrease of the labeling index from 5.1% in the controls to 3.4% in the treated group (Fig. 6a, b). However, the mitotic index in treated animals displays higher values (1.5%) than in the controls (0.25%), suggesting an activation of growth.

TABLE 1

QUANTITATION OF VITAMIN A PALMITATE EFFECT ON KERATINIZATION, DNA REPLICATION, AND MITOTIC ACTIVITY IN THE BLASTEMA EPIDERMIS OF THE NEWT *TRITURUS ALPESTRIS*, AS OBSERVED ON THE 11th DAY AFTER AMPUTATION AND VITAMIN A TREATMENT(@)

Animal group	Number of epithelial cells/defined area	Number of keratinizing cells	Mitotic index (%)	Labeling index (%)
Control	378±6.6	226±3.1 (60)* #	0.25±0.03 #	5.1±0.48 #
Experimental	551±11.3	219±2.3(40)	1.50±0.04	3.4±0.64

(@) Values are expressed as the mean ± standard deviation.
(*) Numbers in parentheses indicate percentage of the total.
(#) Control and experimental values from each column differ significantly at the 0.001 level of statistical confidence, as judged by conventional statistical analysis (t-tests).

Discussion

The notion that epidermis might play an important role in organizing proximal-distal sequence of limb elements during ontogeny and regeneration has been repeatedly supported (Saunders, 1948; Stocum and Dearlove, 1972; Stocum, 1985). However, surgical interventions attempting to identify graded positional values on epidermis have weakened the possibilities for a central role of wound epidermis in pattern formation (Carlson, 1975).

In order to investigate the implication of the wound epidermis in position-specific cell differentiation of the underlying mesodermal cells in *Ambystoma mexicanum*, Maden (1983, 1984) exploited the ability of retinoids to respecify cell positional memory. In these works, the developmental parameters of the wound epidermal cells were not evaluated. Recall that, axolotl, the animal used in those studies, does not exhibit a normal epithelial cell differentiation, since it is among those few amphibians that do not possess a horny layer at the outermost part of their epidermis (Spearman, 1968). Moreover, mitotic rate and differentiation in wound epidermal cells might well be associated with some special functions in the regenerative process (Hay and Fischman, 1961). An additional argument to continue searching for vitamin A effects on epidermal cells is that the early observation that an intimate relationship exists between vitamin A and division and differentiation of epithelial cells (Fell and Mellanby, 1953) is strongly supported by the recent identification of cellular retinoic acid binding protein (CRABP) and b-retinoic acid receptor (b-RAR) in several epithelial cells (Smith *et al.*, 1989).

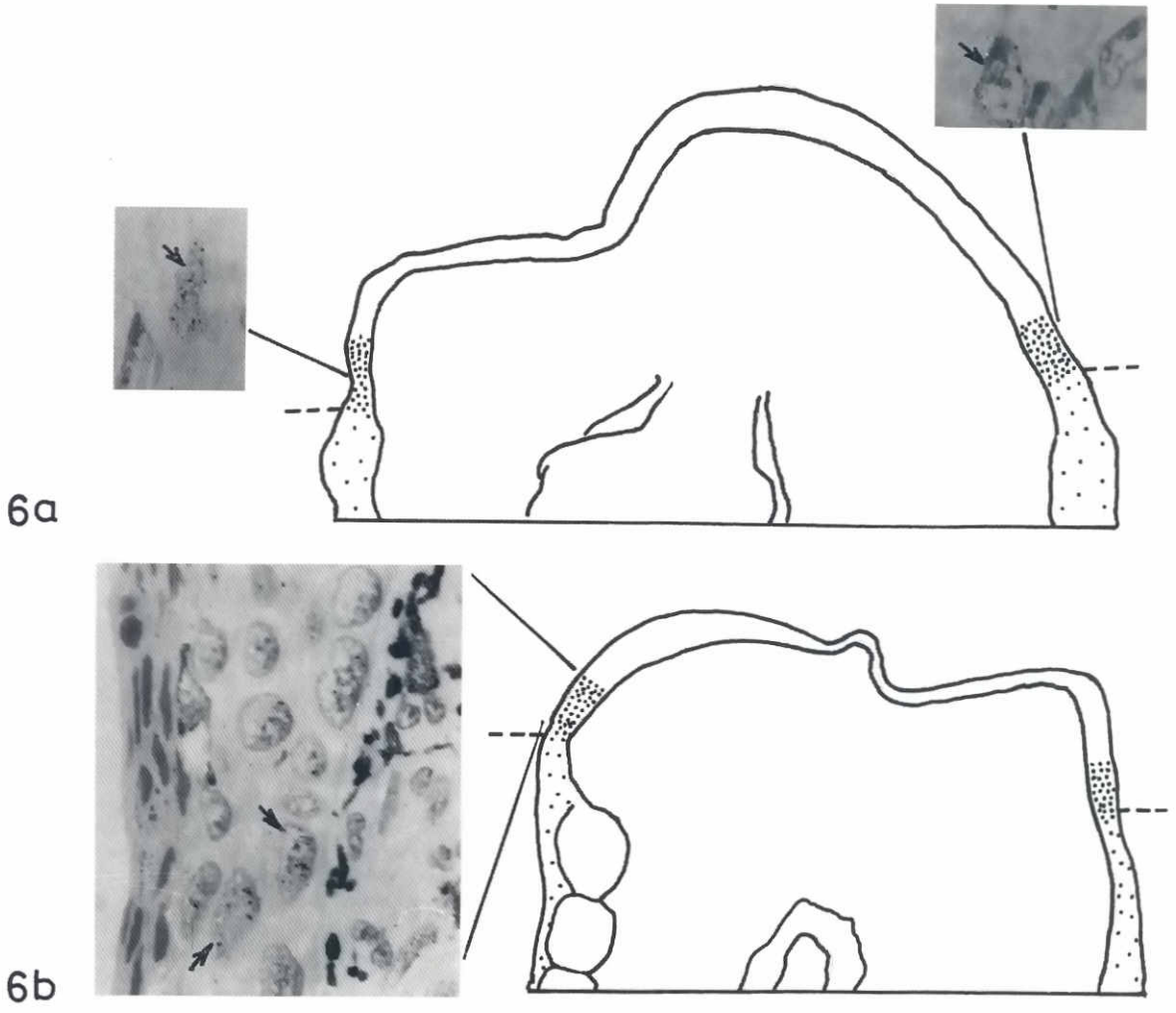

Fig. 6. Outline diagrams of *Triturus alpestris* forelimb blastemata showing incoporation of tritiated thymidine in the wound epidermis of untreated (a), and vitamin A-treated (b), limbs, observed on the 11th day. *Note the intense labeling (black dots) in the epidermis near the end cut of the stump. Arrows indicate labeled nuclei in the epidermis. Dotted lines indicate level of amputation. Most of the labeled and mitotic cells were confined to these boundary regions, in accordance with the background knowledge that wound epithelial cells at the tip of the epidermis do not divide at early stages.*

The experiments described in the present study are the first from a broader research programme attempting to clarify the interactions between blastema mesenchyme and wound epidermis. The aim is to reveal and register the cellular alterations induced in wound epidermis of a regenerating urodele limb by the retinoid vitamin A palmitate. To achieve this goal we chose as experimental animals the terrestrial species *Triturus alpestris*, which displays the normal process of molting through keratinization. Moreover, *Triturus alpestris* does not exhibit blastema growth retardation during vitamin A treatment (Koussoulakos *et al.*, 1988) as axolotl does (Maden, 1983), so that nearly equivalent developmental stages are expected at the same time period between control and treated animals. We also avoided the, at that time, usual method of vitamin A administration, namely immersing the animals into a solution of retinoids. This procedure exposes the epidermis directly to toxic concentrations of the drug (about 50 μM), whereas the physiological concentration of endogenous retinoic acid in chick wing bud amounts to approx. 25 nM. To overcome this difficulty we adopted the oral administration method (Koussoulakos *et al.*, 1988). Vitamin A palmitate, after intestinal absorption, is stored in the liver in the form of long-chain esters of retinyl palmitate. When needed by the body tissues, esters are hydrolyzed and the vitamin is mobilized as free alcohol (retinol), and is transported around the body bound to a specific plasma retinol binding protein and prealbumin. Within the target cells retinol may be required in its alcohol form, or, depending on the cell type, it may be converted to retinoic acid via an aldehyde intermediate. The results obtained by the oral administration procedure should be regarded as reliable, since the drug is expected to act in a near physiological fashion.

We report here that both developmental parameters of the wound epidermal cells, namely differentiation and growth, are significantly affected by the treatment of the animals with vitamin A palmitate. Treated wound epidermis displays a lesser degree of differentiation, as judged by the lower percentage (40%) of the

keratinized cells encountered, in comparison with the untreated ones (60%). Our working hypothesis is that vitamin A delays wound epidermal cell differentiation, and in this way retards mesenchymal cell differentiation, allowing sufficient time for cell proliferation to occur so that a larger cell mass is produced at the distal stump in order to form a proximally duplicated regenerate. There is substantial evidence supporting our hypothesis. Hay and Fischman (1961) have shown that the cells in the apical epidermal cap do not divide during the first days after amputation. It is tempting to speculate that, in this way, cells avoid performing their "quantal mitosis" (Bishoff and Holtzer, 1969), thus delaying differentiation. Tassava and Mescher (1975) and Globus *et al.* (1980) have demonstrated that the mesenchyme underneath the apical cap remains undifferentiated for a longer period of time, and displays, comparatively to other mesenchymal regions, higher cell proliferation. Differentiation of wound epidermal cells is followed by a decrease in mitotic division in the adjacent mesenchymal cells. In the same context, Goss (1969) has shown that differentiation in proximal regenerates is slower than in distal ones, whereas Maden (1976) reported that, for pattern differentiation to commence, the mesenchyme cell mass required is larger, as the level of amputation is shifted proximally.

The effects of retinoids on DNA synthesis, on the one hand, and mitosis on the other, in wound epidermal cells seem at first glance controversial. We found a decrease in the labeling index accompanied by an increase in the mitotic activity. The possibilities that the differences between the labeling indices in control animals (5.1±0.48) and experimental ones (3.4±0.64) occur by chance are less than 0.1%. The density of the silver grains over the nuclei of the treated regenerates is comparable to that of control ones, suggesting that the thymidine incorporation reflects true nuclear DNA synthesis. In a recent work (Koussoulakos and Anton, 1988b) we have reported comparable results concerning mesenchyme, and it has been suggested that vitamin A intensifies DNA synthesis and mitosis in *Triturus alpestris* regenerates. The fewer labeled figures obtained in the present study could be due to faster DNA synthesis, with a concomitant decrease in the duration of the S-phase. The higher mitotic rate in treated animals is also reflected in the larger number of epithelial cells encountered. The increased number of the wound epidermal cells in retinoid-treated animals might be necessary for the expected epidermal expansion to accomodate the growing mesenchyme. In this study it was clearly shown that vitamin A treatment causes several cellular alterations in the wound epithelium. Similar alterations in untreated animals are usually correlated with various changes in the mesenchyme. In this respect, we believe that the observations reported here and in other similar studies (Maden, 1983, 1984; Sharma and Anton, 1986) must be complemented by further investigations on the cellular and biochemical effects of vitamin A on wound epithelium. Then, one could possibly define the precise role of ectodermal-mesodermal interactions in pattern specification.

Materials and Methods

Animals

The experiments were performed on young, postmetamorphic terrestrial newts, *Triturus alpestris* raised *ab ovo* in our laboratory. To avoid cannibalism, animals were housed individually in vented plastic containers. They were fed live Tubifex twice a week, and cleaned every other day. The temperature of the humidified atmosphere was kept at 22±1°C at a 14:10 h cycle of photoperiodism. The animals were used in these experiments once they had reached the age of 11 months, and ranged in length (snout to tail-tip) between 47 and 62 mm.

Operations

During the surgical procedures the animals were anesthesized in 0.5 g/l tricaine methanesulfonate (MS 222 Sandoz A.G., Basel), neutralized by sodium bicarbonate. Limbs were amputated with a sharp razor blade, removing all elements distal to the mid of the radius and ulma. Protruding skeletal elements were trimmed by watchmaker's forceps, to achieve a flat wound surface. Following amputation the animals were separated into two distinct groups: experimental (to be treated with vitamin A), and control (no treatment).

Vitamin A administration

Experimental animals were subdivided into four subgroups (4, 7, 11 and 14), and administered by gastric intubation 250 IU of vitamin A palmitate (Serva Feinbiochemika, Heidelberg, oily concentrate), per gram of body weight per day, for 4, 7, 11 and 14 days postoperation, respectively (Koussoulakos *et al.*, 1988).

Morphological observations

Camera lucida drawings of the regenerates were performed under a relatively high magnification (12X) on alternating days to monitor growth and morphogenesis of control and experimental limbs. Two months after the initial surgery, the limbs were severed at the mid of the stylopod, stained *in toto* by Victoria blue and cleared in methyl benzoate, to reveal skeletal organization of the regenerates. Blastema morphology was also assessed by drawing and comparing equivalent sections.

Histoautoradiography

To assess DNA synthesis in blastema epidermis, two hours before the end of each experimental period, all animals were injected intraperitoneally with 0.37 KBq (0.01 μCi) tritiated thymidine (spec. act. 1MBq/ml) per milligram of body weight. At the end of the incorporation period (2 h) the right regenerates were removed and fixed for 2 h in Lillie's fixative, and then stored in 70% alcohol saturated with non-radioactive thymidine. Semi-thin sections (1 μm) were cut on LKB ultramicrotome after embedding in Spurr's resin (1969). The sections, performed horizontally along the anterior-posterior axis of the blastema, were mounted on glass slides, and dipped in Ilford K2 emulsion. After 4 weeks of exposure in the dark, the sections were processed by Kodak D19 developer and stained by 1% neutral red. For histological observations representative sections were stained by 1% toluidine blue in 10% borax.

Quantitative analysis

A total of 24 specimens were used throughout this experimental series; 3 controls (untreated) and 3 experimentals (vitamin A-treated) from each of the 4 animal groups. Only the right forelimb regenerates were considered in this study. At least three sections from each regenerate were evaluated. Since the diameter of a *Triturus* blastema cell is approx. 15 μm, a median section and the 15th preceding and following ones were included in the analysis. All epithelial cells in each section were counted with the aid of a micrometer grid fitted in the eyepiece of a research microscope (Orthoplan Leitz). In this context, the entire epithelium in each section represents a defined area. 9 such areas were included in each group.

Keratinocytes were easily counted due to their discernable, flattened, darkly stained nuclei, which remain nearly intact during amphibian epithelial cell keratinization (Spearman, 1968; Fox, 1986). Moreover, the matrix of the tonofilaments is heavily stained by toluidine blue (Fox and Whitear, 1978). In counting these nuclei (to obtain cell numbers) care was taken not to include lightly stained or round nuclei of the lower layers of the regenerate epithelim in the keratinized group.

To obtain labeling indices, we divided the number of labeled epithelial cells by the total number of epithelial cells encountered in each section. Enough reduced silver grains in the emulsion above a nucleus indicated a labeled cell. The density of background silver grains in the emulsion was

estimated at a long distance apart from the tissue section, and it was found to be much lower than over the well defined area of a labeled *Triturus* epithelial nucleus. The reduced silver grains could be easily differentiated from melanin granules by the various levels of focus of each. Labeled granules were most sharply in focus in the emulsion above the tissue level.

The mitotic activity in each blastema was estimated by the mitotic index, which represents the ratio of the number of all well recognised phases, from late prophase to telophase, divided by the total number of epithelial cells.

Acknowledgments

The stay of Dr. S. Koussoulakos at the Zoological Laboratory of the University of Cologne was partially supported by a Heinrich-Hertz-Stiftung fellowship.

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Accepted for publication: August, 1990