Progressive dysplasia and aneuploidy are hallmarks of mouse skin papillomas: Relevance to malignancy

(cancer/tumor promotion/chemical carcinogenesis/chromosome number)

Claudio M. Aldaz, Claudio J. Conti, Andres J. P. Klein-Szanto*, and Thomas J. Slaga

The University of Texas System Cancer Center, Science Park-Research Division, Smithville, TX 78957

Communicated by Van Rensselaer Potter, November 21, 1986

ABSTRACT We report a systematic histopathologic study of papillomas at different times during promotion, correlating the results with those from cytogenetic analysis of the same tumors. Papillomas were induced in SENCAR mice by two-stage carcinogenesis (7,12-dimethylbenz[a]anthracene and phorbol 12-myristate 13-acetate). Individual tumors were randomly sampled at different times during promotion, and histopathologic and cytogenetic studies were carried out on every tumor. Early during promotion (10 weeks), most papillomas were well-differentiated hyperplastic lesions with mild or no cellular atypia. No tumors showed severe dysplastic changes. By 20 weeks of promotion, a dramatic drop had occurred in the number of lesions with no dysplasia. Most of the tumors presented moderate dysplasia, and some already showed severe dysplastic changes. At later stages (30–40 weeks), most of the papillomas were classified as moderately or severely dysplastic papillomas, and several were considered to be intrapapillomatous carcinomas. This histopathologic evaluation was supported by nuclear measurements performed on papillomas at different time points. Chromosomal abnormalities followed a similar trend. Papillomas seem to start as diploid lesions, but between 10 and 20 weeks of promotion, hyperdiploid cells can be observed in almost every tumor. In some cases the stem line was taken over by aneuploid clones. At 40 weeks of promotion, all papillomas were aneuploid, most of them with hyperdiploid stem lines. A positive correlation was found between the histological and cytogenetic studies, with the most aggressive and atypical tumors being the more aneuploid. These results support the idea that most, if not all, papillomas are truly premalignant lesions in different stages of the potential progression toward malignancy. Chromosomal abnormalities might play an important role in the sequence of events leading to malignancy.

In spite of the great interest in tumor progression inciting those experiments, some fundamental questions about the biology of this system remain unanswered. It is unknown why, with a regular initiation–promotion protocol, only a small percentage of the papillomas become malignant and what accounts for the higher probability some papillomas have of progressing to malignancy. Some authors have postulated the existence of a subgroup of papillomas, which they called “terminally benign tumors” (2, 8), with little or no chance of progression toward the carcinoma stage. From a morphological point of view, the general assumption is that the putative subtypes of papillomas are indistinguishable from each other.

This assumption is largely based on macroscopic observation of tumors during the experimental period. Histologic examination is eventually performed at the end of tumor-promotion experiments to confirm the nature of the lesions. To our knowledge, a sequential histopathological follow-up of papillomas produced by the two-stage carcinogenesis protocol has not been reported. We recently reported (9) a cytogenetic study in this system; we found that aneuploidy occurs as a general phenomenon during the life of every papilloma and, in some tumors, from very early stages of development (9). Since aneuploidy is almost always related to either dysplastic or fully malignant lesions (10), we considered it important to carry out a sequential histopathologic study of the papillomas during the promotion phase and to classify the tumors following the criteria used for human premalignant lesions (e.g., cervical dysplasia). In this paper we report the results of our detailed histologic study and classification of a set of tumors whose cytogenetic status was already known, comparing both parameters and tumor behavior during the course of tumor promotion.

MATERIALS AND METHODS

Histopathological analysis and nuclear measurements were performed on a set of papillomas and carcinomas that have been characterized cytogenetically (9).

Papillomas and squamous cell carcinomas (SCCs) were induced in the back skin of SENCAR mice by a single dose of 10 nmol of 7,12-dimethylbenz[a]anthracene and repetitive applications of 2 μg of phorbol 12-myristate 13-acetate (PMA) twice a week. Tumors were randomly obtained at 10, 20, 30, and 40 weeks of promotion. Each tumor was divided into two pieces. One was processed for conventional histologic examination (formalin fixation and paraffin embedding). The other piece was prepared for chromosomal analysis using a direct cytogenetic technique described elsewhere (9, 11).

Abbreviations: SCC, squamous cell carcinoma; PMA, phorbol 12-myristate 13-acetate.

*Present address: Fox Chase Cancer Center, Department of Pathology, Philadelphia, PA 19111.
**Histopathological Classification of Papillomas.** We applied criteria and nomenclature similar to those used for the classification of human premalignant lesions to the papillomas. The histological changes that were considered as dysplastic included disturbed cell polarity (mainly in basal cells), basal cell hyperplasia, disturbed maturational sequence, increased number of mitoses, mitoses in suprabasal layers, abnormal mitoses, nuclear hyperchromatism, prominent nuclei, and increased nuclear/cytoplasmic ratio.

We subdivided the papillomas into four groups. Group 1. *Regular papilloma:* well-differentiated hyperplastic lesions with no atypical cells, or with very few atypical cells in the basal layer (Fig. 1A). Group 2. *Moderately dysplastic papilloma:* lesions with atypical cells in the basal and suprabasal layers up to one-third of the thickness of the epithelium. Group 3. *Severely dysplastic papilloma:* lesions with more than two-thirds of the thickness of the epithelium occupied by atypical cells (Fig. 1B). Group 4. *Intrapapillomatous carcinoma:* lesions equivalent to carcinoma in situ, with marked atypia in all layers and lack of differentiation patterns in the surface. The whole epithelium is occupied by neoplastic cells (Fig. 1C). If invasion was observed, the lesion was considered malignant and consequently not included in this subgroup.

Papillomas were classified by two independent observers according to the above criteria. The observers had knowledge of neither the age of the papillomas nor the cytogenetic status of each tumor.

**Nuclear Measurements.** Nuclear areas of cells from the basal layer of control normal skin (five mice), 30 papillomas, and 10 SCCs were measured using a Video Plan image analyzer (Zeiss, Oberkochen, FRG) on direct projections from the histologic sections. One hundred nuclei were measured in each sample. The most atypical areas of each histologic section were selected for the measurements.

**RESULTS**

Once the histopathological grading of the lesions was performed, the tumors were grouped according to the number of weeks of promotion. As can be observed in Fig. 2, at 10 weeks of promoter treatment, 70% (7 out of 10) of the papillomas corresponded to group 1 lesions (regular papillomas; see Materials and Methods) and only 30% to group 2 (moderately dysplastic papillomas). No tumors showed severe dysplastic changes (groups 3 and 4).

At 20 weeks of promotion, a dramatic drop occurred in the number of lesions with no dysplasia (regular papillomas), only 1 out of 11 tumors. Most of the papillomas presented moderate dysplasia, and 27% of the tumors already showed severe dysplastic changes.

Papillomas obtained from the animals at 30 and 40 weeks were considered as a single group because histopathologically and cytogenetically they showed almost identical profiles. In these groups, only 2 out of 16 tumors (12.5%) did not show dysplastic changes. Most of the tumors were classified as moderately or severely dysplastic papillomas, and 4 of 16 (25%) were considered to be intrapapillomatous carcinomas, presenting severe atypia in all layers and no squamous differentiation on the surface (Figs. 1C and 2). In most papillomas the dysplastic changes were focal. It was common to observe the coexistence, in the same histologic section, of areas with slight or no atypia, together with foci of severe atypia.

In an attempt to reduce the subjective element in our histopathological evaluation of papillomas, we measured the nuclear areas of the cells in the basal layer of normal skin, papillomas, and carcinomas. We found that this parameter changed with time, reaching in papillomas at 30 and 40 weeks values similar to those found in carcinomas (Fig. 3). Such progression in nuclear size clearly corresponds with and supports the histopathological grading of the same tumors. The cytogenetic studies performed with the same papillomas were discussed in detail in a previous report (9). For comparative purposes, those data are summarized in Fig. 4. Because of difficulties in chromosomal preparation, it was not possible to analyze cytogenetically every tumor included in Fig. 2. Analyzable metaphases were obtained in 7 of 10 papillomas at 10 weeks, in 10 of 11 at 20 weeks, and in 13 of 16 at 30–40 weeks.

No correlation was found between size of the tumors and histopathological status or degree of aneuploidy.
DISCUSSION

The definition of "premalignant lesion" implies the relatively high probability for a lesion to undergo malignant transformation. This definition does not connote the inevitability of such degeneration or irreversibility in the progression towards the cancerous state. In fact, there is experimental (12) and human epidemiological (13) evidence showing spontaneous regression of premalignant lesions; on the other hand, several cases have been reported of human cervix carcinomas in situ and of severe dysplasias that progressed to invasive carcinomas even up to 20 years after the first diagnosis (13).

If we analyze the information available on the mouse skin system using the two-stage protocol, we observe that the carcinoma incidence almost never reaches a plateau when the experiments are arbitrarily terminated at 40 or 50 weeks (1–3, 8, 14). Even in those cases in which the animals were observed for longer periods, the carcinoma incidence kept rising (3), indicating the possibility of continuous conversion of new papillomas.

Our experimental data show clear histopathological and cytogenetic progression in most of the papillomas, which were randomly selected. Early during promotion, most papillomas were well-differentiated hyperplastic lesions with little or no cellular atypia, and most of them had diploid chromosomal constitution (Figs. 2 and 4). At 30 and 40 weeks, almost every tumor presented a degree of dysplasia and a high proportion of the lesions had reached the carcinoma in situ stage (intrapapillomatous carcinoma) (Figs. 1C and 2), and at this stage diploid tumors were not found (Fig. 4). Our assumption is that if the animals could have lived long enough, we would have seen most of those lesions progress to the invasive stage. It has to be considered that PMA toxicity and concurrent infections prematurely kill a high percentage of the animals (15).

In mouse skin papillomas, not only aneuploidy, but also point mutations, seems to play a fundamental role in the development of malignancy. The Ha-ras gene mutation was postulated to be critically involved in the initiation event (16, 17). Independently we have shown that papillomas, the putative expanded population of initiated cells, bear a high degree of genomic instability, which may increase the probability of a second relevant event occurring in those cells, as postulated by Potter (18) and Moolgavkar and Knudson (4). The nature of such an event or combination of events remains unknown. Our results suggest, however, that such events may be the result of gross chromosomal abnormalities. The biological significance of aneuploidy, the most consistent chromosomal alteration in solid tumors, has been reviewed recently by Oshimura and Barrett (19). They concluded that some of the main effects that could be produced by aneuploidy are genetic disbalance, phenotypic expression of recessive mutations, and changes in genetic stability.

In studies performed to identify the specific chromosomal alterations involved in SCC (unpublished results) the most common abnormality found was chromosome duplication (trisomy). Extra copies of chromosomes may represent not only a mechanism of genetic disbalance but also a form of gene amplification (19). We recently reported the occurrence of other cytogenetic equivalents of gene amplification in the mouse skin system (20). These findings seem relevant if we consider that gene amplification has been reported to be...
Papillomas have already occurred in many tumors, which suggests a close correlation between the occurrence of papillomas and the development of tumors. The sequential appearance of papillomas in different stages of the potential progression toward malignancy is important in understanding the sequence of events leading to the malignant stage.

We are grateful to J. A. Fries for excellent technical assistance, to J. Mayhugh for typing this manuscript, and to J. Riley for the artwork. These studies were supported by National Institutes of Health Grants CA42157 and CA38863.

The observation of the sequential appearance of papillomas and the development of tumors is consistent with the model postulated by Nowell (22), in which the tumors develop in a sequential manner. The sequential appearance of papillomas and the development of tumors is consistent with the model postulated by Nowell (22), in which the tumors develop in a sequential manner. The sequential appearance of papillomas and the development of tumors is consistent with the model postulated by Nowell (22), in which the tumors develop in a sequential manner.

The sequential appearance of papillomas in different stages of the potential progression toward malignancy. Within this scenario, chromosomal abnormalities might play an important role in the sequence of events leading to the malignant stage.

We are grateful to J. A. Fries for excellent technical assistance, to J. Mayhugh for typing this manuscript, and to J. Riley for the artwork. These studies were supported by National Institutes of Health Grants CA42157 and CA38863.