Patterns of Growth and Ribosome Accumulation during 3-Methylcholanthrene-induced Epidermal Hyperplasia¹

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SUMMARY

The growth of mouse epidermis following a single topical application of 1, 2, or 4 μmoles 3-methylcholanthrene (MCA) to the back of a mouse and the pattern of ribosome accumulation associated with this growth were quantitated. All doses of MCA produced cell hypertrophy, cell damage, and basal cell loss, and increased intercellular spacing within 3 days; the extent and duration of these effects were increased with increasing dose. There followed accumulations of epidermal wet weight and protein and increases in the numbers of suprabasal cells and nucleated cell layers; the epidermis was also hyperkeratotic. Ribosome accumulation preceded epidermal growth. Total ribosomal RNA per cell was increased 2- to 3-fold 1 day after all doses of MCA. All doses elicited the same maximum cellular accumulation of ribosomes (3.5- to 4.5-fold) at Days 4 to 7. The accumulation of ribosomes was disproportionate in that it exceeded by over twofold the accumulation of protein and DNA per unit area of epidermis. The proportions of free and membrane-bound ribosomes did not change from normal following treatment (85% free:15% membrane-bound). Ribosome content remained elevated while the epidermis was hyperplastic; it returned toward normal levels as the epidermis approached its normal appearance. All variables measured were at normal levels 21 days after 2 μmoles MCA. These results are interpreted as an integrated sequence of cell damage and loss, ribosome accumulation, and regenerative growth.

INTRODUCTION

The regulation of the accumulation of rRNA and the regulation of cellular growth appear to be highly integrated in a wide variety of growth systems (3, 10, 11, 13, 15). Recent reports from this laboratory (2, 8) have shown that the growth of mouse epidermis induced by abrasion and during the promotion stage of 2-stage skin tumorigenesis is associated with large and disproportionate cellular accumulations of rRNA. In this study, we characterize the pattern of rRNA accumulation associated with the acute epidermal hyperplasia induced by various topically administered doses of the polycyclic hydrocarbon MCA.² A preliminary report of this work has been published (18).

MATERIALS AND METHODS

Chemicals and Reagents. MCA was obtained from Eastman Kodak Co., Rochester, N. Y., and all other chemicals used were reagent grade obtained from commercial sources (8).

Animals and Treatments. Albino CD-1 female mice (40 days old) were obtained from Charles River Farms, Wilmington, Mass., and were maintained as usual (8). Prior to treatment, the hair on the backs of 16 to 24 mice in the resting phase of the hair-growth cycle was removed with a small animal electric clipper (Oster Model A2, size 40, John Oster Manufacturing Co., Milwaukee, Wis.). Only mice showing no signs of hair growth during the next 2 days were treated. One, 2, or 4 μmoles MCA in 0.2 ml benzene were applied with a Biopet (Schwarz/Mann, Orangeburg, N. Y.) to the back of each mouse between 9 a.m. and 11 a.m. After treatment, mice were immediately placed for five min in a cage through which a stream of air was passed to remove toxic fumes as the benzene evaporated. Because our purpose was to compare treatment effects to normal epidermis and not to determine the specificity of MCA effects per se, normal mice (55 days old), clipped immediately after sacrifice, were used as controls.

Isolation of Epidermis and Determination of Its Area. Epidermis was isolated free of dermis by trypsinization at 4° as described previously (8). The total area of skin from which the epidermis was isolated was determined by the tracing technique developed in this laboratory (1). The measurement of any epidermal component (e.g., total mass, rRNA) could be thus normalized to a standard area of epidermis. Because such a measurement could not be determined accurately for the epidermis from the back of 1 mouse (approximately 12 sq cm) but was based upon a pool of epidermis from 16 to 24 mice, it was expressed per 100 sq cm epidermis (e.g., mg RNA per 100 sq cm epidermis). Such an expression was an index of the total quantity of that component on the back of a mouse and an index of the total accumulation of that component after treatment.

Isolation of Ribosomes and Analytical Methods. Ribosomal fractions were isolated from postnuclear supernatants of epidermal homogenates as described elsewhere

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3400 CANCER RESEARCH VOL. 37
(8). RNA and DNA were differentially hydrolyzed and extracted by the method of Schmidt and Thannhauser (20), modified as described previously (18). RNA was measured spectrophotometrically as 1 A_260 unit = 32 μg RNA per ml (4, 9). The purity of the RNA extracts was monitored by their A_260/A_280 ratios. These values occurred within the range of A_260/A_280 = 1.34 to 1.40, indicating relatively pure preparations of ribosomal ribonucleotides (19). DNA and protein were measured as described previously (8).

Histological Examination and Nuclear Counts of the Epidermis. Biopsy specimens of normal and MCA-treated whole skin were fixed in 4% formaldehyde and dehydrated in 1,4-dioxane. Sections were cut in paraffin at 7 μm and stained with hematoxylin and eosin.

The number of interfollicular epidermal basal and suprabasal nuclei and the number of nucleated cell layers were determined under oil immersion (×970). The number of nuclei in 5 fields (0.5 mm) of a square reticule 100-μm long were counted for each of 6 mice for each dose-time treatment combination. Increasing the number of fields counted to 10 did not result in a significant difference in the number of nuclei per mm of interfollicular epidermis. Only sections clearly sectioned perpendicularly to the dorsal surface of the skin were used.

Statistical Analysis. The methods of statistical analyses used have been described (8).

RESULTS

Morphological Observations. The morphological changes of mouse epidermis induced by single applications of various doses of MCA have been described (5, 6, 18) and are in general agreement with our present observations. We have extended these observations to include the effect of 3 doses of MCA on the numbers of both basal and suprabasal cells per mm interfollicular epidermis. One day after treatment with 1, 2, or 4 μmoles MCA, the number of suprabasal cell nuclei per mm interfollicular epidermis increased (Table 1), but the overall appearance of the epidermis was normal (Fig. 1). By Day 3 extensive cell injury was indicated by pyknotic nuclei and hypertrophic cells with very pale cytoplasm (Fig. 2). Furthermore, the number of basal cell nuclei per mm epidermis decreased (Table 1), and intercellular spacing increased markedly. The extent and duration of these effects were dose dependent: a 15% decrease in basal cells for 2 days after 1 μmole MCA, a 24 to 45% decrease for 3 days after 2 μmoles MCA, and a 19 to 54% decrease for 4 μmoles MCA.
days after 4 μmoles MCA. The epidermis then became increasingly hyperplastic (Fig. 3) through increases in the number of basal and suprabasal cells and in the number of nucleated cell layers (Table 1). The basal cells became vertically elongated, and intercellular spaces and bridges persisted among all cells. By 21 days after treatment, the epidermis was indistinguishable from normal except for occasional areas of hyperkeratosis (Fig. 4). There was a mild inflammatory response in the dermis soon after treatment with MCA; however, inflammatory cells were never seen in the epidermis.

**Epidermal Wet Weight, Protein, and DNA per 100 sq cm Epidermis.** Epidermal growth following MCA treatment was further quantitated as the net accumulations of epidermal wet weight, protein, and DNA per unit area of epidermis. Significant accumulations of epidermal wet weight per 100 sq cm epidermis did not occur until Day 2 or 3 after treatment (Charts 1 and 2); there then followed 3- to 5-fold increases over normal. The maximum growth induced by 1 μmole MCA was lesser in magnitude and occurred earlier than that induced by either 2 or 4 μmoles MCA; the latter doses, however, elicited equivalent responses. The kinetics of accumulation of protein per 100 sq cm epidermis was essentially the same for epidermal wet weight.

Homogenate DNA per 100 sq cm epidermis was an index of the accumulation of cells after treatment with MCA. Following 1 μmole MCA, this increased to 1.6 times normal by Day 5 and had begun to decrease by Day 7 (Chart 1). However, values 2.5 to 3.0 times normal were attained after 2 or 4 μmoles MCA by Days 3 and 4, respectively, and these increases were maintained through at least Day 10 (Chart 2).

**Ribosome Accumulation Associated with Epidermal Growth.** The net accumulation of rRNA on the back of a mouse was indicated by total rRNA per sq cm epidermis. There were no significant accumulations until Day 2 after 1 and 2 μmoles MCA and Day 4 after 4 μmoles MCA (Charts 1 and 2). The rate of accumulation increased after Day 3, and the maximum values observed were striking: 5.5 times normal after 1 μmole MCA at Day 5 and over 9 times normal after 2 or 4 μmoles MCA at Day 7. As was the case for epidermal wet weight and protein, 1 μmole MCA produced an earlier but lesser accumulation of rRNA than did 2 or 4 μmoles MCA. The maximum accumulation of total rRNA per 100 sq cm epidermis exceeded by over twofold the accumulations of epidermal wet weight, protein, and DNA. Thus, epidermal growth after MCA treatment was associated with a disproportionate accumulation of ribosomes.

Further characterization of this accumulation as total rRNA per homogenate DNA revealed several distinct features. First, total rRNA per DNA was significantly increased to 2 to 3 times normal 1 day after treatment following all doses of MCA (Charts 1 and 2). Thus, there was a significant
increase in total ribosomes per cell before any significant growth occurred. Second, although total rRNA per DNA continued to increase through Day 4 after 1 μmole MCA, it returned to normal levels by Day 3 after 2 or 4 μmoles MCA. This was followed by a 2nd accumulation of total ribosomes per cell to 2.5 times normal by Day 4 after 2 μmoles MCA; after a lag of 1 day, the same increase occurred by Day 5 after 4 μmoles MCA. Third, ribosome levels remained elevated while the epidermis was hyperplastic. Finally, all 3 doses of MCA elicited approximately the same maximum increase in total ribosomes per cell (3.5 to 4.5 times normal); however, the kinetics of accumulation, as noted above, after 1 μmole MCA was different from those after 2 and 4 μmoles MCA.

Epidermis 14 and 21 Days after Treatment. The data above indicated that the variables observed were returning to normal levels by Day 7 after 1 μmole MCA and by Day 10 after 2 and 4 μmoles MCA. Observations were made 14 and 21 days after treatment with 2 μmoles MCA to determine the relationship between ribosome levels and epidermal growth as the epidermis returned to normal. Fourteen days after treatment, the epidermis was still hyperplastic; epidermal mass, protein, and DNA per 100 sq cm epidermis and total rRNA per homogenate DNA were all increased to about 2 times normal (p < 0.05). By 21 days after treatment, only epidermal wet weight per 100 sq cm skin was still significantly increased (1.7 times normal, p < 0.05). Cellular ribosome content has now returned to normal levels (p > 0.05).

Free and Membrane-bound Ribosomes. The quantities of ribosomes sedimentable through the discontinuous sucrose gradient in the presence of detergent (total ribosomes) and in the absence of detergent (free ribosomes) were determined; the calculated differences between these represented the quantity of membrane-bound ribosomes. Normal epidermis contained predominantly free ribosomes (85% free, 15% membrane-bound), and this distribution was not altered significantly at any time after treatment with MCA. Therefore, the accumulation of total rRNA described above appeared to be a reflection of proportionate increases in free and membrane-bound rRNA. However, the increases in the amounts of membrane-bound rRNA were not, in most instances, statistically significant (data not shown). This was so because the values for membrane-bound rRNA were small and very close to the limits of detection of the techniques used.

Relative Ribosome Yields. Two questions were considered: (a) did the proportion of total homogenate RNA that was rRNA change after treatment, and (b) did the relative yield of ribosomes through the discontinuous sucrose gradient change after treatment? To answer these questions, ribosomes were isolated from the postnuclear supernatant in the absence of a discontinuous sucrose gradient; this fraction contained total cytoplasmic rRNA, i.e., all the sedimentable RNA in the postnuclear supernatant. The proportion of total homogenate RNA that was rRNA (i.e., total cytoplasmic RNA/homogenate RNA) increased from 62% in normal epidermis to 70 to 75% at the times of maximal ribosome accumulation following 1, 2, or 4 μmoles MCA (p < 0.05). The ratio of total rRNA/total cytoplasmic rRNA indicated the relative yield of ribosomes through the discontinuous sucrose gradient. Except for a 33% increase above normal at Day 2 after 1 μmole MCA and a 30% decrease below normal at Day 3 after 4 μmoles MCA, there were no significant changes after treatment in the relative yields of ribosomes (p > 0.05). Thus, at no time after treatment, including the 2 exceptions noted, could the reported 3.5- to 4.5-fold increases in cellular ribosome content be accounted for by changes in relative ribosome yield.

DISCUSSION

These results show that the epidermal growth and differentiation (keratinization) induced by a single application of MCA to the back of a mouse are preceded by and associated with cellular ribosome accumulation. The pattern of these events suggests a regenerative response in that the kinetics of ribosome accumulation and of the subsequent growth induced by the various doses of MCA is related to the basal cell loss produced by each dose of MCA. Initially, total rRNA/DNA increases 2 to 3 times over normal within 1 day following all doses of MCA. Then, increasing dose effects increasing and more persistent cell damage and cell loss. Hence, following 1 μmole MCA, while the epidermis is experiencing a mild cell loss between Days 1 and 3, the rate of accumulation of rRNA/DNA falls off; then after Day 3, there is a sharp increase in this rate for 1 day, at which time the maximum cellular accumulation of rRNA is achieved. Following 2 or 4 μmoles MCA, during the time when there is more severe cell damage and cell loss between Days 1 and 3, the rate of accumulation of rRNA/DNA reverses, and the cellular content of rRNA actually returns to normal levels. There is then a rapid, 2nd accumulation of ribosomes followed by further increases in the number of cells (DNA per 100 sq cm epidermis) and further accumulation of protein and wet weight per 100 sq cm epidermis. This 2nd accumulation of ribosomes following 4 μmoles MCA is delayed 1 day behind that after 2 μmoles MCA; this correlates with the additional day the epidermis appears damaged and sustains a significant decrease in cell number.

The present data support the concept that the accumulation of ribosomal RNA and the regulation of cellular growth are integrated. The times of increased basal cell labeling by [3H]thymidine reported by Bürki et al. (6) correspond to the times of increased cellular ribosome content reported here (Day 1 and Days 4 to 7). Also, mitotic activity (basal cell mitoses per cm epidermis) slowly begins to increase between 1 and 2 days and then rises 4-fold over controls by 3 days after treatment; it remains elevated through at least Day 7 (6).

Bürki et al. (6) have reported morphological and cyto kinetic changes in BALB/c mouse epidermis following treatment with 1.5 or 3 μmoles MCA consistent with the results reported here. In a comparison of effects produced by MCA and some of its metabolites they observed no positive relationship between increases in pyknotic nuclei (cell death) and subsequent hyperplasia (5). The authors suggest that the MCA-induced hyperplasia is not a regenerative response to cell injury, but rather is due to a direct stimulation of the epidermal cells by MCA (5). Although such a direct
stimulation is suggested in these results by the initial, rapid accumulation of ribosomes during the 1st 24 h after MCA treatment, we clearly observe a positive relationship between decreases in basal cell number and the subsequent regenerative response. Iversen and Evensen (12) concluded from an analysis of epidermal cell kinetics, including the actual rate of cell loss, that following MCA treatment the subsequent hyperplasia was the result of a sudden increase in cell death. Skjægestad (22) similarly showed a correlation between epidermal hyperplasia and increased cell loss following single applications of a variety of hyperplasia-producing and nonproducing carcinogens and noncarcinogens. Clearly, the issue merits further clarification.

The maximum relative cellular increases in total ribosomes following MCA treatment of the epidermis are very large (3- to 4-fold). The same relative increases in total homogenate RNA occur in the regenerative epidermis produced after removal of the epidermis by abrasion (2). These relative increases are similar to those that occur in cultured fibroblasts stimulated to divide by replating at subconfluent densities (14) or by serum stimulation (13, 17). These relative increases in ribosomes are much greater than those that occur during liver regeneration (15, 21, 23), drug-induced liver growth (11), compensatory renal hypertrophy (10, 16), or isoproterenol-stimulated salivary gland growth (3). However, each of these organs contains much greater quantities of cellular RNA than does the epidermis or most of the cultured cells studied. Therefore, the lower relative accumulations of ribosomes following stimulation of those organs with larger unstimulated levels of RNA result in roughly the same absolute net increases in cellular rRNA content as that occurring in the epidermis. Perhaps it is this absolute increase in ribosome number that meets the specific quantitative and/or qualitative requirements of the growing cell for protein synthesis.

One striking feature of the accumulation of epidermal RNA following MCA treatment, following abrasion (2), and during phorbol ester tumor promotion (8) is that it is disproportionate by severalfold to the accumulation of protein and DNA per sq cm epidermis. This is also apparent at the cellular level, because the rRNA/DNA and rRNA/protein ratios are also increased. Furthermore, this disproportionate ribosome content is maintained as long as the epidermis is hyperplastic, a period of 1 to 2 weeks following MCA treatment. This feature appears to be unique to the epidermis, because in those other organ systems cited above in which cell proliferation is the principle mechanism of growth, the increases in ribosome content are shorter-lived.

REFERENCES

Fig. 1. Cross-section of interfollicular epidermis of normal CD-1 female mouse. E, epidermis; c, corneum; s, spinosum; b, basal layer; D, dermis. H & E, x 430.

Fig. 2. Cross-section of interfollicular epidermis 3 days after treatment with 2 μmoles MCA. E, epidermis; c, corneum; g, granulosum; s, spinosum; b, basal layer; D, dermis. H & E, x 430.

Fig. 3. Cross-section of interfollicular epidermis 7 days after treatment with 2 μmoles MCA. Symbols as in Fig. 2. H & E, x 430.

Fig. 4. Cross-section of interfollicular epidermis 21 days after treatment with 2 μmoles MCA. Symbols as in Fig. 2. H & E, x 430.
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