Vitamin A alters the internal viscosity of fragments of limb-bud mesenchyme

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SUMMARY

Two techniques were used to examine the effect of vitamin A compounds (vitamin A acid = retinoic acid and vitamin A acetate) upon the relative strengths of adhesion among mouse limb-bud mesenchymal cells. Treatment with retinoic acid \textit{in vivo} and with vitamin A acetate \textit{in vitro} reduced the rate at which the fragments of mesenchyme rounded-up when cultured on a non-adhesive substratum, but these compounds did not alter the behavior of tissues tested in fragment-fusion experiments. These conflicting results indicate that the two tests measure different activities of cells and suggest that treatment with vitamin A alters the property(ies) of cells which regulate the internal viscosity of tissues.

INTRODUCTION

Although the teratogenic potential of vitamin A in rodents has been established for some time (Cohlan, 1953; Kalter & Warkany, 1961; Kochhar, 1967), the mode of its action is still not understood. Excess vitamin A is known to interfere with the biosynthesis of several macromolecules including glycosaminoglycans, glycoproteins, protein and RNA (Kochhar, 1977; Schimmelpfenning, Baumann & Kaufmann, 1972; Solursh & Meier, 1973; DeLuca & Wolf, 1969; DeLuca, Kleinman, Little & Wolf, 1971; Pennypacker, Lewis & Hassell, 1978; Kwasigroch & Neubert, 1978). However, the relationship between these metabolic alterations and the specific and stage-dependent congenital long bone reduction deformities produced in mouse fetuses by hypervitaminosis A (Kochhar, 1977) is not clear. Since vitamin A compounds are able to penetrate lipid monolayers (Bangham, Dingle & Lucy, 1964) and the lipid phase of biological membranes (Mack, Lui, Roels & Anderson, 1972; Nyquist, Crane & Morré, 1971), it is possible that a primary site of action of vitamin A is the cell membrane. Alterations in the integrity and structure of membranes could influence such developmental processes as oriented cell movements and cellular

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aggregations, both of which play a significant role in the development of an organ (Ede & Flint, 1975a, b; Steinberg, 1970).

Earlier we reported that vitamin A reduced the rate of migration of cells obtained from the mesenchyme of embryonic mouse limbs (Kwasigroch & Kochhar, 1975). The present study examines the possibility that vitamin A compounds might also interfere with cell-to-cell adhesion.

MATERIALS AND METHODS

Animals. ICR/DUB mice (Flow Laboratories, Dublin, Va.) were maintained in a controlled environment and fed lab chow and water ad libitum. Virgin females were caged with fertile males between 8.00 a.m. and 12.00 noon and immediately examined for the presence of a vaginal plug. If a plug was found, this was designated the 1st day of pregnancy.

Pregnant females were killed on the 12th day of development. Embryos were removed from the uterine horns and their forelimbs were cut off adjacent to the flank and placed into culture medium (Ham's F-12 + 10% fetal calf serum and containing 150 μg/ml ascorbic acid, 12.5 μg/ml streptomycin and 7.5 μg/ml penicillin-G). Epithelium was cut away as previously described (Kwasigroch & Kochhar, 1975) and fragments of mesenchymal tissue were prepared from the proximal, central and distal areas of forelimbs, as indicated in Fig. 1.

Tissue treatment. The following treated and untreated fragments of limb mesenchyme were tested: (1) control – from untreated limb buds, (2) retinoic acid-treated – from limbs of embryos exposed in utero to retinoic acid (100 mg/kg of maternal body weight given orally via gavage) on day 11½, and 3) vitamin A-acetate-treated – fragments from previously untreated limbs cultured in the presence of 10 i.u./ml of vitamin A acetate.

Fragment rounding. To determine the length of time required for central limb mesenchymal fragments to round-up (become ball-shaped) while in culture, a hanging-drop culture method was used. Fragments of limb mesenchyme (0.2–0.3 mm in diameter) were placed in individual 0.2 ml droplets of culture medium on the under surface of the top of 100 mm Falcon plastic petri dishes. Each dish had about 10 of these drops. In addition, the bottom of the dish contained 3 ml of culture medium to maintain a moist atmosphere. All cultures were maintained in an atmosphere of 10% CO₂, in air, at 37°C.

The rate of rounding-up of control and treated fragments was estimated from periodic observations of hanging-drop cultures.

Fragment fusion and envelopment. The method of Steinberg (1964) was used to determine relative strengths of intercellular adhesion. With this technique, a hierarchy of tissue adhesiveness can be established based on the behavior of tissue fragments when tested in pair combinations. Fragments of proximal, central and distal limb mesenchyme were prepared (Fig. 1) and allowed to
round up for 12–24 h as described. To test for the relative strengths of adhesion of treated and untreated tissue types, pairs of fragments were placed in contact in hanging-drop cultures and allowed to fuse (Figs. 2–5). The following tissue combinations were tested:

- control proximal – paired with – control proximal
- control proximal – paired with – control distal
- control central – paired with – control central
- control proximal – paired with – retinoic acid proximal
- control distal – paired with – retinoic acid distal
- control central – paired with – retinoic acid central
- control proximal – paired with – vitamin A acetate proximal
- control distal – paired with – vitamin A acetate distal
- control central – paired with – vitamin A acetate central

In each pair tested, one of the fragments has been cultured in the presence of 0.5 µCi/ml [3H]thymidine (specific activity 6 C/m-mole, Schwarz/Mann, Rockville, Md.) for 12 h during the time of rounding-up. The labeled fragments were washed for several hours in fresh culture medium containing 10 µg/ml unlabeled thymidine prior to being placed into hanging-drop culture with an unlabeled fragment. Cultures of fragment pairs were maintained for up to three days to permit complete fusion to occur.

Fragments were either fixed in Bouin’s solution, dehydrated and embedded
FIGURES 2-5

Drawings that demonstrate the hanging drop culture technique (see materials and methods) used to produce and fuse rounded fragments of control (C) and vitamin A-treated (T) limb-bud, mesenchymal tissue. $M = 0.2$ ml culture medium.

Fig. 2. At 0-time in culture, the fragments were irregular in outline.

Fig. 3. After 12–24 h in culture, the fragments had become spheroids.

Fig. 4. Pairs of pre-rounded fragments were placed in contact to permit fusion to occur.

Fig. 5. Single fragments, formed through complete fusion of two parent fragments, resulted.

in paraffin, or fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) postfixed in 2 % OsO$_4$, dehydrated and embedded in Spur ERL 4206. Paraffin sections were cut at 7 $\mu$m and processed for radioautography using Kodak NTB-2 emulsion. Plastic sections were cut at 3 $\mu$m and radioautograms were prepared with 3:2 diluted Ilford K-2 emulsion (Ilford Limited, Great Britain). Developed slides were stained with either hematoxylin and eosin (paraffin) or toluidine blue solution (100 ml distilled water, 1 gm toluidine blue 0, 1 gm borax) (plastic).

The sections of the fragments were examined and photographed using a 20 x objective. The location of labeled cells was determined with oil immersion optics and marked on accompanying photographs to determine which of the following possibilities occurred (For detailed description and explanation of each possibility see Steinberg, 1963):

(a) Fusion without envelopment (Fig. 6) or random intermixing (Fig. 7). These would indicate that there was no detectable difference in the relative cohesive strengths of the tissues tested.

(b) Complete (Fig. 8) or partial envelopment (Fig. 9) of one fragment by the other, which would indicate that the tissue types involved had different
Schematic representations of tissue spheroids indicating the final arrangements of cells within tissues which can be obtained when pre-rounded fragments are fused in pair combinations in vitro, as diagrammed in Figs 3 and 4. Cells from each parent (○, represents cells from one parent fragment; ●, cells from the other) remain together to form hemispheres of the final fragment.

Fig. 6. Fusion without envelopment or mixing. Such a distribution of cells indicates that the tissues tested were equally cohesive.

Fig. 7. Fusion with random intermixing. Cells from each parent are scattered throughout the resulting fragment in an apparently random manner. Cells producing this arrangement would be equally adhesive.

Fig. 8. Fusion with complete envelopment. Cells from one fragment becomes covered by cells from the other fragment. The centrally located tissue has stronger intercellular adhesive strengths than the externally segregated tissue.

Fig. 9. Fusion with partial envelopment. Cells of one fragment are capped by cells of the other. Again, the internal tissue is the more adhesive one.

strengths of adhesion. The surrounding tissue would be less cohesive than the surrounded tissue.

RESULTS

Fragment rounding. Outline drawings of representative fragments were prepared (Figs 10–13). These sketches served as ‘standard drawings’ against which the subsequent cultured fragments were compared to estimate the rate of their rounding-up.

Control and treated (retinoic acid- or vitamin A acetate-treated) fragments in hanging-drop cultures were observed at various times during an initial 24 h in culture. First examinations were conducted at 2, 4, 6 and 8 h post-culture; subsequent observations were made at 4 h intervals. More frequent
Example of outline drawings prepared from photomicrographs of an untreated fragment observed at various times during rounding-up while in culture. Subjective values of roundness (\% of roundness) were given to the shapes after viewing several hundred fragments during the rounding-up process. These standard figures were used for the estimation of roundness of subsequent fragments – both control and treated.

Fig. 10. 2 h in culture. Estimated to be about 20 \% rounded.
Fig. 11. 4 h in culture. 50 \% rounded.
Fig. 12. 8 h in culture. 75 \% rounded.
Fig. 13. 12 h in culture. 100 \% rounded.
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Fig. 14. Comparison of the average roundness of control and treated fragments during a 24 h period while in hanging-drop culture. The cultured fragments were examined for their relative roundness (% of roundness) at increasing time intervals by comparing them to outline configurations (Figs 10–13) representative of progressively rounding fragments. Both retinoic acid- and vitamin A acetate-treated fragments showed a delay in rounding-up when compared to controls. ●, control; ●, retinoic acid; □, vitamin A acetate.

Observations at room temperature were avoided in order to minimize shock caused by frequent alteration in the temperature of the culture medium in which the fragments were grown. At each examination, a value of the roundness (expressed as percentage of total roundness) of each fragment was determined by comparing the shape of the fragment with the 'standard drawings'. Figure 14 depicts a subjective estimate of the average percentage of roundness of control and treated fragments at increasing time intervals.

Control fragments. Fragments from control limb buds tended to round-up early in culture, and rounding progressed rapidly so that by 12 h nearly all control fragments were fully rounded (Fig. 14). However, a small proportion (6 %) of the control and 10 % of treated fragments failed to round-up completely even when the culture period was extended to 24 h or beyond.

Treated fragments. Retinoic acid-treated fragments showed a delay of about 2 to 4 h in the onset of rounding (Fig. 14). After initial signs of rounding, there was a stationary or lag phase between the 6th and 12th hours in culture. This was followed by a period of about 4 h during which the fragments rapidly improved in their state of roundness. During the last 4–8 h of the observation period, the rate of rounding-up once again slowed down considerably. Most
fragments did not become fully rounded until 24 h in culture, i.e. twice as long as was required for control fragments. An almost identical pattern of rounding-up was observed in the vitamin A acetate-treated fragments, except that the lag period was shorter (4 h or less, between hours 4 and 8 of culture) (Fig. 14). The time required for completion of rounding was twice as long as for control fragments (12 h for controls and up to 24 h for vitamin A acetate fragments).

**Fragment fusion.** Figures 15–28 and Table 1 indicate the fragments of each pair-type and the percentage of fragments which demonstrated: (a) fusion without envelopment (Fig. 6), (b) intermixing of labeled and unlabeled cells in an apparently random manner (Fig. 7), or (c) envelopment – either partial or complete (Figs 8, 9).

**Control v. Control.** Untreated (control) fragments from the proximal region of limbs (CP) were fused either with similar fragments (CP) or with fragments removed from the distal region of other control limbs (CD). Fusion between fragments removed from the central aspect of the limb was also performed (CC). After fusion, one of the two patterns of cell distribution was observed in most fragments: fusion accompanied by random intermixing of cells (Figs 15, 22; Table 1) and fusion with no envelopment or mixing of cells. In one

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**Figures 15–21**

Fragments formed by fusion of two rounded-up fragments that were placed in contact in hanging drops and cultured for 3 days. The fragments were paired in various control v. control and control v. treated recombinations. Drawings of resulting configurations are illustrated in Figs 22–28.

Fig. 15. Fragment formed by fusion of a control proximal (CP) and a control distal (CD) fragment. Labeled CP cells and unlabeled CD cells are intermixed in an apparently random manner.

Fig. 16. Fragment formed by fusion of CP (labeled) with CD. Internally segregated CP cells are within a chondrogenic center and are surrounded by CD cells. This occurred in 33% of the CP/CD trials.

Fig. 17. Fragment formed by fusion of a labeled CD fragment and unlabeled retinoic acid-treated distal fragment (RAD). Intermixing was the most prevalent result.

Fig. 18. Fragment formed by fusion of a labeled CP fragment and unlabeled retinoic acid-treated proximal fragment (RAP). CP covered RAP in 5% of the cases.

Fig. 19. Fragment formed by fusion of CD (labeled) and RAD fragments. Each tissue maintained its integrity as a hemisphere within the fused fragments.

Fig. 20. Fragment formed by fusion of labeled CD and unlabeled vitamin A acetate-treated distal (VAD) fragments. Cells were intermixed in all fragments tested.

Fig. 21. Fragment formed by fusion of control and vitamin A acetate fragments obtained from the center of the limb. In this single instance unlabeled VAC cells were internally segregated as a chondrogenic nodule surrounded by labeled control cells.
FIGURES 22–28

Outline drawings of the photomicrographs presented as Figs. 15–21 (drawing 22 corresponding to Fig. 15, etc.).

Fig. 22. Indicates the location of labeled cells which were scattered randomly throughout the daughter fragment formed by fusion of a previously labeled CP fragment with an unlabeled CD fragment.

Fig. 23. Identifies labelled CP cells which were covered by a cap of CD cells.

Fig. 24. CD and RAD cells were intermixed.

Fig. 25. Complete envelopment of cells of RAP fragment by CP cells occurred in only a few cases.

Fig. 26. No adhesive differences between CD and RAD cells were detected, as indicated by the labeled and unlabeled hemispheres produced in this fragment.

Fig. 27. This fragment of CD and VAD cells showed intermixing.

Fig. 28. A chondrogenic nodule formed by cells from the centre of a vitamin A-treated limb was surrounded by cells from a control fragment from a similar limb region.
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Table 1. Extent of envelopment in fragments fused while in hanging-drop culture in various paired combinations

<table>
<thead>
<tr>
<th>Type of fused fragment (treatment and limb position)</th>
<th>No. of fragments</th>
<th>% of fused fragments in each category</th>
<th>Fusion without envelopment</th>
<th>Intermixing or partial envelopment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control v. control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP/CP</td>
<td>20</td>
<td></td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>CP/CD</td>
<td>36</td>
<td></td>
<td>44</td>
<td>22</td>
</tr>
<tr>
<td>CC/CC</td>
<td>32</td>
<td></td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Retinoic acid v. control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP/RAP</td>
<td>38</td>
<td></td>
<td>21</td>
<td>74</td>
</tr>
<tr>
<td>CD/RAD</td>
<td>36</td>
<td></td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>CC/RAC</td>
<td>32</td>
<td></td>
<td>63</td>
<td>25</td>
</tr>
<tr>
<td>Vitamin A acetate v. control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP/VAP</td>
<td>32</td>
<td></td>
<td>19</td>
<td>81</td>
</tr>
<tr>
<td>CD/VAD</td>
<td>24</td>
<td></td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>CC/VAC</td>
<td>96</td>
<td></td>
<td>38</td>
<td>60</td>
</tr>
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</table>

CP, Control proximal; CD, control distal; CC, control central; RAP, retinoic acid-treated proximal; RAD, retinoic acid-treated distal; RAC, retinoic acid-treated central; VAP, vitamin A acetate-treated proximal; VAD, vitamin A acetate-treated distal; VAC, vitamin A acetate-treated central.

* CD partially surrounded CP. However, CP was chondrogenic in all cases.
† CP capped RAP.
‡ RA-Treated partially covered Control. However, some dead RA-Treated cells were seen.
§ CC partially covered VAC.

third of CP/CD fused fragments, however, a partial envelopment of CP tissue by CD cells was observed (Figs 16, 23; Table 1). In each of these cases the 'internal tissue' (CP) was chondrogenic. These results indicate that regional differences in limb tissue adhesiveness do not exist until differentiation occurs, and that with the onset of chondrogenesis intercellular adhesiveness increases.

Control v. Retinoic acid-treated. Pre-rounded fragments from the proximal region (CP), distal (CD) or central aspect (CC) of untreated limbs were paired with fragments obtained from the same regions of retinoic acid-treated limbs (retinoic acid proximal = RAP, distal = RAD or central = RAC). In most resulting fused fragments there was random mixing of labeled and unlabeled cells (Figs 17, 24; Table 1). In a small number of cases (5% for CP/RAP, 12% for CC/RAC combinations, Table 1) varying degrees of envelopment were seen (Figs 18, 25). Some instances of fusion without envelopment, resulting in a labeled and an unlabeled hemisphere (Figs 19, 26; Table 1), occurred.

Control v. Vitamin A acetate-treated. Most combinations of control and vitamin A acetate-treated tissues (VAP or VAD) resulted in fused fragments that exhibited random intermixing of labeled and unlabeled cells (Figs 20, 27; Table 1). However, a few rare (2%) cases of partial envelopment of treated tissues were observed (Figs 21, 28; Table 1). These fragment fusion results
failed to demonstrate any consistent differences in adhesive behavior between control and treated tissues.

**Discussion**

Steinberg (1962a, b) proposed that tissue fragments, consisting of cells that are free to move, resemble liquids. As a liquid droplet on a nonadhesive surface tends to round-up, so does a homogeneous tissue fragment. While rounding-up, the cells within the fluid-like fragment move past each other, making and breaking bonds. The process continues until cells exhibit maximum adherence to each other. Because of the liquid-like nature of the tissue, the driving force in the movement of cells within the fragment would be expected to be surface tensions. The rate of rounding-up of fragments would then be a function of the tissue surface tensions and internal viscosity of the tissue (resistance of the cells to slippage) (Phillips & Steinberg, 1978).

In the present studies it was found that there was a delay in the onset and rate of rounding-up of limb mesenchymal fragments treated with either retinoic acid or vitamin A acetate. These differences could have been caused by alterations in surface tensions or in internal viscosity (or perhaps both mechanisms working together) of treated fragments.

When two immiscible liquid droplets are brought into contact with a third immiscible (liquid) phase, one droplet will surround the other (Steinberg, 1962a, b). In a similar manner, when two tissue fragments possessing different intercellular adhesive strengths are placed in contact in a culture system where they are enclosed within a third phase, the liquid culture medium, they will demonstrate the phenomenon of envelopment. This fragment fusion technique indicates relative differences in surface tension between the pairs tested, as indicated by their final position within the fused fragment (Steinberg, 1970). In the fragment fusion experiments conducted, no consistent difference in behavior of control and treated tissues was detected. Apparently, treatment with retinoic acid or vitamin A acetate does not influence those factors which control the surface tension of tissue fragments. Therefore, the altered behavior of treated fragments, seen as a retarded rate of fragment rounding, must be via an effect(s) on those properties of cells which regulate the internal viscosity of tissues and may be another manifestation of those factors which are responsible for the reduced rate of movement observed in cultures of vitamin A-treated cells (Kwasigroch & Kochhar, 1975).

It is true that the *in vivo* corollary of these *in vitro*-observed changes in cellular adhesiveness and migration are unknown. It is always difficult to assume that observations made *in vitro* are an accurate reflection of effects that have been produced *in vivo* (Packard, Skalko & Menzies, 1974). However, in studies of the Talpid chick mutant, reduced cell migration and changes in cell adhesiveness have been suggested to play a role in the abnormal outgrowth of the limbs (Ede & Flint, 1975; Bell & Ede, 1978). Problems of interpretation
are also encountered when evaluating results of *in vivo* toxicology studies. For example, the specific influence upon limb development of each of the observed vitamin A-induced cellular effects that have been described *in vivo* (i.e. interference with cell proliferation and the synthesis of glycosaminoglycans, glycoproteins, protein and RNA, the production of cell death and the reduction of cell migration; Kochhar, 1977) has yet to be determined. Further study, is needed, therefore, so that the role of each of these cell activities in normal development may be defined. Only then can vitamin A teratogenesis be fully understood.

In this study, two techniques were used to compare intercellular adhesiveness of control and treated tissues. The results obtained seem to conflict. Fragment-rounding experiments indicated that there was a difference between control and treated cells, while fragment fusion experiments showed no difference between the tissues. A similar conflict has been described in the literature in investigations of Talpid chick mutants. In the Talpid₃ mutant chick, limb-bud morphogenesis is abnormal. Alterations in the property of cell adhesion have been proposed as a basis for the observed malformation (Ede & Kelly, 1964). Trypsin-dissociated Talpid₃ cells formed smaller aggregates than cells from non-mutant chick limbs, suggesting that Talpid₃ cells are more adhesive to each other (Ede & Agerbak, 1968; Ede, 1971, 1972). The size, shape and internal architecture of aggregates produced in rotation cultures using a Couette viscometer confirmed that there indeed was a difference in the intercellular adhesion of Talpid₃ and normal chick wing-bud mesenchymal cells (Ede & Flint, 1975a, b). However, using fragment fusion and sorting out experiments with Talpid₂ (whose limb bud appears similar to that of Talpid₃) and normal chick limb-bud tissue, no detectable difference in adhesion could be observed (Niederman & Armstrong, 1972). The possibly conflicting results obtained with Talpid₃ and Talpid₂ limbs could be due to variance in the cellular make-up of limbs of the respective mutants, or the two techniques have dissimilar sensitivities and the fragment fusion method was not capable of demonstrating a difference in the behavior of Talpid₂ limb-bud cells. Similarly, the fragment fusion technique may be incapable of demonstrating subtle differences in cellular behavior induced by vitamin A treatment. The capabilities of these two techniques should, therefore, be more critically examined and defined.

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