High-Resolution Autoradiographic Localization of 3,4-Benzpyrene-$^3$H in Mouse Skin

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SUMMARY

Tritiated 3,4-benzpyrene was traced at intervals between 1 hr and 21 days by autoradiography with both light and electron microscopes after topical application to the skin of mice. The sebaceous glands were the most heavily labeled site 1 and 4 hr after application. Electron microscopy showed labeling of a variety of cellular components in both sebaceous and epidermal cells. No one organelle appeared to be exclusively associated with the tracer. Nuclei were labeled in proportion to their cytoplasm, but generally to lesser degrees. The findings are consistent with previous work on the binding of hydrocarbon carcinogens to soluble proteins and DNA. The data also support the hypothesis that the binding of carcinogen is proportional to cellular synthetic activity.

INTRODUCTION

The cellular site of initiation of chemical carcinogenesis remains unknown. A variety of methods have been used to try to localize carcinogens in cells and tissues. These have included analysis of chemical constituents (1, 7, 9), subcellular fractionation of organelles (6), fluorescence microscopy (2, 4, 10, 14), and ARG (11, 13). Fluorescence microscopy and ARG have produced conflicting data, primarily concerning the localization of polycyclic hydrocarbon carcinogens in the nucleus as well as in other organelles. ARG has certain advantages over fluorescence techniques. There is no problem of metabolism to nonfluorescent compounds or with quenching of fluorescence with ARG.

In order to obtain higher levels of resolution than those previously reported in the cellular localization of hydrocarbon carcinogens, ARG was performed on OsO$_4$-fixed, plastic-embedded tissues and studied by both light and electron microscopy.

MATERIALS AND METHODS

The backs of 8-week-old adult female Swiss mice were shaved with electric clippers 5 days prior to treatment. Those showing no hair growth were selected. Each of 16 received 500 $\mu$Ci (31.5 $\mu$g) BP-$^3$H, 4 Ci/mmole, (Amersham/Searle) in 70 $\mu$l benzene by syringe, topically over an area of 1.5 sq cm. Eight mice received 0.2 of the above dose. Control mice were given equivalent doses of nonradioactive BP in benzene. Groups of 3 mice (2 treated with 500 $\mu$Ci, 1 treated with 100 $\mu$Ci) were sacrificed with chloroform after 1, 4, 24, or 48 hr through 5, 8, 14, or 21 days. Specimens of treated skin and untreated skin (ear) were fixed for 1 hr in 1% OsO$_4$ with Millonig's sodium phosphate buffer (12) containing glucose, pH 7.4, 328 milliosmoles. Tissues were rinsed 10 times for 5 min each in the phosphate-glucose buffer, then dehydrated in ethanol and embedded in Epon after propylene oxide.

ARG. One-$\mu$m sections were cut and applied to glass slides that had been previously coated with a solution containing 0.5% gelatin and 0.05% chrome alum. These were dipped in Ilford L-4 liquid emulsion, diluted to produce a monolayer of silver halide (3). After drying, they were stored in taped boxes at 7°. Controls for negative and positive chemography were included. After 22 to 44 days, they were developed at 22° in Kodak D-19 for 3.5 min and, after fixation, stained with 1% toluidine blue in 1% borax.

Electron Microscope ARG. The 4-hr specimens were chosen for electron microscope ARG because they contained the most radioactivity. Thin Epon sections (pale gold) were mounted on uncoated or Formvar-coated nickel grids which were placed face down on thin carbon films floating on water. These were picked up on gelatin-chrome alum-coated slides, dried, and dipped in Ilford L-4 emulsion diluted to produce a monolayer of silver halide (3). After drying, they were stored in taped boxes at 7°. Controls for negative and positive chemography were included. After 22 to 44 days, they were developed at 22° in Kodak D-19 for 3.5 min and, after fixation, stained with 1% toluidine blue in 1% borax.

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2 The abbreviations used are: ARG, autoradiography; BP, 3,4-benzpyrene.

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RESULTS

Radioactivity was detected to some extent in all components of the skin, but labeling of the epithelial cells was greater than in the other tissues. Fat cells in the subcutis were lightly tagged; mature mast cells and underlying muscle were virtually free of radioactivity. The dermis was lightly labeled. There was no difference in the pattern of labeling between mice that had received 500 μCi and mice that had received 100 μCi, although the exposures in the latter were weaker.

Sebaceous Cells. The sebaceous cells showed the most intense degree of labeling of any skin component within the first 4 hr following the application of the carcinogen. These cells at 4 hr were more heavily labeled than at 1 hr, but the pattern remained the same. More primitive basal cells, as well as differentiated cells, in these glands showed heavy tagging, and nuclei, in addition to the cytoplasm, contained considerable radioactivity (Fig. 1).

In the autoradiographs of the 4-hr specimens examined with the electron microscope, cytoplasmic radioactivity was associated with a variety of organelles. Occasionally, the radioactivity could be localized to mitochondria or large granules; it was sometimes seen in conjunction with lipid vacuoles. Most often, however, the sites of exposure were over the cytoplasmic matrix without definite association with specific organelles (Figs. 2 to 6).

By 24 hr, the sebaceous cells were decidedly less heavily labeled than earlier, and the extruded sebum appeared more intensely radioactive. During the next 6 days, the amount of tracer present in these cells diminished; hardly any was detected in them by 8 days. There were no degenerative changes seen in the sebaceous cells other than those associated with holocrine secretion.

Hair Follicle Epithelium. The follicular epithelial cells above the levels of the sebaceous duct were labeled to much greater degrees than those below this level. The former were less intensely radioactive than the sebaceous cells at 1 and 4 hr. However, they became the most heavily labeled components 24 and 48 hr after treatment. This was made more striking by the concentration of tracer in the cells closest to the lumen of the follicles, as compared with those farther away (Fig. 7). The sebum surrounding the hair shafts was particularly radioactive in the 5- and 8-day specimens (Fig. 8).

Epidermis. The labeling of the epidermis at 1 and 4 hr was similar to that of the hair follicle epithelium, being rather evenly distributed, with the 4-hr samples more heavily labeled than those taken at 1 hr. As in the sebaceous cells, electron microscopy of the epidermis showed labeling to be associated with a variety of cellular components (Fig. 9). By 48 hr, overall labeling had decreased, with some accumulation towards the surface. This aggregation was not nearly as intense as in the hair follicle epithelium, where the luminal aspect of the cells was heavily tagged at this time.

The epithelium surrounding the ostia of the hair follicles was more heavily labeled in the 2- to 8-day specimens than the interfollicular epidermis. The surface keratin appeared to be heavily labeled up to 8 days (Fig. 10).

Nuclei. In general, nuclei of all cell types were labeled in proportion to their cytoplasm, but usually to a somewhat lesser extent. In other words, it was exceptional to find an unlabeled nucleus with heavily labeled cytoplasm, or a heavily tagged nucleus with lightly labeled cytoplasm. The labeling was in both the nuclear internum and near the nuclear membrane (Fig. 2). Nucleolar labeling was also present, but not to marked degrees.

Untreated Skin. Radioactivity was found in the skin of the ears between 4 and 48 hr after administration. The concentration was highest at 24 hr, although much less than that at the site of original application. The tracer was principally in the sebaceous cells, and present to a lesser extent in hair follicle epithelium. The overlying keratin was only faintly labeled (Fig. 11).

DISCUSSION

The findings obtained in the present study are in general agreement at the tissue level with observations made by fluorescence microscopy (4) and lower-resolution ARG (11, 13). The present data, however, do establish firmly that labeled polycyclic hydrocarbon carcinogen is detected in nuclei following OsO₄ fixation. The degree of nuclear radioactivity seems to be roughly proportional to the degree of cytoplasmic labeling, and holds true as early as 1 hr after administration of carcinogen. This observation is contrary to the findings of fluorescence microscopy which, as a rule, has not demonstrated nuclear localization of polycyclic hydrocarbons (14). The results obtained here are of further interest since there is complete quenching of cellular hydrocarbon fluorescence following OsO₄ fixation (14). Autoradiographic findings of nuclear localization of hydrocarbon have been more consistent with data acquired by subcellular fractionation (6) and identification of carcinogenic hydrocarbons bound to DNA (7, 9). Ethanol dehydration can lead to nucleolar uptake of BP (14), but this probably cannot account for most of the nuclear radioactivity observed.

The limitations of fluorescence microscopy on the intraacellular localization of hydrocarbon include the alteration of hydrocarbon to nonfluorescent metabolites, the quenching of fluorescence when hydrocarbon is bound to nucleic acids (5), and the necessity for discernible differences in fluorescent intensities from adjacent regions, which might make uniformly distributed hydrocarbon poorly visible (14).

ARG also has several disadvantages. It does not permit the examination of live cells. The label may represent metabolic derivatives of the original material. Positive and negative chemography can cause spurious results. Fortunately, these can be recognized with proper controls. Resolution can be impaired by a variety of factors in both the specimen and the emulsion.

The autoradiographic data obtained with the electron microscope indicate that the high cytoplasmic concentration of BP in sebaceous cells is not merely caused by its dissolution in the lipid secretory vacuoles, but also because of association with various cytoplasmic structures. These observations show that in both the epidermal and sebaceous cells no single organelle is responsible for cytoplasmic
localization. This is at variance with other work, which indicates concentration of carcinogens in lysosomes (2). The multiplicity of localizations is consistent with evidence that a major site of binding is soluble proteins of the cell (6). In addition, the prominent nuclear labeling supports evidence that the carcinogen can bind to DNA (7).

The holocrine nature of secretion of sebaceous cells probably accounts for the rather prompt transfer of radioactivity from the glands to the lumens of the pilosebaceous apparatus and its subsequent journey to the surface of the skin. This phenomenon may also account for the high concentration of tracer in the cells adjacent to the lumens of these ducts and around their external orifices, since the label might be reabsorbed from the sebum. It perhaps explains the high radioactive content associated with the surface keratin, since the tracer may actually be in the coating sebum. The presence of the label at an untreated site might be due to contact contamination from other treated parts or other mice, but the lack of epidermal labeling versus high sebaceous gland labeling in the skin of the ear better supports blood stream transport (Fig. 11).

A possible explanation for the prominence of bound BP or its metabolites in sebaceous cells is that the degree of binding is proportional to the synthetic activity of the cell. The very nature of holocrine secretion, with its complete destruction of the cell and necessary renewal, indicates that these cells are probably more able to synthesize proteins and lipids than are other components of the skin. The data are thus consistent with a hypothesis that binding of carcinogen is dependent on an active synthetic apparatus, and may be proportional to synthetic activity. This alone need not be synonymous with carcinogenesis. It may merely make more carcinogen available to one cell over another, thus increasing the chance of a carcinogenic event, i.e., initiation, to occur.

The relation between sites of carcinogen localization and sites of mouse epidermal tumors has been described (8, 13). In view of the intensity of carcinogen localization, it might be expected that sebaceous adenomas should be even more common than they are under experimental conditions. However, the vast majority of sebaceous cells are naturally destroyed and extruded during holocrine secretion. Thus, even if sebaceous cells incur the most frequent number of carcinogenic events compared to all other cells of the skin, they are promptly removed from the body. These glands might be considered to serve a function of binding polycyclic hydrocarbon carcinogens and excreting them without leaving many altered cells behind.

REFERENCES

Note: The light photomicrographs (Figs. 1, 7, 8, and 11) are of autoradiographs of skin from mice given 500 μCi BP-3H. These preparations were exposed for 44 days, developed in Kodak D-19 for 4 min, and stained with toluidine blue.

Fig. 1. Sebaceous gland and hair follicle 1 hr after application of BP-3H. Sebaceous cells are more heavily labeled than is hair follicle epithelium. Nuclear labeling is evident in both types of cells. h, hair shaft. X 1300.

Fig. 2. Sebaceous cells with nuclear and cytoplasmic labeling (arrows). N, nucleus; L, lipid vacuoles; m, mitochondrion containing typical dense granules; a, artifacts. Lead citrate stain, X 6000.

Fig. 3. Part of a sebaceous cell with 4 exposed grains lying in relation to lipid vacuoles. N, nucleus. Lead citrate stain, X 9000.

Fig. 4. Portion of a sebaceous cell with 6 exposed grains overlying cytoplasm. m, mitochondrion; L, lipid vacuoles. Uranyl acetate and lead citrate stains, X 17,000.

Fig. 5. Three exposed grains are in perinuclear cytoplasm of a sebaceous cell. N nucleus; L lipid vacuoles; m mitochondrion. Lead citrate stain, X 115,000.

Fig. 6. Portions of cells lining (D) sebaceous duct. The exposed grains (arrows) lie mainly over cytoplasm and are not associated with any large organelles. N, nucleus. Uranyl acetate and lead citrate stains, X 10,000.

Fig. 7. Hair follicle close to epidermal surface from mouse given BP-3H 24 hr earlier. Labeling is of greatest intensity in cells adjacent to follicular lumen. Most cells continue to show nuclear labeling. h, hair shafts. X 1300.

Fig. 8. Hair follicle near surface 8 days after BP-3H application. Sebum coating 2 hair shafts is heavily labeled. Most epithelium is free of label. Larger black deposits in cytoplasm of epidermal cells are keratohyaline granules. X 800.

Fig. 9. Portions of epidermal cells. Exposed grains lie mainly over cytoplasm. N, nucleus. Uranyl acetate and lead citrate stains, X 8500.

Fig. 10. K, heavily labeled keratinous layer, overlies E, cytoplasm of superficial epidermal cell. Uranyl acetate and lead citrate stains, X 8500.

Fig. 11. Sebaceous gland, h, hair follicle with hair shaft and e surface epidermis, in the skin of the ear from a mouse 24 hr after application of BP-3H to the back. Pattern of labeling similar to sites of direct application. Nuclear labeling proportional to that of cytoplasm. Epidermis and surface keratin sparsely labeled. The distribution is more consistent with tracer reaching this site via blood stream, rather than by contact contamination from treated parts. X 1300.
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1. Image of tissue section with autoradiographic labeling.
2. Detail showing labeled structures, possibly nuclei (N) and other cellular components.
3. Enlarged view of labeled structures, possibly nucleoli (N).
4. Another view showing labeled nuclei (N) and other labeled structures.
5. Further magnified view of labeled nuclei (N) and cellular components.
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