Virchows Arch [Pathol Anat] (1986) 409:47–59

Virchows Archiv A

Malignant melanoma – its precursors and its topography of proliferation

DNA-Feulgen-cytophotometry and mitosis index*

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Summary. DNA-content and size of the nuclear areas in different zones of malignant melanomas of different histological types and in dysplastic naevi were measured in order to provide information on the histogenesis and proliferative behaviour of human malignant melanoma. The results were compared with those from normal epidermis, common naevi, and reactive melanocytic hyperplasias. The mitotic index of melanomas – divided into different topographic zones in an analogous way – was also determined. The DNA-histographs of all naevi and reactive melanocytic hyperplasias showed a diploid maximum, but the dysplastic naevi had a larger proportion of nuclei with hyperdiploid and tetraploid DNA-content, indicating an increased proliferative activity. The mean values ($\bar{X}$) of nuclear areas in dysplastic naevi (DN) were about the same as in common naevi (CN) and slightly lower than in superficial spreading melanomas (SSM). The coefficient of variability (cv) as an indicator of anisokaryosis was markedly higher in DN (27.8) and SSM (29.3) than in CN (20.2). In DNA-content we found similar results: almost no difference in mean values, but DN taking an intermediate position between CN and SSM with respect to cv (CN: 12.3; DN: 21.0; SSM: 36.6). There was no unequivocal evidence in these data for DN being a precancerous stage. Superficial melanomas with a nodular component ("SSM/NM") differed from SSM and NM by increased DNA-content and greater variability of nuclear areas and showed the clearest features of malignancy in their DNA-histographs. The mitotic indices had rather low values in SSM and intraepidermal marginal zones of "SSM/NM" on one hand and markedly higher values in NM and nodular parts of "SSM/NM" on the other. The highest mitotic counts were found in the three investigated metastases.

* We gratefully acknowledge the generous support of the Hamburger Stiftung zur Förderung der Krebsbekämpfung

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Key words: Malignant melanoma – Precursors – Topography – Mitotic index – Feulgen-cytophotometry

Introduction

Both benign and malignant melanocytic lesions arise in the epidermis and tend to invade the dermis. Whereas the common naevocellular naevus is totally harmless, malignant melanoma, a tumour with recently increased incidence (Magnus 1981; Lee 1983) has a poor prognosis depending upon the degree of dermal invasion more than on any other factor (Breslow 1970; Sober et al. 1983). The controversal problem of the histological position of dysplastic naevi as questionable precursors of melanomas has been widely discussed (Ackerman 1980; Crucioli and Stilwell 1982; Elder et al. 1980; Kraemer 1983; Kraemer et al. 1983; McKie 1981; Rhodes et al. 1983), and this text addresses the problems of introducing objective criteria into this discussion.

The degree of nuclear pleomorphism can be quantified by measurement of nuclear area. The coefficient of variability of nuclear planimetry is a parameter of varying size of nuclei that does not depend on the degree of chromatism. The determination of the nuclear DNA-content by Feulgen-cytophotometry in the same cells gives evidence of their nuclear chromatism and allows differentiation between normo-(di-)ploidy, benign hyperdiploid proliferation, and malignant DNA-distribution patterns like aneuploid tumor cell stem lines (Boehm and Sandritter 1975).

In this paper we use the term "anisokaryosis" with respect to variability of nuclear areas and "nuclear atypia" only if there are objective indicators of malignancy like aneuploid DNA-patterns.

Without regard to nuclear or other cellular criteria there are unequivocal structural/architectural patterns of melanocytic lesions that permit the diagnosis of "malignant melanoma". The most important of these are an asymmetrical growth pattern and intraepidermal/dermal destructive infiltration (Ackerman 1981). Whether or not the several types of malignant melanoma like "superficial spreading melanoma" (SSM) and "nodular melanoma" (NM) determine the prognosis per se, independent of their level of invasion, is uncertain. Ackerman suggests that the melanoma subtypes introduced by Clark and coworkers are only different time stages in melanoma ontogenesis (Ackerman 1980; Ackerman 1981; Clark et al. 1969; Clark et al. 1977).

We divided the melanomas we investigated into three subgroups:

1. Superficial spreading melanoma (SSM)
2. Superficial spreading melanoma with a nodular component ("SSM/NM")
3. Nodular melanoma (NM).

This was not done in order to find a new classification but only to define our melanomas descriptively instead of by a histogenetic concept. The aim of our investigation was the distribution pattern of objective parameters of anisokaryosis (varying nuclear size) and cellular DNA-content in

topographically well defined regions of malignant melanoma, dysplastic naevus and common naevocellular naevus in comparison with melanocytic hyperplasia (concommitant with dermal histiocytoma) and normal epidermis. The results were compared with the topography of mitotic index, being correlated with the prognosis of malignant melanoma (Schmoeckel and Braun-Falco 1978; Sober et al. 1983).

Material and methods

A total of 73 melanomas and naevi from patients who had been diagnosed and operated in the Dermatological Clinic of the University of Hamburg were investigated. The histological diagnoses of melanomas were made according to the classification of Clark and coworkers. The criteria for the diagnosis of dysplastic naevi were as follows:
- structural patterns like lentiginous nests of different sizes and shapes
- horizontal extension of melanocytes within the epidermis beyond the bulk of the naevus at the dermo-epidermal junction
- melanocytes with usually abundant and pale-staining cytoplasms containing scanty dusty melanin
- nuclei with a relatively higher degree of anisokaryosis compared with common naevi
- prominent collagen around the rete ridges
- lymphocytic infiltrate of varying degree in the upper dermis.

1. *Index of mitosis.* The mitotic index was determined in 38 melanomas (14 NM, 18 SSM – 7 of which were considered as a distinct subgroup because of their marked nodular component – 3 unclassifiable melanomas and 3 metastases).

The tissue was fixed in 4% neutrally buffered 4° C cold formalin for 48 h. After dehydration it was embedded in paraffin wax. 5 µm thick sections were cut and stained with haematoxylin-eosin in the usual manner.

For the determination of the mitotic index the tumours were subdivided into different regions, NM into 3 horizontal zones:
1. the superficial zone
2. the central zone
3. the zone of the deepest invasion.

In each region an average of about 1,100 tumour cells was counted at 1,000 X magnification and the number of mitoses was determined. A clear distance between the different regions investigated was deliberately monitored. In some NM only two areas as described under 1. and 3. could be examined due to the small vertical diameter of the lesions.

SSM were divided into 2 different zones:
1. the basal cell layer or the layer close to the subcutis
2. the suprabasal part of the lesion.

SSM with a nodular component ("SSM/NM") were divided in their two components in analogy to SSM and NM. The choice of these regions was due to the idea that there might be zones of high proliferation e.g. at the lower border of the lesion.

The others (unclassifiable melanomas and metastases) were divided into two to five zones depending on their size and shape. For statistical evaluation of the results we performed Student's *t*-test.

2. *DNA-content and nuclear planimetry.* 10 melanomas (2 NM, 5 SSM, 2 "SSM/NM", and 1 metastasis), 15 common naevi, 15 dysplastic naevi, and 5 reactive melanocytic hyperplasias were investigated with respect to their DNA-content and nuclear areas. The melanomas were divided into different zones: NM as described above, SSM into the marginal zones and one or more central zones, "SSM/NM" into intraepidermal marginal zones and the superficial and the deep zone of the nodular part, the metastasis into 4 marginal and 1 central zones.

A minimum of 50 nuclei per region or lesion was measured. The results were compared to normal epidermal cells which served as a standard. 7.5 µm thick sections were cut using

a Reichert-Jung microtome and Feather® disposable microtome blades. The sections were mounted on slides and deparaffined. After hydrolysis in 1 n HCl (pH 1.2) for 15 min at 60° C they were stained with Schiff's reagens for 90 min at 25° C and then put into $Na_2S_2O_5$ for 30 min. Following dehydration sections were mounted in Eukitt® (refractive index: 1:1.494).

A section thickness of 7.5 µm appeared to be useful for our measurements, as these slides contained a sufficient number of complete nuclei and not too many overlapping nuclei, factors which are prerequisites for cytophotometrical analysis. By focussing the nucleus it was ascertained that the nucleus was entirely in the slide. The measurement of transmission was performed at the level of maximum length and breadth of the nuclei which served for the determination of DNA-content and was also taken as parameter of anisokaryosis (variation of nuclear size).

The measurements were performed with a microscope-photometer MPV 3 (Leitz, Wetzlar) using the plug-method at a wavelength of 587 ± 9.5 nm under constant optical and electronic conditions. In each nucleus measured the length and breadth was determined and so the nuclear area was calculated using the formula $F = \pi \times a \times b$. This is correct only for a perfectly ovoid nucleus but as the heterogeneity of nuclear shape of the cells investigated was relatively small, it seemed to be sufficient for our purpose. The transmission of the Feulgen-stained nucleus was measured and used for the calculation of the extinction according to $e = \log 1/T$. The product of extinction and nuclear area gave as a result the DNA-content in arbitrary units (au).

The interpretation of the DNA-distribution patterns is shown in the histographs. In addition each group of nuclei was divided into 3 ploidy-levels for statistical evaluation:
1. hypodiploid and diploid ($\leq 2\,n$)
2. hyperdiploid and tetraploid ($>2\,n$–$4\,n$)
3. hypertetraploid ($>4\,n$)
and compared with each other using the Chi^2-test.

Results

1. Mitotic index

The results of the melanomas investigated are summarized in Tables 1 and 2. 11 of them were SSM, 7 superficial spreading melanomas with a nodular component ("SSM/NM"), 14 nodular melanomas (NM), 3 metastases and 3 could not be classified. The subdivision into different topographic regions was made as described above. Per region an average of 1,131 (232–

Table 1. Mitotic index (in percents) of SSM, "SSM/NM", and NM with respect to topographical analysis

Hist. type	*n*	Cells counted	Mitotic index in percents					Total
			SSM		NM			
			Upper zone	Lower zone	Superf. zone	Central zone	Deep zone	
SSM	11	1,182 (774–1,773)	0.22 (0–0.5)	0.33 (0–0.6)				0.25
"SSM/NM"	7	936 (232–1,837)	0.27 (0–0.65)	0.24 (0–0.62)	0.52 (0.28–0.84)	0.5 (0.31–1.12)	0.41 (0.26–0.45)	0.42
NM	14	1,260 (568–2,407)			0.59 (0.2–1.38)	0.54 (0.12–1.2)	0.55 (0.18–0.72)	0.56

Table 2. Mitotic index (in percents) of metastases and unclassified melanomas with respect to topographical analysis

Hist. type	*n*	Cells counted	Mitotic index in %		Total
			Central Zone	Marginal Zone	
Metastasis	3	1,134 (662–1,864)	0.92 (0.79–1.04)	0.93 (0.69–1.16)	0.93
Unclassif. melanoma	3	1,097 (763–1,856)	0.92 (0.26–1.18)	0.84 (0–1.52)	0.86

2,407) cells was counted and the number of mitoses in each region was determined. There was almost no difference between the values in the different topographic zones in the same tumour with the exception of the "SSM/NM", which showed rather low values in the intraepidermal marginal zones and markedly higher ones in their nodular parts. Using Student's *t*-test, there was a statistically significant difference between SSM and NM with $t=4.3393$ (DF$=66$, $\alpha=0.1$), and between "SSM/NM" and NM with $t=2.5781$ (DF$=19$, $\alpha=1$). The highest values were found in the 3 metastases and the unclassified melanomas.

2. DNA-cytophotometry and nuclear planimetry

The histographs of all common naevi and reactive melanocytic hyperplasias showed a normoploid distribution pattern with only very few values exceeding the diploid range and none above the tetraploid value. The mean values of DNA-content and the coefficients of variability were similar to those of normal epidermis, which served as a (diploid) standard. The histographs of the dysplastic naevi also had diploid peaks, but a markedly higher number of nuclei between the diploid and tetraploid regions. This finding is usually correlated with a greater fraction of cells in S-phase as an expression of an increased proliferative activity (Boehm and Sandritter 1975). The variability of nuclear areas was evidently higher in DN than in CN (27.8 compared to 20.2) and reached almost the value found in SSM (29.3). So the degree of nuclear pleomorphism in DN was almost as pronounced as in malignant melanomas of the SSM-type and clearly higher than in common naevi. The data is summarized in Table 3. Some typical examples of DNA-histographs are shown in Fig. 1.

The different topographic zones in SSM and in NM showed different results, but no constant rules for these differences could be recognized. In the 2 "SSM/NM" investigated we found aneuploid stem lines in the intraepidermal marginal zones of both tumours. In one case the same stem line was also found in the nodular part.

In the melanomas investigated there were surprisingly few features of malignancy assessed by means of DNA-distribution patterns. Many of them had diploid peaks in their histographs and only few nuclei had a DNA-

Table 3. Nuclear areas, Feulgen-cytophotometrical measured DNA-content and ploidy levels of melanocytic lesions regarding topographical analysis (X = mean value, cv = coefficient of variability)

	n	Nuclear area (μ^2)		DNA-content (au)			Ploidy level (%)	
		X	cv	X	cv	2 n	2 n–4 n	4 n
Epidermis	250	20.85	18.7	2.73	11.7	99	1	–
React. melan. Hyperplasia	750	22.75	21.7	2.94	12.7	98.4	1.6	–
Common naevi	750	23.4	20.2	2.84	12.3	98.2	1.8	–
Dyspl. naevi	750	22.53	27.8	2.93	21.0	93.5	6.5	–
SSM total	780	25.66	29.3	2.89	36.6	85.9	12.8	1.3
SSM margin.	330	25.46	28.3	2.85	32.5	87.3	11.1	0.6
SSM central	450	25.81	30.0	2.92	39.3	84.9	13.3	1.8
"SSM/NM" total	350	35.33	40.2	4.16	44.3	48.3	44.6	7.1
"SSM/NM" intraep.	150	35.06	39.3	4.11	45.0	47.3	45.3	7.3
"SSM/NM" nodular	200	35.53	41.0	4.20	43.8	49	44	7
NM total	900	35.61	30.5	3.60	39.0	66.4	29.8	3.8
NM superficial	300	39.1	27.6	3.80	46.8	60.7	35.3	4
NM central	300	34.89	32.6	3.74	39.3	59.7	35.3	5
NM deep	300	32.85	28.6	3.24	37.5	79	18.6	2.3
Metastasis	250	26.23	30.7	2.82	31.0	87.2	12.8	–

content beyond the tetraploid range. Aneuploid stem lines could only be found in some cases, but never in all parts of the tumour. Figure 2 gives an example of the histiographs of the different topographic regions and their sum in one "SSM/NM". The results of DNA-cytophotometry and nuclear planimetry are summarized in Table 3.

For statistical evaluation the different groups were arranged in three ploidy levels:

1. hypodiploid and diploid (≤ 2 n)
2. hyperdiploid and tetraploid (>2 n–4 n)
3. hypertetraploid (>4 n)

and then compared with each other using the Chi^2-test. The only significant difference between different topographic zones within the same tumour was found between the deep zones of NM and the central and superficial zones of these tumours, the deep zone showing a more monomorphic distribution. The comparison of the different lesions revealed statistically significant differences between DN and CN ($Chi^2 = 20.2961$; $DF = 1$; $\alpha = 0.1$) as well as between DN and reactive melanocytic hyperplasias ($Chi^2 = 9.0919$; $DF = 1$; $\alpha = 1$).

There were also differences between the three groups of melanoma investigated:

SSM vs. "SSM/NM": $Chi^2 = 177.0631$ ($DF = 2$; $\alpha = 0.1$)
SSM vs. NM: $Chi^2 = 85.7409$ ($DF = 2$; $\alpha = 0.1$)
"SSM/NM" vs. NM: $Chi^2 = 34.1923$ ($DF = 2$; $\alpha = 0.1$).

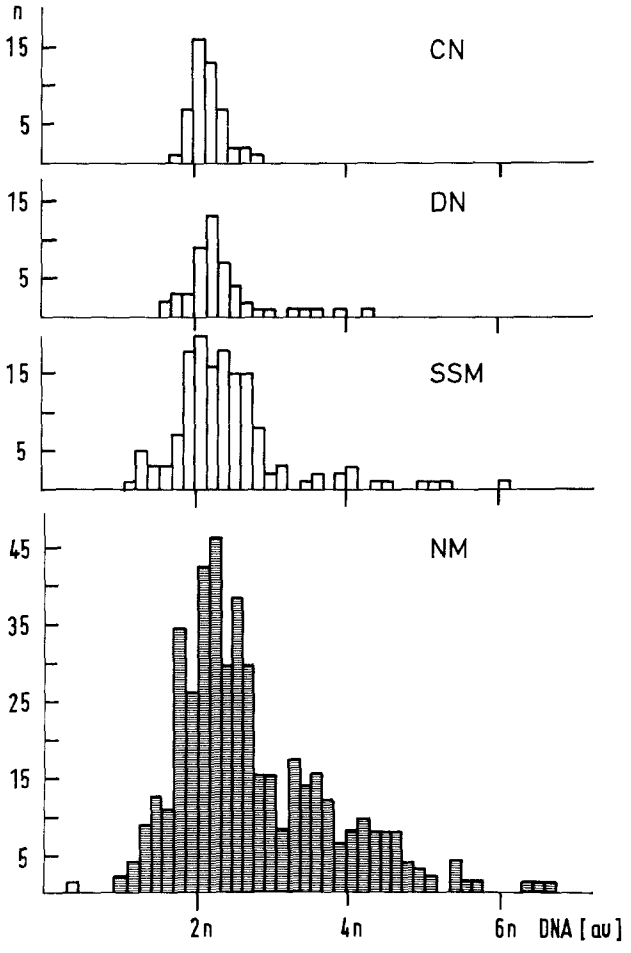

Fig. 1. Typical examples of DNA-histographs: Common naevi (*CN*) with diploid DNA-distribution, dysplastic naevi (*DN*) with signs of increased proliferation, superficial spreading melanoma (*SSM*) and nodular melanoma (*NM*) with signs of malignancy (broadened peak, cells beyond the 4n-range, and an aneuploid stem line at about 3n in the (*NM*) (ordinate: n = number of cells)

Finally DN as possible precursors of malignant melanoma were compared to SSM. They differed significantly from each other:
DN vs. SSM: $\text{Chi}^2 = 27.5795$ ($\text{DF} = 2$; $\alpha = 0.1$).

Discussion

This investigation had two aims. The first was whether the different histological types of melanoma defined according to the usual classification have basically different DNA-distribution patterns, nuclear areas, and mitotic indices. Secondly we investigated whether the so called dysplastic naevi – as opposed to common naevi and reactive melanocytic hyperplasia – would

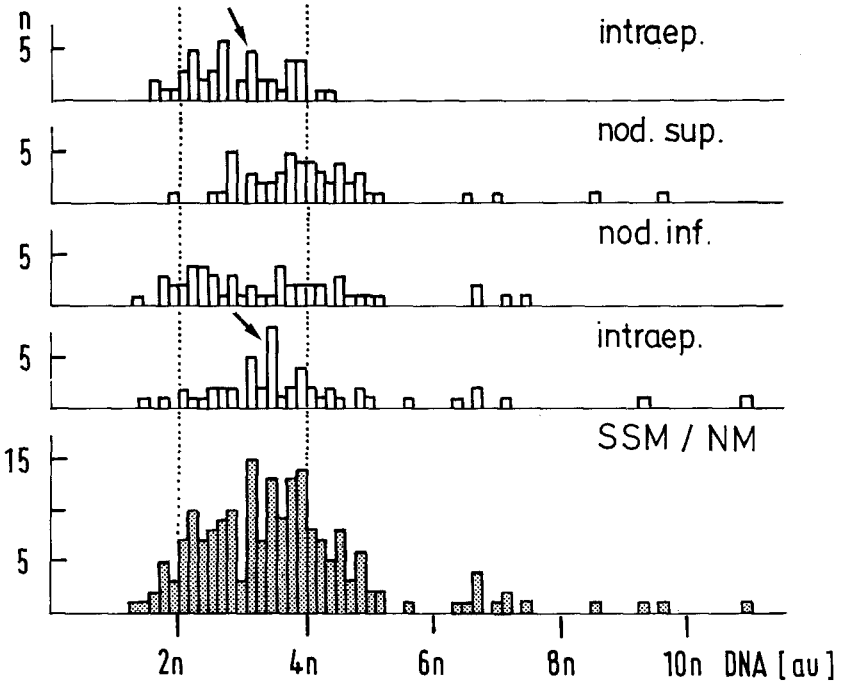

Fig. 2. Topographical pattern of DNA-distribution in a "SSM/NM": Two different aneuploid stem lines at about 3n in the intraepidermal zones (*arrows*), one of which can also be found in the upper zone of the nodular part (nod. sup.). Tumour cell stem lines which are only present in one zone may not be detected in the DNA-histograph presenting the whole tumour (see "SSM/NM") (ordinate: n = number of cells)

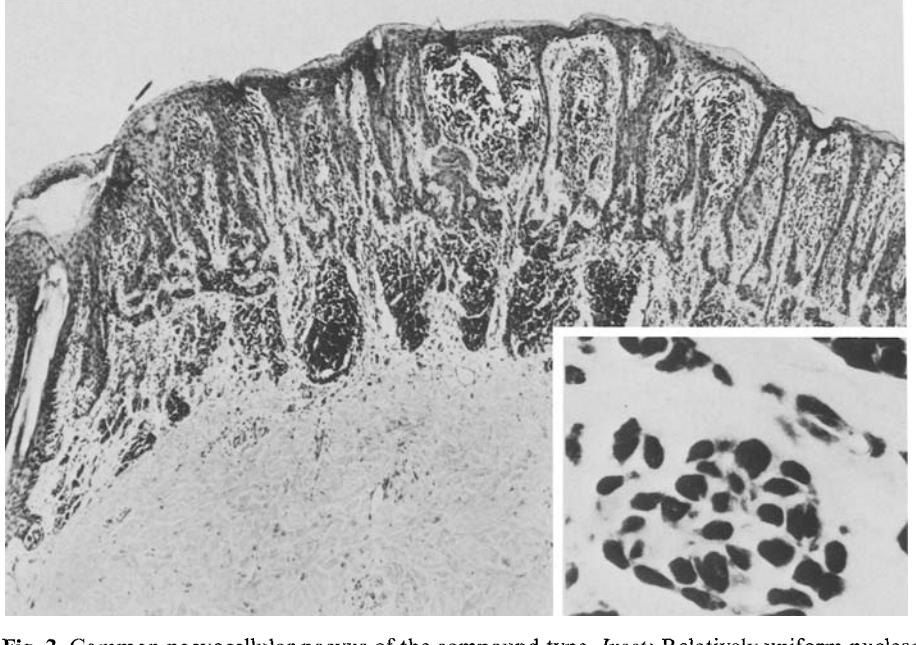

Fig. 3. Common naevocellular naevus of the compound type. *Inset*: Relatively uniform nuclear size of the melanozytes

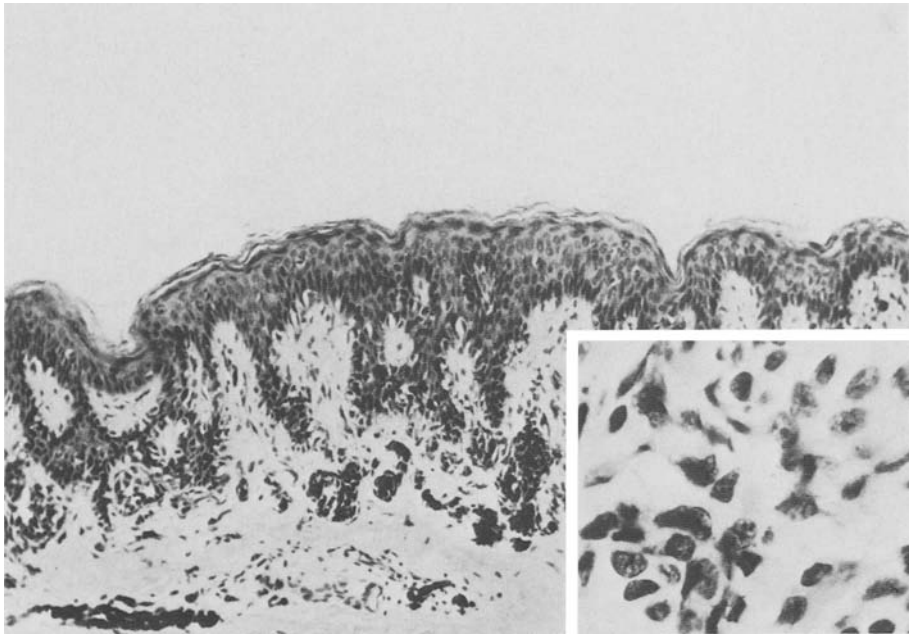

Fig. 4. So called dysplastic naevus of the lentiginous type. In this case no inflammatory reaction can be shown. *Inset*: Compared to the common naevus (Fig. 3) there is more variability in nuclear size (see Table 3)

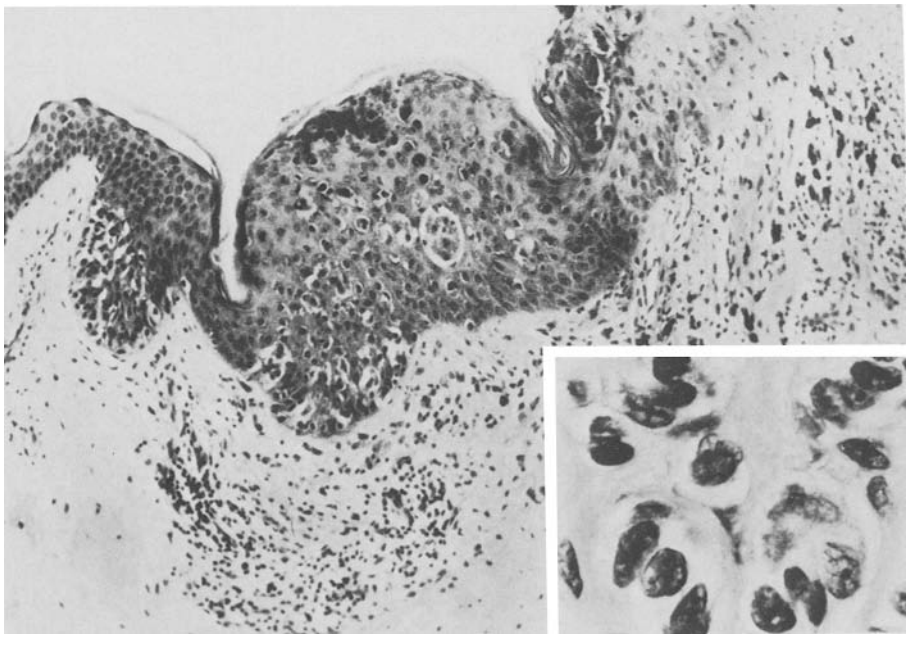

Fig. 5. Periphery of a superficial spreading melanoma (*SSM*) with large pagetoid cells infiltrating the upper parts of the epidermis and a dense inflammatory reaction. *Inset*: Marked anisokaryosis of melanoma cells (see Table 3)

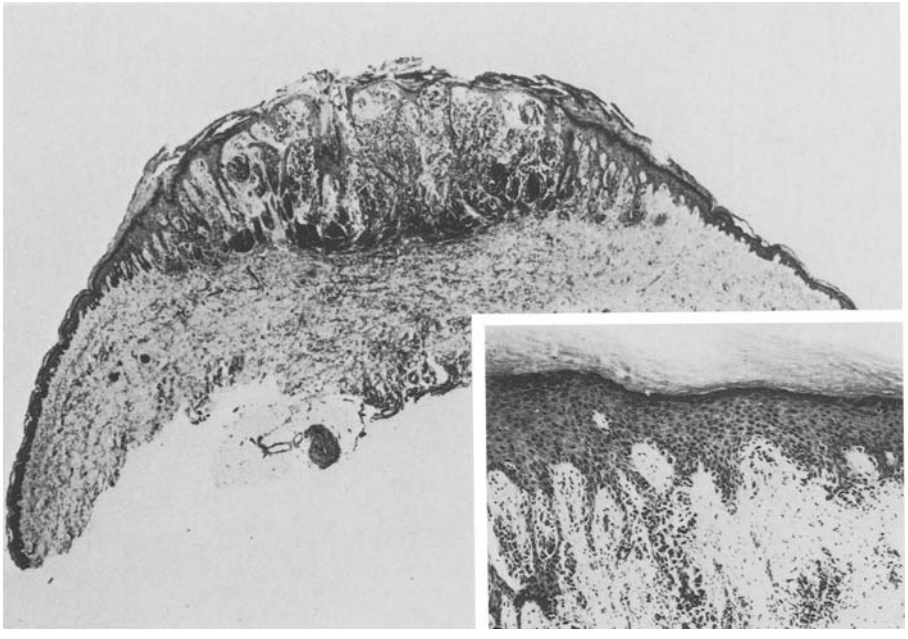

Fig. 6. Overview of a "SSM/NM" with an obvious intraepidermal tumour margin (*inset*) on each side and a big nodule in the center of the melanoma

provided data indicating a histogenetic relationship to malignant melanoma by means of the first two variables listed above.

For the first question it seemed important to classify the melanomas investigated using not only the "classical" histological criteria (Ackerman 1982; Clark et al. 1969, 1977), but also descriptive ones. For this purpose we studied the group of melanomas which showed a typical nodular component as well as a flat superficial part. We called them superficial spreading melanoma with nodular components ("SSM/NM").

With respect to mitotic index we found that the flat parts of "SSM/NM" did not differ from those which showed exclusively superficial growth patterns (SSM). The mitotic indices of the nodular parts were generally higher than those of the superficial ones, whether they were present in the same melanoma or not. The three metastases investigated showed the highest mitotic index. The number of mitoses in dysplastic naevi and in common naevi was so low that we did not quantify it. From the results the conclusion can be drawn that the number of mitoses when quantified in a reproducible way in relation to a defined area, can only be related to the growth pattern and may be different in one melanoma with two different components.

The problem of the mitotic index as a prognostic factor (Schmoeckel and Braun-Falco 1978; Sober et al. 1983) could not be considered in this study, as the time of follow-up is not long enough. We would like to point out, however, the fact that more than 100,000 cells were evaluated for this study and not just one high power field per case.

The determination of nuclear areas and their coefficient of variability within a melanocytic lesion revealed interesting results. The so called dysplastic naevus has a significantly higher nuclear variability compared to the common naevus. We linked this variability to the term "anisokaryosis". Anisokaryosis of dysplastic naevi almost reaches the degree of that found in SSM. Nodular melanoma shows markedly larger nuclear areas than superficial spreading melanoma. Nuclear areas and their variability did not reveal any difference between common naevi, melanocytic hyperplasia, and normal melanocytes.

Dysplastic naevi also differed from common naevi with respect to DNA-content. The DNA-distribution pattern of dysplastic naevi gives evidence for an increased melanocytic proliferation only, but in none of the 15 cases investigated was an indication of malignancy recognized. There were no cells with a DNA-content beyond the 4n range and no tumour stem lines could be found. It may be pointed out however, that in the reactive melanocytic hyperplasias accompanying a dermal histiocytoma an increase of proliferation derivable from the DNA-histograph could not be shown. But 13 out of 15 dysplastic naevi investigated had this sign of proliferation.

It should be noted that not all of the dysplastic naevi investigated by us were derived from patients with a so called "dysplastic naevus syndrom" (Greene 1984). Among our cases there are also patients with pigmented lesions which were clinically atypical, which turned out to be atypical melanocytic hyperplasias or dysplastic naevi in the histological examination. So we separate the dysplastic naevus only by means of description from the common naevus and consider this histological diagnosis independent of the dysplastic naevus syndrome. Our results concerning dysplastic naevi lead to the conclusion that in the cases investigated by us a clear feature of malignancy could not be recognized with the methods we used. This however does not question the fact that there is a higher incidence of melanoma in patients with dysplastic naevus syndrome, but this cannot yet be related to a single histologically examined dysplastic naevus.

The investigation of nuclear areas and DNA-distribution patterns in superficial spreading and nodular growing components of melanoma lead to the result that there is a variable distribution in "SSM/NM". Whereas SSM frequently show only discrete criteria of malignancy they are more distinct in the nodular parts. The metastasis investigated by us presented a relatively monotonous picture with predominance of the 2n-range. The heterogenous composition of a malignant tumour with respect to chromosomal analysis (Nowell 1978; Whang-Peng et al. 1970) and DNA-distribution (Manocha et al. 1969) is well known and can be demonstrated by subdividing the melanomas into components with a different growth pattern. The relatively uniform picture of metastases can be related to the fact that a small number of tumour cells may be responsible for their origin.

The investigation of superficial spreading melanoma with a nodular component ("SSM/NM") turned out to be especially interesting. All criteria of malignancy like an aneuploid stem line and also a broad distribution of DNA-values up to 11n could be demonstrated in the intraepidermal mar-

ginal component. In one case a continuation of this stem line from the intraepidermal to the nodular part could be found. The fact that in both "SSM/NM" in contrast to the other SSM there was an aneuploid stem line intraepidermally, allows the speculation that such a change in DNA-distribution pattern may regularly be found if a vertical growth pattern develops within a primarily horizontally growing SSM. By analogy with the histogenetic concept of cervical cancer (Boehm and Sandritter 1975) this might possibly indicate the selection of a tumour cell population with an increased malignant potential. We are presently investigating a greater number of this type of melanoma in order to get more information about this point.

The results presented by us lead to the conclusion that dysplastic naevi ordinarily show an increased proliferation of melanocytes as well as a greater anisokaryosis when compared with common naevi, but no criteria of malignancy.

Furthermore, we can say that superficial spreading melanomas without nodular component showed unequivocal features of malignancy remarkably seldom. These criteria could be found in most cases of melanomas with a nodular growing component, no matter if there was a superficial spreading component in the same melanoma or if it was a melanoma of the primarily nodular type.

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Accepted December 20, 1985