Two-Stage Tumorigenesis of Dermal Melanocytes in the Back Skin of the Syrian Golden Hamster Using Systemic Initiation with 7,12-Dimethylbenz(a)anthracene and Topical Promotion with 12-O-Tetradecanoylphorbol-13-acetate

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ABSTRACT

Tumor formation in terms of a two-stage mechanism after initiation with 7,12-dimethylbenz(a)anthracene (DMBA) and promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) has been investigated in the Syrian golden hamster. Forty hamsters were initiated intragastrically by two applications of DMBA (50 mg/kg body weight each), followed by repeated topical promotion with 20 nmol TPA. In comparison with the corresponding control groups (no treatment, DMBA initiation only, and TPA treatment only), a clear-cut two stage mechanism was observed to operate in the perifollicular dermal melanocytes, leading to the early appearance of a high incidence of benign melanomas. Upon transplantation to normal hosts, these tumors rapidly underwent malignant growth and developed pigmented metastases in several organs. The morphology of melanoma formation resembled that described for DMBA-mediated melanocytic carcinogenesis in hamsters. The effect of TPA alone consisted of both a reactivation of nonfunctional melanocytes and an enhanced proliferation of the perifollicular melanocytic population. Thus, like epithelial two-stage carcinogenesis, pigmented cell tumor promotion seems to be linked to a hyperplasia of the target cell under consideration.

Although evidence for two-stage carcinogenesis also exists for some internal organs (i.e., esophagus, forestomach, liver), the dorsal epidermis did not show morphological alterations worth mentioning, despite the relatively high amounts of TPA applied. Even epidermal hyperplasia, normally the consequence of TPA application to mammalian skin, could scarcely be observed. Consistent with the view of an interdependence of hyperplastic and promoting capacity of phorbol esters, no tumors developed in the dorsal epidermis. In accordance with recent findings on two-stage carcinogenesis mechanisms in various organs of different animal species, melanocytic two-stage carcinogenesis in the hamster not only strengthens the general validity of the principle but also provides an excellent model for the study of melanotic tumors closely resembling those of humans.

INTRODUCTION

The classical application schedule of the 2-stage carcinogenesis experiment in mice, consisting of a carcinogenic polycyclic hydrocarbon used as an initiator and croton oil or TPA used as promoter, invariably causes tumors to arise in the dorsal epithelium of the animals. Almost all initiation-promotion experiments have been performed in the mouse, and numerous modifications at both the level of initiation and the level of promotion have emphasized the particular sensitivity of the dorsal epithelium as a target organ. In order to demonstrate, however, the general validity of the 2-stage principle and, more importantly, to distinguish relevant effects from side effects, the experiment required reproduction in species other than the mouse. Since earlier attempts had been unsuccessful (25), it appeared as if the experiment in its classical form was species and organ specific.

Only recently has the 2-stage principle been reevaluated with the demonstration of 2-stage mechanisms of carcinogenesis in internal organs of species other than the mouse by means of initiator-promoter combinations other than DMBA-TPA (5, 12, 16, 17, 19, 22, 30). Using the DMBA-TPA combination in the mouse, we have also been able to demonstrate in lifelong experiments that, apart from the dorsal epithelium, several internal organs had undoubtedly undergone tumor formation in terms of a 2-stage mechanism, although it was less pronounced than in dorsal skin (7-10, 24). Recently, we could show that upon appropriate systemic initiation and promotion the forestomach epithelium of the mouse exhibited the same high selectivity with regard to tumor formation as did the dorsal epithelium (11).

Therefore, we thought that it was mandatory to take up again the earlier unsuccessful DMBA-croton oil or TPA-mediated 2-stage experiments in other species, including the rat, rabbit, guinea pig, and hamster.

The present paper deals with the results in the Syrian golden hamster. Previous experiments have shown that the pigmented system of the hamster is particularly susceptible to neoplastic transformation by DMBA (15, 20). Melanocytic tumors in the hamster, having some features in common with cellular blue nevi of humans (21), occur in response to topically applied DMBA doses well below those capable of inducing epithelial tumors in this species (4). It was therefore of interest to investigate the behavior of both the dorsal integument and the dorsal pigmented cells upon systemic DMBA initiation and subsequent topical TPA promotion.

MATERIALS AND METHODS

One hundred 6-month-old female Syrian golden hamsters (Mesocricetus auratus), of a strain bred in the German Cancer Research Center and weighing approximately 100 ± 5 (S.D.) g, were used in the experiment. The animals were kept singly...
under specific-pathogen-free conditions in Macrolon type II cages and fed Altromin R10 Standard food pellets (Altromin, Lage/Lippe, Germany) with water available ad libitum. The dorsal skin of all animals was shaved routinely once per week over the total time of observation. The animals were assigned by random distribution to one of the following 4 experimental groups.

Group 1 (n = 20): Control Group. The animals received 0.2 ml acetone 3 times/week on the dorsal skin.

Group 2 (n = 20): TPA Group. Twenty nmol TPA in 0.2 ml acetone were applied topically to each animal 3 times/week. Maximum application time was 26 weeks. TPA was kindly provided by E. Hecker and his group, Institute of Biochemistry, Heidelberg.

Group 3 (n = 20): DMBA Group. Each animal was initiated within 1 week by 2 applications of DMBA, 50 mg kg body weight. The interval between the first and the second DMBA application was 3 days. The DMBA in the appropriate concentration was dissolved in sesame oil and administered i.g. by means of a stomach tube (11).

Group 4 (n = 40): DMBA-TPA Group. Each animal was initiated as described for Group 3 and promoted 1 week after the second DMBA administration according to the treatment of Group 2.

All animals sacrificed at the end of the experiment, as well as animals which died in the course of the experiment, were autopsied. Skin, tongue, trachea, esophagus, stomach, intestine, liver, lung, spleen, adrenal gland, uterus, ovaries, and bladder were histologically examined. Three benign melanomas, approximately 0.8 cm in diameter, were dissected under sterile conditions from 3 of the animals of Group 4 that had died spontaneously. One-half of each tumor was used for histological examinations; the remaining half was finely minced with scissors and suspended in 5 volumes of Hanks' balanced salt solution (Flow Laboratories, Meckenheim, Germany). From each cell suspension of the 3 tumors, 0.1 ml per animal was injected into the s.c. tissue of 5 recipient animals of the same strain.

RESULTS

Group 1. Five animals died spontaneously within 211 to 336 days after the commencement of the experiment. The remaining animals were sacrificed after 345 days of observation. Six animals had developed cholangiomas of the liver (Table 1), which is a current finding in our strain. Abnormalities of the pigimentary system, the dorsal integument, the internal epithelia, and the other organs investigated could not be observed (Table 1).

Group 2. Thirteen animals died spontaneously within 138 to 189 days (median, 159 days) after the commencement of the experiment. Thus, only 7 animals received TPA applications over the intended period of 26 weeks (182 days). The surviving animals were then killed after 2 days. The cause of death in animals that died spontaneously was mainly bronchial pneumonia and renal damage with pronounced albuminuric nephrosis. In addition to the strain-inherent frequency of cholangiomas, 3 animals had developed cholangiomas (Table 1). A frequent finding was the occurrence of amyloidoses of the spleen (8 of 20) and kidney (11 of 20). Six weeks after TPA treatment, more than one-half of the animals showed pronounced perifollicular melanoses in the treated area (Fig. 1); this was invariably preceded by a diffuse and moderate hyperpigmentation, which was also visible in animals that did not develop melanoses. Only a few animals showed signs of slight hyperplasia in the interfollicular epidermis, whereas moderate hyperkeratoses occurred more frequently. Esophageal hyperplastic response was comparable to that in interfollicular epidermis, whereas the forestomach seemed to be particularly susceptible (Table 1).

Group 3. All animals of this group died spontaneously within 118 to 161 days (median, 153 days). The strongest response to the systemically applied DMBA, including hyperplasia, hyperkeratoses, and formation of papillomas and carcinomas, occurred in the esophageal and forestomach epithelium; however, the skin was also affected in that 4 weeks after DMBA initiation one-half of the animals showed the beginning of hair depigmentation which progressively and irreversibly extended over the entire hair coat (Fig. 2). This alteration was less pronounced, yet visible, in the rest of the animals. Four animals of this group developed small, discrete melanotic melanomas arising from preceding perifollicular melanoses (Table 1).

Group 4. In this group, the survival time was even more reduced than in Group 3; all animals died spontaneously within 92 to 116 days (median, 111 days). Consequently, none of the animals received the total intended dose of TPA. The combined DMBA-TPA treatment led to a significant increase in the number of animals with benign esophageal alterations and to a slight increase in the number of animals with malignant tumors of the forestomach. The absolute number of animals with benign tumors of the forestomach was not higher than in Group 3; however, the multiplicity of these tumors was drastically augmented (Table 1).

Lesions in the back skin (i.e., depigmentation, melanoses, and epidermal alterations) of this group were in the same range...
as in Group 3; however, considerable differences occurred with regard to melanoma formation. Not only was the latent period of melanoma development reduced, but also the multiplicity of the tumors was significantly increased. Whereas each melanoma-bearing animal of Group 3 had developed, on an average, only 1 to 3 small tumors (mean diameter, 0.25 to 0.5 cm; mean latent period, 100 to 120 days), tumor-bearing animals of Group 4 consistently showed 6 to 9 tumors of increased size and volume (mean diameter, 0.5 to 1.0 cm; mean latent period, 40 to 60 days) (Table 1; Figs. 3 and 4).

Histological examination of the material to be transplanted revealed well-encapsulated benign melanotic tumors. In none of the 15 recipient animals of the first passage did rejection of the inoculated material occur. The survival time of the animals was in the range of 8 to 10 weeks. During this time, the tumors grew up to a size of approximately 1.5 to 2.0 cm in diameter at the site of inoculation and led to the formation of metastases in the regional lymph nodes and in the lungs (Figs. 5 and 6). Presently, the transplantable tumor is carried in the fourth generation, and its growth rate is slightly accelerated without showing signs of morphological alteration.

DISCUSSION

Unlike results obtained from experiments in the mouse skin, early experiments on tumor promotion in the rat, rabbit, and guinea pig did not lead to skin tumor formation by means of a 2-stage mechanism (25). Furthermore, using both topical DMBA initiation and croton oil promotion in another species, the Syrian golden hamster (4, 18), neither the dorsal epithelium nor the melanocytic system exhibited tumor formation that could be interpreted as 2-stage carcinogenesis (4).

In contrast, our modified application scheme consisting of systemic DMBA initiation and topical TPA promotion, although leaving the dorsal epithelium almost unaffected, strongly indicates a 2-stage carcinogenesis mechanism with regard to cutaneous melanoma formation. The rate and especially the incidence of tumors per animal in the DMBA-TPA-treated group were significantly higher when compared with the corresponding control groups (Table 1). Moreover, as is typical for 2-stage carcinogenesis, the latent period of tumor formation was drastically shortened.

The sequential morphological features of tumor formation in this new target cell susceptible to promotion followed in principle those features described in detail for the DMBA-mediated melanoma development in hamsters and other rodents (3, 4, 14, 20).

As a first demonstrable abnormality, a generalized and irreversible depigmentation of the hair coat was noted (Fig. 2). Almost concomitantly with the hair depigmentation, a slight and diffuse hyperpigmentation was visible (3, 13) in the TPA-treated area which readily turned into focal perifollicular melanoses (3, 4, 15, 20) consisting of fairly sharply defined, moderately pigmented spots (Fig. 1). When viewed from the dermis, melanomas developing from these lesions showed up as well-circumscribed, coal-black nodules (Fig. 3). In vertical sections, the tumors could be seen as well-demarcated, encapsulated lesions located within the apparently uninvolved dermal connective tissue. Even in cases where the tumor was immediately subjacent to the dermal-epidermal junction, the overlying epidermis never seemed to be morphologically altered (Fig. 4).

It is known that the pigmentary system of the Syrian golden hamster consists of 3 different melanocytic populations (14). The dysfunctional amelanotic junctional melanocytes (type 1) have been shown to be insensitive to DMBA treatment (14), and it is now generally accepted that the partly yet potentially functional melanocytes, occupying mainly the perifollicular dermal regions (type 2), represent the primary target cell of DMBA-induced melanomas (4, 14, 15, 20). There has been some uncertainty with regard to a possible participation of the functional melanocytes associated with the hair matrix (type 3) in tumor formation (4, 15, 20, 29). However, from our observation that systemically applied DMBA leads to irreversible hair depigmentation, a possible involvement of this melanocytic population in the hamster may be excluded.

Consistent with findings of others (4, 15, 20), the cutaneous tumors showed all criteria of benign melanomas. Neither infiltration of adjacent connective and epidermal tissue nor metastases in other organs was noted. However, after transplantation of 3 tumors to untreated hosts, the melanomas rapidly underwent, even in the first passage, invasive growth and developed metastases in the lymph nodes (Fig. 5) and lungs (Fig. 6). The capacity to produce pigment was retained in the transplanted tumors (Fig. 5). It is known that both human and rodent melanomas of this type show a biphasic growth pattern characterized by an initial radial growth phase followed by a subsequent vertical growth phase (3). As a rule, metastases and invasion do not develop until the vertical growth phase has evolved (3, 15, 20).

It is conceivable that, due to the strongly reduced survival time in the DMBA-TPA-treated group (Group 4), the tumors did not reach the phase of malignancy, but the malignant phase was seen after transplantation and probably enhanced by the transplantation procedure. In turn, the reduction in life expectancy was due to the apparently overdosed initiator, leading to tumor formation in internal organs, sometimes suggestive of a 2-stage mechanism in Group 4 (Table 1; Fig. 7), which shortened the survival time of the animals. The reduction of the survival time of the TPA-treated animals is more difficult to explain. In general, the animals showed signs of preaging (i.e., the high incidence of amyloidoses), and it may be that TPA strongly interferes with the general metabolism in this species.

The reaction of the pigmentary cells to TPA treatment alone throws some light on the promoting effect of the phorbol ester. Whereas the pigmentary system in the hair follicles remained unaffected (1), a fairly visible hyperpigmentation was seen, followed by a pronounced perifollicular melanosis shortly after the first TPA applications (Fig. 1 and 8). In spite of continuing treatments for 26 weeks, melanocyte networks retained their structural integrity without progressing to tumor formation. This observation has also been reported after croton oil treatment of hamsters (18). The perifollicular melanoses obviously consist not only of a reactivation of otherwise nonfunctional dermal melanocytes but also, to a considerable extent, of an enhanced proliferation of this cell population (3, 4, 14, 18, 20). Thus, like epithelial (2, 6, 26) and hepatic (26) 2-stage carcinogenesis, the cutaneous melanoma formation is intimately connected with a hyperplastic response of the corresponding target cell during the promotion period, and it may be assumed that the TPA-inducible enhanced melanosis is responsible for the reduction of the latent period of the tumors.

In sharp contrast to the strong response of the pigmentary
cells to TPA treatment is the apparent inactivity of the dorsal epidermis (Table 1; Fig. 8), which consistently shows hyperplastic transformation in other mammals [i.e., guinea pig (25), rat, rabbit], including humans (28). Preliminary results indicate that TPA doses even as high as 80 nmol in no way lead to a measurable increase in DNA synthesis and mitotic activity. Consistent with the current view of a relationship between hyperplasogenic and tumor-promoting effects of phorbol esters (2, 6, 23, 27), no papillomas or carcinomas developed in the dorsal epithelium of the hamsters (Table 1). The particular refractoriness of the dorsal epidermis of the hamster, which is no thicker than that of the mouse, is difficult to explain, especially since the squamous epithelia of the esophagus and the forestomach showed a strong hyperplasogenic response. The proliferative and cellular events in both the dorsal epidermis and dermal perifollicular melanocytes after single and repeated applications of TPA, as well as after other chemical and mechanical stimuli, are the subject of current investigations in our laboratory.

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CANCER RESEARCH VOL. 40

K. Goerttler et al.


2 K. Goerttler, H. Loehrke, J. Schweizer, unpublished observations.


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Fig. 1. Dorsal skin of an animal of Group 2 six weeks after application of TPA, showing numerous spots of peri follicular melanosis. Lesions occur mainly in slightly hyperpigmented areas. x 3.

Fig. 2. Right, generalized hair depigmentation 6 weeks after DMBA initiation in an animal of Group 3. The animal on the left (Group 1) received only sesame oil. x 0.5.

Fig. 3. Multiple melanomas in a DMBA TPA-treated animal (Group 4) viewed from the dermis. Arrow, lesion in an advanced state of melanosis at the transition to benign, encapsulated melanoma. x 2.
Fig. 4. Transverse section through a melanoma of an animal of Group 4. Despite the low magnification, it can be seen that the epidermis overlying the tumor is not essentially altered in comparison to the uninvolved tissue. H & E, x 25.

Fig. 5. Malignant melanotic melanoma of the I. passage 5 weeks after transplantation, with axillary lymph node metastasis. x 2.

Fig. 6. Same animal as in Fig. 5 showing heavily pigmented metastases in the lung. x 3.

Fig. 7. a, multiple papillomas in the forestomach of an animal of Group 3. x 3. b, multiple papillomas and carcinomas in the forestomach of an animal of Group 4. x 3.

Fig. 8. Transverse section through dorsal skin of an animal of Group 2 5 weeks after TPA application, showing perifollicular melanosis and slight hyperpigmentation in the dermis. Note the thin hyperkeratotic epidermis. H & E. x 150.
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