Localization of Stratum of Maximum Mitotic Frequency in Epidermal Methylcholanthrene Carcinogenesis in Mice*

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The purpose of the major cancer research project of Barnard Hospital is to measure the properties of epidermis during epidermal carcinogenesis induced by methylcholanthrene, to integrate the findings and thus to construct a comprehensive account of what happens (6).

Mitotic frequency is one of these properties and has been investigated by Cooper and Reller (5) and by Reller and Cooper (12). They have observed that 18 hours after the initial application of methylcholanthrene there is a distinct increase in mitotic frequency over that exhibited by normal epidermis, that this increase grows in magnitude to 16 days, that it is maintained fairly constant to 51 days, when it decreases only to increase again about the time that cancers make their appearance in the series. Since these observations were based on counts of mitoses and of nondividing nuclei made on preparations of whole epidermis mounted with proximal, that is dermal, surface uppermost which were viewed vertically, it was not possible to identify the epidermal strata in which the mitoses occurred. The counts were therefore total for whole epidermis, not differential for its several strata.

Differential counts of mitotic frequency can be made only on stained sections of epidermis which are vertical to the surface and which cut across the strata at right angles. But the epidermis of the area chosen for treatment with carcinogen is normally so thin (2 to 3 strata) that in it the strata, easily identifiable in the early hyperplastic stages of carcinogenesis, are not represented. Consequently, it was necessary to make differential counts of mitotic frequency on much thicker epidermis of mice, which normally is stratified, in order to secure data on the basis of which to interpret differential counts of epidermis in our carcinogenic series. Cowdry and Thompson (9) selected for this purpose epidermis of the hind foot pads of mice of the same New Buffalo strain used in the major project. They produced a summation of mitoses by injecting the mice with colchicine and discovered that the maximum mitotic frequency was localized in the middle third of the suprabasal part of the epidermis.

The next step reported in this paper was, employing the same technic, to make similar differential counts of mitotic frequency in the early hyperplastic stages of carcinogenesis to supplement the total counts of Cooper and her associates. It was thought that the normal level of maximum mitotic frequency might be shifted during carcinogenesis because in a few preliminary counts Cowdry and Paletta (7) found more mitosis in the basal than in the spinous layer and also for the reason that Thuringer (18) had reported that it can be shifted in cats by the simple expedient of electrical stimulation.

MATERIALS AND METHODS

Male New Buffalo mice, 6 weeks old, were divided into two groups of 5 each. Mice of the first received on the back 3 cutaneous applications of 0.6 per cent methylcholanthrene in benzene in the uniform manner already described in numerous reports on the major project and were killed 10 days after the initial treatment; whereas those of the second received 6 applications and were killed after 20 days. Eight hours before killing, all mice were injected with 0.01 per cent aq. colchicine as prescribed by Paletta and Cowdry (10).

Uniformly hyperplastic-looking samples of skin from the treated areas were excised, fixed in Bouin's fluid and serial 6 micron paraffin sections were stained with hematoxylin and eosin. The samples of epidermis counted were carefully selected as typical of the several specimens. The thickness, of course, was in general greater in the 20 day mice than in the 10 day mice and there was a little variation, noticeable on naked eye inspection, between individual mice of the same group. Epidermis dipping into hair follicles, or unusual in respect to mechanical injury, or particularly massive leukocytic invasion, or for some other reason, was not included.

Epidermis backed by a basement membrane, which in section was approximately a straight line, proved

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most satisfactory because in it the epidermal cells were arranged with some regularity. The stratum basalis was regarded as composed of cells actually in touch with the basement membrane. Identification of such basal cells was a simple matter. It was possible to resolve the stratum spinosum into several rows of cells and to count the number of mitoses and nondividing nuclei in each. Rows of squares ruled on a disc inserted in the ocular of the microscope served as guides and prevented counting the same nuclei twice. Nuclei in the more distal stratum granulosum were counted, but were not included in Table I because none of them was in mitosis.

The procedure in making the counts was to orient the section so that the large square on the disc (itself subdivided into the small ones mentioned in the preceding paragraph) covered the stratum basalis and the

OBSERVATIONS

In the upper half of Table I are data relative to mice under treatment with carcinogen for 10 days and in the lower half to mice treated for 20 days. Passing in columns from left to right the numbers assigned to individual mice are given, then the numbers of mitoses (M) and nondividing nuclei (N) counted in the stratum basalis and in 1 to 7 rows of cells of the stratum spinosum. The figures are totaled and the ratios of the totals are listed in the last lines of the upper and lower halves of the table.

The first and most obvious fact is that the total ratios of mitoses to nondividing nuclei were greater in the stratum basalis at both 10 and 20 days than they were in any row of cells of the stratum spinosum.

It is clear also that the epidermis was in general somewhat thicker in the 20 day than in the 10 day

TABLE I: COUNTS OF MITOSIS AND NONDIVIDING NUCLEI IN EARLY EPIDERMAL CARCINOGENESIS

Mice, 10 days No.	Stratum basalis		Stratum spinosum													
			Row 1		Row 2		Row 3		Row 4		Row 5		Row 6		Row 7	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N
1	52	2,573	9	1,688	1	1,311	1	829	0	533	0	111	0	14	0	0
2	34	3,241	10	1,984	2	1,261	0	498	0	140	0	30	0	3	0	0
3	79	4,202	11	2,581	0	1,418	0	264	0	9	0	0	0	0	0	0
4	49	2,691	9	1,819	3	1,178	1	437	0	72	0	7	0	0	0	0
5	65	3,752	21	2,470	6	1,562	1	505	0	51	0	22	0	0	0	0
TOTALS	279	16,459	60	10,742	12	6,730	3	2,533	0	805	0	170	0	17	0	0
Ratios	1:	58.99	1:	179.03	1:	560.83	1:	844.33								
Mice, 20 days																
1	43	4,684	16	3,359	5	2,367	3	1,284	1	427	0	86	0	13	0	7
2	32	4,017	12	2,797	3	1,759	2	916	0	338	0	93	0	24	0	1
3	66	3,679	16	2,136	0	1,013	0	229	0	20	0	2	0	0	0	0
4	35	1,544	8	1,022	4	698	1	399	0	139	0	35	0	4	0	0
5	80	3,908	38	2,940	21	2,316	15	1,678	2	928	1	341	0	80	0	21
TOTALS	256	17,832	90	12,254	33	8,153	21	4,506	3	1,852	1	557	0	121	0	29
Ratios	1:	69.66	1:	136.15	1:	247.06	1:	214.57	1:	617.83	1	557				

suprabasal layers. Since all of the included mitoses and nuclei were counted, the figures given in the table under M (mitosis) and N (nondividing nuclei in the layer of basal cells and in the rows of spinous cells (Nos. 1 to 7) give a fairly satisfactory measure of relative cellularity on the basis of one cell per mitosis or nucleus.[1] The basal cells were the smallest and therefore the most numerous in all areas of epidermis covered by the large square.

All the counts were made by B. B. G. under an oil immersion objective with a mechanical counter. It was hard work for one person but gave uniformity to the counts, which would have been lacking had several people done the counting.

specimens, for in the former only was a seventh row of cells encountered in the spinosum of 3 out of 5 mice. Moreover, the sixth and even the fifth rows were better represented at 20 than at 10 days. The mitoses were found to extend further up into the epidermis at 20 days and the ratios in the second and third rows of spinous cells were noticeably greater than at 10 days.

Though this shift was paralleled by an increase in thickness, the over-all ratio of frequency of mitosis for basal and spinous cells was decreased from (354: 37.456) 1:105.7 at 10 days to (404:45.304) 112.1 at 20 days, whereas Cooper and Reller (5) observed in their mounts of whole epidermis a ratio of 1:166.66 at 9 days and one of 1:106.9 at 23 days.

From the data presented it is a simple matter to calculate the ratios for separate mice at the various levels. The individual differences in the extension of mitosis into the several rows of the spinous layer are

[1] However the presence of epidermal cells possessed of more than one nucleus must be taken into consideration in arriving at an accurate measure of cellularity. These will soon be described by Gopal-Ayengar, A. R., "The Occurrence of Bi- and Multinucleated Cells in Epidermis." Anat. Rec. (In Press)

more clearly presented in Table II than in Table I. In 10 day mouse 3 and 20 day mouse 3 mitoses were not found distal to the first row. Variability in extension was noted in both groups of mice.

In the stratum basalis at 10 days the range was from 1:49.4 in mouse 1 to 1:94.4 in mouse 2; while at 20 days it was from 1:44.1 in mouse 4 to 1:126.1 in mouse 2. In 10 day mouse 2 the ratio of 1:63.5 in the second row of spinous cells was greater than that of 1:94.4 in the basal cells. Because of such variability we do not consider the difference in observed total ratios for basal cells between 1:58.99 at 10 days and 1:69.66 at 20 days (Table I) as necessarily significant.

DISCUSSION

Evidently this localization of maximum mitotic frequency in the stratum basalis of the epidermis 10 days after the first application of carcinogen is in interesting contrast to the localization of maximum mitotic frequency more distally in the middle third of the suprabasal epidermal layer of the hind foot pads of mice of the same strain.

But the mice, the foot pads of which were studied by Cowdry and Thompson, were younger than those used in this experiment, being only 10 days old, and it is of course possible that, had the counts been made on the foot pads of older mice, the level of maximum frequency might have been found to be more proximally situated than in the young ones. In other words, an age factor in the determination of this level may exist, but there is as yet no evidence whatever that it does exist. It is easy to be wise after the event. It would have provided a more direct comparison had Cowdry and Thompson employed 6 weeks old mice, but for us to have selected 10 day old mice, as they did, would have interfered with the directness of the comparison of the data herein described with other available data on properties of the hyperplastic epidermis 10 days after the initiation of treatment with the carcinogen. Even as it is, the evidence presented by Thuringer (6-8) that there are normally more mitoses in the spinous than in the basal strata in human beings and cats, in conformity with a similar location in young mice, suggests the probability that the usual location of maximum mitotic frequency in thick mouse epidermis is more distal than it is at 10 days in our carcinogenic series.

However this may be, proximal concentration of mitoses at 10 days is a property of the reacting epidermis contemporaneous with the presence of a new chemical equilibrium in it, details concerning which have been recently summarized (6). It is to be noted also that at 20 days the maximum mitotic frequency, still located in the stratum basalis, remained substantially the same as it was at 10 days just as the new chemical equilibrium endures without significant change for weeks. We could have made these differential mitotic counts at 30 and even at 40 days, but it would have been with increasing difficulty because the rows of cells become more irregular as hyperplasia advances and it did not seem necessary to prolong this fatiguing and monotonous kind of work. The essential task was to cover the early stages of hyperplasia during which many new properties appear, which, with remarkable uniformity, are maintained until about the time when a few cells break through the basement membrane, display malignant behavior, and form tumors.

It is not a case of the level of maximum mitotic frequency shifting, in consequence of the applications of carcinogen, to a position in close apposition to the underlying blood vessels from a more distal location. Rather is it the expression of a tendency on the part of mitotic division to continue in the descendants of the basal cells, in which it was originally centered when the epidermis consisted only of 2 or 3 layers of cells and before it became hyperplastic. With increasing hyperplasia more cells are displayed toward the surface and some of them undergo mitosis; but, up to 20 days in our series, the level of maximum mitotic frequency is still in the basal cells. Why is this level

TABLE II: INDIVIDUAL M/N RATIOS

Mice, 10 days No.	Stratum basalis	Stratum spinosum				
		Row 1	Row 2	Row 3	Row 4	Row 5
1	1:49.4	1:188.4	1:131.1	1:829.0	—	—
2	1:94.4	1:196.4	1:63.5	—	—	—
3	1:53.1	1:234.6	—	—	—	—
4	1:54.9	1:202.1	1:392.6	1:437.0	—	—
5	1:57.7	1:117.6	1:260.3	1:505.0	—	—
Mice, 20 days						
1	1:108.4	1:209.9	1:433.4	1:428.0	1:427.0	—
2	1:126.1	1:233.0	1:586.3	1:458.0	—	—
3	1:55.7	1:133.5	—	—	—	—
4	1:44.1	1:127.7	1:174.5	1:399.0	—	1:341.0
5	1:48.7	1:77.3	1:110.2	1:111.8	1:617.3	1:557.0

so proximal in location in contrast with its more distal situation in the spinous layer of normal epidermis of about the same thickness?

Perhaps the frequency of mitosis is conditioned by the action of methylcholanthrene and/or its products as mitotic stimulants or as inhibitors. Simpson and Cramer (13, 14) followed the carcinogen into the skin by fluorescence microscopy, and, by making fluorescence spectra, they noted significant changes in it. In stages of moderate hyperplasia, resulting from several paintings, the amount of fluorescent material is generally greatest in the distal strata, near the surface, and decreases as the basal layer is reached. If all of this fluorescent material is equally active carcinogenetically the basal layer may receive less of a stimulus than the spinous layer. If, further, we are dealing with an agent which in high concentration inhibits mitosis, or is less of a mitotic stimulant, the greater mitotic frequency observed in the basal layer would be understandable. This, however, is speculation. So, also, is the idea that the decomposition products of methylcholanthrene, which Simpson is detecting in the dermis, may be responsible for the greater mitotic frequency in the basal cells, which are more immediately exposed to dermal influences than the spinous ones, if, in the concentrations available to the epidermis, these products are mitotic stimulants.

It is likewise possible that the new chemical equilibrium, imposed on the epidermis in some way by methylcholanthrene and/or its products, does not influence equally the cells of the basal and spinous layers. In case the decreases in calcium, copper, cholesterol, biotin and other materials work greater hardship on the spinous cells than on the basal ones, which latter are nearer the source of supply in the vasularized dermis, living conditions compatible with mitoses may be less favorable for them than for the basal cells the higher mitotic frequency of which we are trying to explain.

More data are urgently needed on the localization of chemical alterations within the reacting epidermis. Analyses have been made chiefly of the whole epidermis of the area treated with carcinogen, indeed, usually of several specimens taken together to give sufficient amounts of tissue. The range in calcium content and in other properties between different epidermises similarly treated and between different parts of and different levels of a single epidermis, is not known.

All we have at present are certain microscopic observations of little quantitative value which suggest a greater change in the spinous than in the basal layer. These observations were that in the spinous layer the nuclear chromatin and nucleoli are more easily displaced than are those in the basal layer by equal ultracentrifugal force (8); the increase in volume of spinous cells is greater than that of basal cells (7); and that microincineration preparations sometimes show less mineral residue in the spinous cells than in the basal ones (11) suggesting that the cells of the spinous layer may be less well provided with minerals than those of the basal layer.

A major task ahead is to obtain these desired quantitative data on the individual chemical compositions of the basal and spinous layers. It will be necessary first to separate the two layers so that each may be analyzed by itself. By chemical means it is possible to split the epidermis into suprabasal and basal layers (1). This does not help much; for exposure to the solution modifies the composition of the tissue. The method holding greatest promise seems to be to stretch the skin and make thick sections of fresh frozen epidermis parallel to the surface. It might then be feasible to collect sufficient material for direct measurements of the components, calcium, cholesterol, etc., in the basal and spinous layers of the epidermis respectively. The chances are that it would be possible both to try other microchemical methods and to get contrasting data on enzyme composition of the two layers in such thick sections by Linderstrøm-Lang technics.

If evidence is secured that, during these early stages of carcinogenesis, the spinous cells are more subjected to deprivations of various sorts and are exposed to harsher living conditions than the basal cells, the fact that they show less mitotic activity than the basal cells would not be surprising. In the event of an actual struggle for existence among the spinous cells, not felt to the same degree in the basal ones, more favorably placed in respect to food supply and removal of waste, Spencer's (15) concept of "Cancer as a survival process" would receive support. There is reason to think that some cancer cells are tougher and have a greater ability to survive than normal cells from which they originate (3). Since only very few of the myriads of cells in the reacting epidermises undergo a malignant transformation, which thereafter appears to be indelibly engraved in the organization of their descendants so that by multiplication they form cancers, the chance that in our experimental series the malignant transformation is a kind of mutation, may not be lightly dismissed.

Unfortunately in the investigation of Biesele and Cowdry (2) observations of chromosome abnormalities had to be carried out on whole cells of impression preparations; because the sections, which would have given topographic information, might include only parts of the chromosome clumps. If the chromosome abnormalities are mainly in the spinous layer, as we think is the case, in view of the large size of cells and nuclei, the suggestion of a possible mutation therein would have

greater weight. Evidently before reaching a tentative conclusion, we must await not only accurate measurements of the contrasting properties of the spinous and basal layer but also more detailed microscopic observations than have yet been made on the actual location of the malignant transformation, and not be content with the tacit assumption that it is somewhere in the spinous layer.

Caution is necessary in basing conclusions on the observed differences in chemical composition of the resulting cancers and of the whole hyperplastic epidermises in which they originate. In this paper we report pronounced differences between the spinous and basal epidermal layers relative to mitotic frequency and we mention other possible differences especially in chemical composition. It is possible that the malignant change occurs in an epidermal layer, the composition of which resembles that of the cancers much more closely than does that of whole hyperplastic epidermis.

Evidently in this major project of Barnard Hospital, attempts are made to integrate observations bearing ever closer on the true sequence of events in the line of carcinogenesis; first, the properties of whole reacting epidermis; second, those of the particular epidermal layer in which the malignant transformation takes place (for the others are not in the direct line); third, those of the very few of many cells in the layer which undergo malignant change and finally, those of the components of the cells which condition their new malignant behavior. Eight years ago the keel of the project was laid; the launching is just in prospect.

SUMMARY

Of 5 male New Buffalo mice, 6 weeks old, the backs of which were treated 3 times with 0.6 per cent methylcholanthrene in benzene, 4 showed a greater mitotic frequency in the basal epidermal layer than in any of the rows of spinous cells, while 1 showed a greater frequency in the basal layer than in the first row of spinous cells and less than in the second row 10 days after the initial treatment.

Five similar mice showed maximum mitotic frequency in the basal layer 20 days after the first application of carcinogen. Though considerable variability was observed in individual ratios of mitoses to nondividing nuclei the conclusion is drawn that under the conditions of the experiment maximum mitotic frequency is predominately in the single layer of basal cells in touch with basement membranes.

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