Antitumor Activity of Vitamin A Acid and Fluorouracil Used in Combination on the Skin Tumor, Keratoacanthoma  

Lawrence Prutkin  
Department of Anatomy, New York University Medical Center, New York, New York 10016  

SUMMARY

A study was undertaken to observe the gross and ultrastructural effects of vitamin A acid and fluorouracil used in combination and in different concentrations on the skin tumor keratoacanthoma. Both fluorouracil and vitamin A acid used separately and applied locally to tumors had an effect on tumor regression. However, when fluorouracil is given concomitantly with vitamin A acid, then tumor regression is markedly enhanced depending on the concentration of both drugs. Those tumors treated with 0.3% vitamin A acid and either 2 or 5% fluorouracil exhibited many large autophagic vacuoles in the epithelial cytoplasm. Vitamin A, a known labilizer, may increase the release of degradative enzymes into the cytoplasm and could account for the increased tumor regression.

INTRODUCTION

The keratoacanthoma is a skin tumor that can be produced in experimental animals (8, 9, 13—17) and man (8, 18). The tumor is characterized by rapid growth, excessive keratinization, and spontaneous regression. Complete regression (full clinical remission) in man usually averages several weeks to months while, in the rabbit, complete regression usually averages 3 weeks to 1 month.

In a previous study in which vitamin A acid was topically applied to keratoacanthomas in rabbits, regression was noticeably accelerated (14). In another study, vitamin A acid was applied locally to 60 human patients, which treatment resulted in partial or complete regression of premalignant and malignant skin tumors (2). The inhibition of keratinization in certain epithelia by an excess of vitamin A has been well documented (5—7). One resultant phenomenon of hypervitaminosis A is a mucous metaplasia in the treated epithelium (5, 14).

It is well known that antimetabolites can affect epidermal structure and function. Fluorouracil has been used in the treatment of superficial basal cell epitheliomas (11, 12) and has been shown to produce an accelerated regression of the keratoacanthoma (10).

Prompted by observations that vitamin A acid can affect both keratinization and skin tumors and that 5-FU2 has been selectively used as a successful cytotoxic agent in certain skin tumors, a study was undertaken to observe the gross and ultrastructural effects of these 2 drugs used in combination and in different concentrations on the keratoacanthoma.

MATERIALS AND METHODS

Fifty-three albino male rabbits (average weight, 1 kg) had the inner surface of their left ear auricles painted twice weekly with DMBA in equal parts of lanolin and mineral oil. After 2 weeks of carcinogen application, the follicular ostea were very prominent. After 6 weeks, all the rabbits had developed keratoacanthomas on their ears (average diameter, 0.5 to 1 cm), with an average yield of 4 to 6 tumors per ear. After the 6th week (12 applications), the procedure of applying carcinogen to the ears was stopped with 45 of the 53 animals. The 53 animals were separated as follows: 4 groups consisted of 5 animals per group, 5 groups consisted of 6 animals per group, and 3 rabbits were kept separate.

The 4 groups of 5 animals per group received either (a) 2% 5-FU alone or 5% 5-FU alone in acid mantle cream; (b) 0.05% vitamin A acid alone or 0.3% vitamin A acid alone; (c) continued applications of DMBA; or (d) no further treatment after the 6 weeks of carcinogen applications.

The 5 groups each consisting of 6 animals with tumors received a specific dose and concentration of vitamin A acid and 5-FU in combination in a standard cream vehicle with a water-washable base. The 5 different concentrations were as follows: 0.05% vitamin A acid and 1% 5-FU, 0.05% vitamin A acid and 2% 5-FU, 0.1% vitamin A acid and 2% 5-FU, 0.3% vitamin A acid and 2% 5-FU, and 0.3% vitamin A acid and 5% 5-FU, all kindly supplied by Hoffmann-La Roche, Inc., Nutley, N. J.

Each application of combined drugs (averaging 0.25 g) was applied to a specific tumor by means of a wooden stick spatula, resulting in a thin film of drugs over the surface of the tumor. The drugs were applied daily for a period of 6 to 9 days. These procedures are summarized in Table 1.

In a preliminary study, 3 separate rabbits received 9 weeks of DMBA applications (18 applications). Each rabbit received either 0.3% vitamin A acid alone, 5% 5-FU alone, or 0.3% vitamin A acid and 5% 5-FU in combination, applied during the 9th week of DMBA applications. These procedures are summarized in Table 2.

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2 The abbreviations used are: 5-FU, 5-fluorouracil; DMBA, 1% 7,12-dimethylbenzanthracene.
Table 1
A protocol of the experimental materials, methods, and results

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment or drug</th>
<th>No. of tumors</th>
<th>Duration of treatment (consecutive days)</th>
<th>No. of tumor regressions</th>
<th>Day of biopsy (after drug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2% 5-FU</td>
<td>13</td>
<td>7</td>
<td>3 markedly regressing</td>
<td>7th day of drug application</td>
</tr>
<tr>
<td>2</td>
<td>5% 5-FU</td>
<td>14</td>
<td>7</td>
<td>5 markedly regressing</td>
<td>7th day of drug application</td>
</tr>
<tr>
<td>3</td>
<td>0.05% vitamin A</td>
<td>9</td>
<td>7</td>
<td>2 markedly regressing</td>
<td>7th day of drug application</td>
</tr>
<tr>
<td>4</td>
<td>0.3% vitamin A</td>
<td>12</td>
<td>7</td>
<td>7 markedly regressing</td>
<td>7th day of drug application</td>
</tr>
<tr>
<td>5</td>
<td>0.05% vitamin A and 1% 5-FU</td>
<td>24</td>
<td>9</td>
<td>2 completely regressed</td>
<td>9th day of drug application</td>
</tr>
<tr>
<td>6</td>
<td>0.05% vitamin A and 2% 5-FU</td>
<td>28</td>
<td>7</td>
<td>8 markedly regressing</td>
<td>7th day of drug application</td>
</tr>
<tr>
<td>7</td>
<td>0.1% vitamin A and 2% 5-FU</td>
<td>30</td>
<td>7</td>
<td>3 completely regressed</td>
<td>7th day of drug application</td>
</tr>
<tr>
<td>8</td>
<td>0.3% vitamin A and 2% 5-FU</td>
<td>28</td>
<td>6</td>
<td>19 completely regressed</td>
<td>3rd day after 6 days of drug application</td>
</tr>
<tr>
<td>9</td>
<td>0.3% vitamin A and 5% 5-FU</td>
<td>35</td>
<td>6</td>
<td>30 completely regressed</td>
<td>3rd day after 6 days of drug application</td>
</tr>
</tbody>
</table>

a Markedly regressing refers to a 50% reduction (or more) in mass of the tumor.
b Completely regressed refers to full clinical remission.

Table 2
A protocol of the experimental materials, methods, and results when DMBA was continually applied

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment or drug</th>
<th>No. of tumors</th>
<th>Duration of treatment</th>
<th>No. of tumor regressions</th>
<th>Day of biopsy (after drug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Continued applications of DMBA</td>
<td>20</td>
<td>7 wk (14 applications) to 10 tumors</td>
<td>3 markedly regressing</td>
<td>1st day after 5 days of drug application</td>
</tr>
<tr>
<td>11</td>
<td>Continued (18) applications of DMBA and 0.3% vitamin A</td>
<td>6</td>
<td>5 consecutive days of vitamin A applications</td>
<td>1 markedly regressing</td>
<td>1st day after 5 days of drug application</td>
</tr>
<tr>
<td>12</td>
<td>Continued (18) applications of DMBA and 5% 5-FU</td>
<td>5</td>
<td>5 consecutive days of 5-FU applications</td>
<td>5 markedly regressing</td>
<td>1st day after 5 days of drug application</td>
</tr>
<tr>
<td>13</td>
<td>Continued (18) applications of DMBA, 0.3% vitamin A, and 5% 5-FU</td>
<td>6</td>
<td>5 consecutive days of combined drug applications</td>
<td>1 completely regressed</td>
<td>1 wk after last carcinogen treatment</td>
</tr>
<tr>
<td>14</td>
<td>No further applications after initial 6 wk of DMBA applications</td>
<td>18</td>
<td></td>
<td>3 markedly regressing</td>
<td></td>
</tr>
</tbody>
</table>

a See footnotes to Table 1.

The corresponding right ear auricles of all of the rabbits served as controls. Ten additional animals (5 groups of 2 animals per group) also served as controls; the inner surface of their ear auricles were painted with the same amount (0.25 g) and schedule of drug combinations as was used on the ears with tumors. Three separate rabbits had their ear auricles painted with the cream vehicle only.

Biopsies were taken of the treated tumors as well as of the control ears. The biopsies were sliced into 1-cu mm pieces and placed into 3% glutaraldehyde in phosphate buffer for 1.5 hr, followed by fixation in 2% osmium tetroxide buffered to pH 7.4. The tissues were dehydrated in graded strengths of ethanol and embedded in Epon 812. Sections 1 μm thick and ultrathin sections were cut on a Reichert ultramicrotome, stained with uranyl acetate and then with lead citrate, and examined in an RCA Type EMU-2E electron microscope.

RESULTS

Gross Results. After DMBA has been applied to the inner surface of rabbit ear auricles for 6 weeks, keratoacanthomas are produced. If the carcinogen treatment is stopped, the tumors will spontaneously regress in a period of 3 to 4 weeks. The tumors regress by the shedding of a central keratinous plug and by a subsequent reduction in the hyperplasia of the stratum spinosum of the epidermis and hair follicles. If the carcinogen treatment is continued for extended periods, some tumors become ulcerated and necrotic, while most of the other tumors enlarge.

When tumors are treated with 2% 5-FU alone, an average of 3 of 13 tumors markedly regress. The term “markedly regressing,” as used in this study, refers to an average tumor diameter of 0.25 cm or less. The average diameter of an untreated keratoacanthoma is 0.5 to 1 cm. When the tumors are treated with 5% 5-FU alone, 5 of 14 tumors markedly regress.

When the tumors are treated with either 0.05 or 0.3% vitamin A acid alone, the latter is the optimal dose (7 of 12 tumors markedly regressing), rather than the former dosage (with which only 2 of 9 tumors markedly regress).

When the tumors are treated for 7 to 9 consecutive days with the lower concentrations of vitamin A acid and 5-FU in combination (Table 1, Experiments 5 and 6), the tumors...
exhibit a small reduction in size with very little erythema and a very small amount of exudate. On the control ears, a slight exudate is also observed (Fig. 1).

With a higher concentration of vitamin A acid and 5-FU (Table 1, Experiment 7), exudate which previously (15) and again in this study was determined to be mucus (Mayer's mucicarmine stain and periodic acid-Schiff stain) is present on the tumors by the 6th day. At that time, most of the tumors are undergoing regression. Twenty-four of 30 tumors averaged 0.25 cm in diameter, down one-half from the average diameter of 0.5 cm. Three tumors completely regressed. "Completely regressed" refers to full clinical remission with a resultant scar at the site of the former tumor.

After treatment with the 2 highest concentrations of drugs (Table 1, Experiments 8 and 9), an examination on the 4th day discloses much mucus on the tumors (Fig. 2). By the following day, copious amounts of mucus which had begun to cake was present (Fig. 3). Caked mucus was also seen on the control ears (Fig. 4). On the 9th day (3 days after the last drug application), most of the tumors were completely regressed (Fig. 5).

Among untreated tumors examined 1 week after the last carcinogen treatment, only 1 of 18 had completely regressed.

**Microscopic Results.** The ultrastructural morphology of the keratoacanthoma has been described (13). The epidermal cells contain an increased rough-surfaced endoplasmic reticulum and Golgi with no lysosomes present until the regressing stage of the tumors are examined. Tumors treated only with vitamin A acid have mucinogen droplets in the cytoplasm of the epidermal cells. Tumors treated only with 5-FU have cytoplasmic vacuolation and pycnotic nuclei in the keratinocytes.

At the ultrastructural level, the keratinocytes of the tumors treated with lower concentrations of the combined drugs have an increased rough-surfaced endoplasmic reticulum and Golgi and sparse amounts of mucinogen droplets. However, those tumors treated with 0.3% vitamin A acid and either 2 or 5% fluorouracil show mucinogen droplets in the epithelial cytoplasm, lysosomes, and many large autophagic vacuoles (Figs. 6 and 7). The autophagic vacuoles are sparse in the untumorous epithelium of the control ears subject to the higher concentrations of the drugs.

**DISCUSSION**

Vitamin A acid has been shown to have a selective effect on tumor regression. Bollag (1) noted that vitamin A acid had no inhibitory effect on transplantable tumors but caused chemically induced papillomas to regress. 5-FU, a well-known cytotoxic agent in the treatment of premalignant actinic keratoses, inhibits DNA synthesis (4). Its application can cause rapid regression of the human keratoacanthoma (10), as well as superficial basal cell carcinoma (11, 12).

In this study, neither 5 nor 2% 5-FU, used alone, ever caused as much as 50% of the total number of tumors to undergo a marked regression. When 0.3% vitamin A acid (the optimal dose) was used alone, almost 60% of the total number of tumors markedly regressed. However, the purpose of these studies is to show that the 2 drugs in combination are more effective than either drug alone. The results that are obtained when 0.3% vitamin A acid and 5% 5-FU are used in combination achieve this goal by demonstrating that over 85% of the total tumors have completely regressed.

In a previous study, vitamin A acid-treated keratoacanthomas had an increased number of lysosomes, compared with normal epithelium (17). In this study, large lysosomes, as well as autophagic vacuoles in keratinocytes treated with large concentrations of vitamin A acid and 5-FU, were observed. When treating mammary gland carcinomas with cyclophosphamide and vitamin A, Brandes et al. (3) noted that the vitamin A enhanced the antitumor effect of cyclophosphamide. There was an increased number of lysosomes and numerous autophagic vacuoles. They speculate that vitamin A, a known labilizer, may increase the release of degradative enzymes into the cytoplasm and thus account for the increased tumor regression. This same phenomenon may have occurred in this study and may account for the enhanced tumor effect observed when 5-FU and vitamin A acid were used in combination.

**REFERENCES**

12. Klein, E., Stoll, H. L., Milgrom, H., Traenkel, H. L., Graham, S.,


Fig. 1. Arrows, mucus on control ear treated with a low concentration of vitamin A acid and 5-FU for 7 days.

Fig. 2. Black arrow, caked mucus on a regressing tumor, white arrow, mucus on a regressing tumor. Mucus appears as a glistening exudate. Both tumors were treated with a high concentration of vitamin A acid and 5-FU.

Fig. 3. Regressing tumors possess a caked mucus on their surface.

Fig. 4. Mucus which has caked is present on the control ear of a rabbit subjected to a high concentration of vitamin A acid and 5-FU.

Fig. 5. Arrows, tumors that have almost completely regressed.
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Fig. 6. Arrows, autophagic vacuoles in a tumor keratinocyte treated with a high concentration of vitamin A acid and 5-FU. × 21,775.

Fig. 7. Arrows, large autophagic vacuoles containing mitochondria (M1, M2, and M3) and other cell organelles in a tumor keratinocyte treated with a high concentration of vitamin A acid and 5-FU. × 21,775.
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