Retinoic Acid-binding Protein in Experimental Tumors and in Tissues with Metastatic Tumor Foci

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SUMMARY
Screening for retinoic acid-binding protein (RABP) in experimental tumors revealed the presence of this protein in three mammary tumors, two metastatic colon tumors, B16 melanoma, Lewis lung carcinoma, Ridgway osteogenic sarcoma, and keratoacanthoma. RABP was below the limits of detection in two weakly metastatic colon tumors and in Sarcoma 180. After s.c. implantation of RABP-containing tumors into mice, this protein could be traced in the lungs due to pulmonary metastasis. Following implantation of Lewis lung tumors, RABP was detected in the lung on the 6th day. On the 15th day after implantation, RABP was present in lung and brain, but not in other tissues where this protein was normally lacking. In primary cultures of Lewis lung carcinoma, the lower limit for detection of RABP by sucrose gradient sedimentation technique corresponded to 0.12 mg protein that was extractable from 3 x 10⁶ cells. Both chick embryo skin and rabbit ear skin extracts contained RABP; the level of cellular retinol-binding protein was high in chick embryo skin but only marginal in rabbit ear. The amounts of these proteins on chick embryo skin and rabbit ear skin correlate with the biological potency of retinol and retinoic acid, as observed by others.

INTRODUCTION
The importance of retinoids in epithelial differentiation and in the control of epithelial cancer has been reviewed (3, 28). Vitamin A (retinol) has been reported to have both prophylactic and inhibitory effects in experimental carcinogenesis (7–9, 22). The biological activity of retinoic acid parallels that of vitamin A except that retinoic acid has no role in vision or reproduction (1, 10). There are numerous reports that retinoic acid inhibits tumor-inhibiting activity. Retinoic acid administered systemically to rats repressed DMBA-induced skin papillomas and carcinomas but had little activity on transplanted tumors (4, 5). Actinic keratoses and basal cell carcinomas regressed completely following topical application of retinoic acid (6). In addition, the prophylactic application of retinoic acid delayed or retarded the growth of chemically induced skin papillomas and carcinomas (5). 13-cis-Retinoic acid, which has the same growth-promoting activity as does retinoic acid (31, 32), inhibits carcinogen-induced bladder cancer in rat (29). Keratoacanthomas produced on the ears of rabbits by DMBA (21) or keratinized metaplasia produced on chick embryo skin (30) and hamster trachea (27) can be treated with retinoic acid to reverse the keratinized metaplasia or lesions into mucus-producing epithelium.

The general action of retinol and retinoic acid may be mediated through their respective cellular binding proteins, CRBP and RABP (2, 25). A general correlation has been observed between the binding ability of retinoic acid analogs to RABP and their biological properties (26). RABP has since been detected in many epithelial tissues of rat, mouse, and chick embryo (15, 23, 24) as well as in some experimental and human tumors (17, 18, 24). Although no RABP could be detected in normal lung or colon, Lewis lung carcinoma and 2 metastatic colon carcinomas contained this protein in large amounts (24).

If RABP is essential for mediation of retinoic acid action, then the presence of this binding protein may be used as a positive biochemical parameter for retinoic acid therapy in primary tumors, but not necessarily in transplantable tumors. We have presently examined various experimental tumors and tissues with metastatic tumor foci for the presence of RABP.

MATERIALS AND METHODS
Retinoic acid and [11,12-3H]retinoic acid (1.28 Ci/mmmole) were gifts from Hoffmann-La Roche, Inc., Nutley, N. J. [15-3H]Retinol (1.66 Ci/mmmole) was purchased from New England Nuclear, Boston, Mass. DMBA was obtained from Sigma Chemical Company, St. Louis, Mo. Collagenase was purchased from Worthington Biochemical Corp., Freehold, N. J.

Most of the murine tumors that were used in these studies are in serial passage at our Institute. Trocar fragments (20 to 200 mg) were implanted s.c. in the mice as indicated: Ridgway osteogenic sarcoma in AKR mice, Sarcoma 180 in Swiss albino mice; Lewis lung into C57BL x DBA/2 F₁ (hereafter called BD2F₁) mice; Colon Tumors 26, 36 and 51 in BALB/c mice; B16 melanoma and Colon Tumor 38 in C57 mice. The hosts were sacrificed between the 15th and 20th days after implantation of the tumor. Lung and colon tissues from the corresponding normal strain of mice were removed. The tissues and tumors were
homogenized in 30 mM sodium phosphate (pH 7.2)-100 mM NaCl with a VirTis Teflon homogenizer, centrifuged at 100,000 x g for 60 min, and stored at -60° until used.

For a study of the sensitivity of detection of RABP in Lewis lung carcinoma cells and in various tissues that might contain tumor cells due to metastasis, BD2F mice were implanted s.c. with 20-mg trocar fragments of Lewis lung carcinoma. Two mice were sacrificed each day up to the 14th day, and their lungs were removed. On the 15th day, 6 mice were sacrificed; their hearts, spleens, livers, lungs, colons, brains, and blood were isolated, and soluble protein extracts were prepared as described and stored at -60°. The tumors developed at the primary site on these mice were excised and divided into 3 equal parts on the basis of weight. One part was homogenized, and soluble protein extracts were prepared as previously described. The other 2 parts were used to prepare primary cell cultures according to the procedure described by Owens (19) and Owens et al. (20). The tumor slices, after incubation for 18 hr at 37° in the presence of collagenase at 1 mg/ml in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were centrifuged at 1000 x g for 10 min. The pellet was resuspended in the same medium without serum and mixed for 10 sec on a Vortex mixer; portions were then counted with a Model B Coulter counter.

Keratoacanthomas on the ears of rabbits, 1.5 kg body weight, were produced by painting the inner surface of the ears 3 times weekly with 1% DMBA in equal parts of lanolin and mineral oil (21). After 5 weeks of DMBA applications, the rabbits bearing the tumor were sacrificed and the ears were removed. Skin from the inner surface was peeled off and the epithelial layers containing the tumor were excised. The epithelial layers of skin from the inner surface of the ears of normal rabbits were also collected. The normal skin and skins bearing the tumors were homogenized in a Polytron homogenizer equipped with a P-10 generating head. The homogenates were lyophilized to a dry powder and extracted with 30 mM sodium phosphate (pH 7.2)-100 mM NaCl as described previously. Skin extracts from 13-day-old chick embryos were prepared as described previously (25, 26).

Analysis for RABP was accomplished on 5 to 20% (w/v) sucrose density gradients as described earlier (25, 26). The extracts were incubated with [3H]retinoic acid (300 pmoles) or [3H]retinol (300 pmoles) and sedimented on sucrose gradients at 180,000 x g for 18 hr. The radioactive peak with a sedimentation value of 2 contained RABP, whereas the 5 S peak represented [3H]retinoic acid bound to serum albumin (26).

RESULTS

The results of screening by sucrose density sedimentation for RABP of various experimental tumors, tissues containing metastatic tumor foci, and the corresponding normal tissues are presented in Table 1. RABP was detected in the 3 mouse mammary tumors that were tested, in the 2 highly metastatic murine colon tumors (Tumors 26 and 51), in B16 melanoma, in Lewis lung carcinoma, and in mouse lungs bearing the metastatic foci of Lewis lung carcinoma and B16 melanoma after s.c. implantation. As reported earlier (24), this protein could not be detected in the weakly metastatic Colon Tumors 36 and 38. On the 15th day after s.c. implantation of Colon Tumor 26, the lungs and colon of the animals were analyzed for RABP. Although RABP could be detected in the lung, it was not found in the colon. Whereas Ridgway osteogenic sarcoma contained RABP, it was not present in detectable quantity in Sarcoma 180.

Attempts were made to evaluate the limit of detection of RABP on a cell number basis, with Lewis lung as a model. A wet weight of 1 g Lewis lung tumor in primary cell culture contained, on the average, 10^6 cells as counted by the Coulter counter. The total extractable protein from the same quantity of tumor was 36 mg. The lower limit for detection of RABP by the appearance of the 2 S peak after sucrose gradient sedimentation corresponded to 3 x 10^4 cells in the primary cultures of Lewis lung carcinoma and corresponded to 0.12 mg of extractable protein (Chart 1). The RABP peak was not evident below this level. RABP in normal rat lung extracts even at 3 mg of protein remained below this limit of detection.

After s.c. implantation of the Lewis lung carcinoma, a study for the appearance of RABP in the lungs possibly due to metastatic tumor cells on successive days after implantation was accomplished by the sucrose gradient sedimentation technique. With 3.0 mg protein of these lung extracts, no RABP peak was evident up to the 5th day after implantation. On the 6th day, appearance of the RABP peak due to pulmonary metastasis was detected. With the same amount of protein, pronounced RABP peaks were observed from the 9th day of implantation. In normal animals, tissues such as heart, spleen, liver, colon, brain, and blood do not contain RABP (24). On the 15th day of s.c. implantation of Lewis lung carcinoma, these tissues were isolated and their extracts were screened for RABP. None of the tissues, except for brain, showed any detectable amount of RABP during tumor progression and metastasis.

<table>
<thead>
<tr>
<th>Tumor or tissue extracts (5 mg protein)</th>
<th>RABP</th>
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<tr>
<td>Mammary tumor C3H 13/C/24 (metastatic)</td>
<td>++</td>
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<tr>
<td>Mammary tumor C3H 04/A/64 (metastatic)</td>
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<tr>
<td>Mammary tumor C3H 16/C/13 (metastatic)</td>
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<td>Lungs with metastatic Lewis lung foci</td>
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<td>Normal lungs</td>
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<td>Colon Tumors 26 and 51 (metastatic)</td>
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<td>Colon Tumors 36 and 58 (nonmetastatic)</td>
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<td>Lungs with Metastatic foci from Colon Tumor 26 and 51</td>
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<td>Normal colon</td>
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<td>Ridgway osteogenic sarcoma</td>
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<td>Sarcoma 180</td>
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<td>B16 melanoma</td>
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<td>Lungs with metastatic B16 melanoma foci</td>
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*a ++, readily detectable; +, detectable; -, not detectable.*
Retention Acid-binding Protein in Tumors

Chart 1. Limit of detection of RABP in Lewis lung tumor extracts. Varying amounts of the tumor extracts were incubated with 300 pmoles of [3H]retinoic acid as described in the text and examined for the 2 S RABP peak from the radioactivity profiles after sucrose density gradient sedimentation. The lower limit for detection of RABP was 0.12 mg protein of the extracts.

Spontaneously regressing keratoacanthomas produced on the ears of rabbits can be treated with retinoic acid to convert the keratinized lesions into mucus-producing epithelium (21). In our laboratories, the same effects were produced by retinoic acid on keratoacanthomas, but retinol failed to produce the mucus epithelium (L. J. Wilkoff, D. L. Hill, and J. D. Prejean, unpublished results). Since it is postulated that retinoic acid and retinol action is mediated through RABP (25, 26) and CRBP (16), respectively, it was desirable to examine normal rabbit ear skin and skin bearing keratoacanthomas for both these proteins. Chart 2A illustrates that normal ear skin shows a predominant RABP peak, whereas CRBP could be only marginally detected in these preparations. In keratoacanthoma extracts, the RABP level remained similar to the level found in the normal rabbit skin. However, the CRBP level was slightly elevated in the tumor extracts as compared with normal skin (Chart 2A). Skin extracts from 13-day-old chick embryos contained both RABP and CRBP peaks, the RABP peak being predominant (Chart 2B).

DISCUSSION

Numerous reports that are presently available indicate that retinoids may be used to prevent cancer by arresting or reversing the biological processes that lead to cancer. However, the various transplantable murine tumors, including Lewis lung carcinoma (W. R. Laster, unpublished results), that were tested to date are unresponsive to retinoic acid therapy (4) with the single exception of Swarm chondrosarcoma (11). The intracellular binding proteins that are present in the retinoid responsive tissues are the likely candidates to mediate the biological effects of retinoids. Conversely, the tissues that contain specific retinoid-binding proteins and the primary tumors in their development from those tissues may be responsive to retinoid therapy.

Of the various experimental tumors that were presently examined for RABP, 3 mammary tumors, 2 metastatic colon tumors (Tumors 26 and 51), B16 melanoma, Lewis lung carcinoma, and Ridgway osteogenic sarcoma contained RABP; whereas in Sarcoma 180, RABP was below the limits of detection. In similar screening studies by Ong and Chytil (17), RABP was found in Sarcoma 180. Hence it is not safe to assume that all experimental tumors of a particular type are identical in this respect. We have noticed that, among the 4 colon tumors tested, 2 highly metastatic ones contained RABP, whereas 2 weakly metastatic ones contained undetectable amounts. In support of our earlier findings, where we noticed RABP in lungs containing metastatic Lewis lung foci (24), we have presently observed the appearance of RABP in the lungs of mice implanted with B16 melanoma and the 2 metastatic colon tumors. The fact that, even after the 15th day of implantation of Lewis lung tumor, RABP could not be detected in spleen, liver, colon, heart, or blood indicates that the minimal number of tumor cells required for detection of RABP has not metastasized into these tissues. During tumor progression, RABP appeared in brain and lung where normally this protein is below the limits of detection in these tissues. It is known that, at 0.35 g, the primary Lewis lung tumor has a doubling time of 2.4 days (14) and that, upon s.c. implantation of the tumor on the hind limb and after subsequent amputation of this limb on the 8th day (12, 13) or after the surgical removal of the primary tumor on the 6th day (14), the animals die because of metastasis. Although we have shown that, after implantation of Lewis lung tumors, RABP could be detected in mouse lungs (25), the extent of metastasis and the sensitivity of detection in terms of RABP appearance were not known. The present observation that after such implantation RABP could be detected in the lung on the 6th day agrees with the data presented by others (12–14) on the pulmonary metastatic ability of Lewis lung tumor cells.

The sensitivity of detection of RABP in terms of cell number and protein concentration is an important factor in assessing the potential of RABP appearance in detecting
certain tumors or precancerous lesions in tissues. The preliminary assessment made in this direction shows that 0.12 mg protein that could be extracted from 3 x 10⁶ cells or 3 mg wet weight of Lewis lung tumor is the lower limit for detection of RABP with sucrose sedimentation techniques. More sensitive methods in which immunological probes are used must wait until RABP is purified to homogeneity and specific antisera is produced against it.

Although there is no direct evidence as to how the CRBP and RABP are involved in the action of retinoic acid or retinol, earlier observations (16, 25, 26) strongly suggest that they have a role in mediating the effect of retinoids. Demonstration of the localization of RABP in the nuclei of chick embryo skin and of some experimental tumors (23) is particularly important in elucidating the mechanism of retinoic acid action. We have presently found both RABP and CRBP in the chick embryo skin. However, adult rabbit ear skin shows high levels of RABP, but very little CRBP. The earlier observations that chick embryo skin explants responded to both retinoic acid and retinol to produce mucous metaplasia (30) and that keratoacanthomas produced on rabbit ear responded only to retinoic acid, but not to retinol, is in agreement with the above findings. It is increasingly essential to define more accurately the dependence of the binding proteins to express the biological functions of the retinoids in order to elucidate the mechanism of action of retinoid-protein complexes within the cell.

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