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PCNA EXPRESSION AND NUCLEOLAR ORGANIZER REGIONS IN MALIGNANT MELANOMA AND NEVUS CELL NEVUS

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SYNOPSIS

The histogenesis of malignant melanoma with particular reference to the role of melanocytic nevus is still controversial in oncological pathology. In order to differentiate between the proliferative activity of malignant melanomas and benign melanocytic tumors, immunohistochemical analysis was carried out.

We used monoclonal antibody PC10 to identify the Proliferating Cell Nuclear Antigen (PCNA), a highly conserved 36 KD acidic nuclear protein which correlates with cell proliferation, and the AgNOR method to stain the Nucleolar Organizer Regions (NORs) whose number and configurations may reflect protein synthesis activity.

47 cases of primary melanoma, 63 metastatic melanoma lesions, and 23 cases of nevi are included in this study. Spitz nevi showed a higher index of cell proliferation compared with other common benign nevi but a much lower index compared with malignant melanoma cells.

The metastatic lesions of malignant melanomas had a higher index of proliferation and activity of cells than the primary lesions. Skin-metastatic lesions had higher indexes of cell proliferation and NOR activity than lymphnode-metastatic lesions.

These results support the idea that metastatic melanoma cells are derived from a more advanced stage of tumor progression.

These data suggest that the combination of PCNA and AgNOR is an useful marker for differentiating benign melanocytic tumors from malignant melanomas.

INTRODUCTION

A longstanding histogenetic controversy in oncological pathology is the histogenesis of malignant melanoma with particular reference to the role of the melanocytic nevus. As many as 40% of all primary cutaneous melanomas have histologic remnants of nevomelanocytic nevi adjacent to the tumor. There is increasing evidence that dysplastic nevi are a clinically predisposing lesion for melanoma development. Spitz nevi are not recognized as having such an association, but are noteworthy because of their histologic similarity to malignant melanoma [23].

In the last decade the use of monoclonal antibodies in immunohistochemical technique has proven to be valuable in identifying indeterminate tumor masses to be of melanocytic origin. Melanoma-specific antigen exists and has been isolated. Recent developments in molecular biology techniques have shown that carcinogenesis is a multistep process where genetic changes of oncogenes and tumor suppressor genes accumulate [1].

Proliferating Cell Nuclear Antigen (PCNA), an evolutionary highly conserved 36 KD acidic nuclear protein, is involved in DNA synthesis and the gene for human PCNA has been cloned [17]. PCNA is regulated in a complex manner with the gene being transcribed efficiently in both quiescent and proliferating cells, but PCNA mRNA usually only accumulates in proliferating cells. Accumulation of the PCNA mRNA and the synthesis of high levels of the protein is stimulated by growth factors, notably platelet-derived growth factor (PDGF), but is not necessarily associated with DNA synthesis. PCNA accumulates in the presence of hydroxyurea, which inhibit DNA synthesis [3, 15]. Immunostaining with monoclonal antibody (PC10) to this antigen has been shown in the proliferative compartment of normal tissues [11] and to correlate well with the proliferation, progression and prognosis

of tumors in various organs [2, 9, 10, 11, 14, 21, 22, 24, 25]

Nucleolar organizer regions (NORs) are loops of DNA which occur in the nucleoli and transcribe to ribosomal RNA (rRNA). rRNA genes are transcribed by RNA polymerase I, and are of vital significance in the ultimate synthesis of protein [12]. Thus the numbers and/or configurations of NORs may reflect all kinds of general activities of cells. The AgNOR method, which stains NOR-associated protein, provides a useful means for the examination of nucleolar structure and variations in nucleolar activity [12]. Using these two methods, we investigated the differences in proliferation and metabolic activity of cells in primary and metastatic leisons of malignant melanoma as well as in different kinds of nevi such as intradermal, junctional, compound, dysplastic and Spitz nevi.

MATERIALS AND METHODS

47 cases of primary lesions of malignant melanoma, 63 lesions of metastatic melanoma from 31 cases, and 23 nevi, from Department of Dermatology, Kobe University School of Medicine and Department of Anatomic Pathology, Faculty of Medicine, University of Indonesia, were included in this study.

PCNA staining

Five micrometer sections from paraffin blocks were air dried overnight at room temperature and immunostained with monoclonal antibody PC10 at a dilution of 1:200 as previously described [11, 22], using an immunoperoxidase method (Vectastain ABC kit from Vector Laboratory Inc.) with 3-amino-9-ethylcarbazole (AEC) as the final substrate for development of the coloured reaction product.

A minimum of 1,000 tumor cells in random microscopic fields were counted. The PCNA index was defined as the number of cells with strong nuclear staining, divided by the total number of cells counted, expressed as a percentage.

Nucleolar Organizer Region (NOR) staining

Sections were cut at 5 micrometer thickness from paraffin blocks. They were dewaxed in xylene, then hydrated in ethanol. The AgNOR staining solutions were prepared by dissolving gelatin in 1 % aquaeous formic acid at a concentration of 2 %. This solution was then mixed 1:2 by volume with a 50 % aquaeous silver nitrate solution to give the final working solution. This was poured over the tissue sections and left for 60 min at room temperature. The silver colloid was then washed off with deionized water and the sections were counterstained with Mayer's Haematoxylin. The sections were hydrated in ethanol and mounted.

Intra and extra nucleolar AgNORs were counted at a magnification using x 100 oil immersion lens involving 100 cells of each specimen [5]. The average of the AgNOR number in one cell was calculated.

RESULTS

PCNA staining was almost entirely confined to the nucleus and showed a diffuse or granular pattern or a mixure of both, and the positive cells were generally scattered.

In all specimens, NORs were clearly visible as black dots of varying sizes in the nuclei. In general, these were present over nucleoli, but often smaller NORs could be seen lying away from main nucleolar structure.

The NORs were easily counted. Although cytoplasmic melanin stained black, this did not obscure the NORs.

Malignant Melanomas

Primary malignant melanomas-including 23 acral lentiginous melanomas (ALM), 4 superficial spreading melanomas (SSM) and 20 nodular melanomas (NM).

The PCNA index and the number of AgNORs were 32.6(range: 2.85-55.29) and 6.84(range: 1.69-12) for ALM, 26.99(range: 19.46-31.24) and 7.09(range: 6.25-7.62) for SSM, and 43.03(ranage: 22.32-55.32) and 8.18(range: 6.21-11.62) for NM, respectively (Table I). The total PCNA index and AgNORs for primary melanomas were 34.21 and 7.37 respectively.

Metastatic melanomas were divided into two categories, satellite metastases in the skin, and lymphatic lymphnode metastases. Skin metastases had a higher PCNA index as well as higher numbers of AgNORs than lymphnode metastasis (66.88 to 29.75, 67.97 to 58.08 for the PCNA of ALM and NM; 12.43 to 10.68 and 8.68 to 6.81 for the AgNORs of ALM and NM) (TableII). Since we did not identify skin metastasis for SSM we could not compare the two categories for SSM. Total PCNA indices for the skin metastasis and lymphnode metastasis of melanoma were 67.42 and 49.72 respectively. The AgNORs numbers were 10.55 in skin metastasis and 8.47 in lymphnode metastasis.

Nine patients with both primary and metastatic lesions were included in this study (TableIII). The rates of PCNA were higher in metastatic lesions than in primary lesions except 3 cases but the number of AgNORs did not show a significant difference between the two.

Nevi

We included 5 intradermal nevi, 4 junctional nevi, 4 compound nevi, 4 Spitz nevi and 6 dysplastic nevi. Common benign nevi in general had a low PCNA index and low number of AgNOR.

Either the PCNA index or the number of AgNORs were slightly increased in junctional, compound, Spitz, and dysplastic nevi compared with intradermal nevi. In these nevi, the Spitz nevi had the highest PCNA index [2, 24] even higher than dysplastic nevi (2.88). The number of AgNOR showed no significant differences among the nevi, except intradermal nevi, wich had the lowest numbers (Table IV).

Table I. PCNA and AgNOR in primary malignant melanoma.

Type of melanoma	Number of cases examined	% PCNA	AgNOR
ALM	23	32.60	6.84
SSM	4	26.99	7.09
NM	20	43.03	8.18
TOTAL	47	34.21	7.37

Table II. PCNA and AgNOR in metastatic malignant melanoma.

Type of primary melanoma	Sites	Number of lesions	% PCNA	AgNOR
ALM	Skin	4	66.88	12.43
	LN	7	29.75	10.68
	Subtotal	11	48.88	11.55
SSM	Skin	0	——	——
	LN	9	41.33	7.92
	Subtotal	9	41.33	7.92
NM	Skin	23	67.97	8.68
	LN	20	58.08	6.81
	Subtotal	43	63.02	7.74
TOTAL	Skin	27	67.42	10.55
	LN	36	49.72	8.47
	Total	63	58.57	9.51

Table Ⅲ. PCNA and AgNOR in patients with both primary and metastatic melanoma.

CASE	Primary melanoma		Metastasis		
	% PCNA	AgNOR	Number of case	% PCNA	AgNOR
1. A L M	8.75	1.69	3 (LN)	18.33	4.79
2. A L M	20.22	6.32	1 (LN)	21.30	5.48
3. A L M	46.32	9.25	1 (LN)	47.48	7.77
4. S S M	27.98	6.25	1 (LN)	25.58	6.94
5. N M	47.73	8.14	2 (LN)	77.73	9.00
6. N M	51.24	8.67	1 (Skin)	67.42	9.21
7. N M	45.21	6.94	2 (LN)	63.64	7.45
8. N M	35.18	7.32	1 (LN)	70.31	9.16
9. N M	31.52	9.19	1 (LN)	81.61	8.24
average	34.90	7.08	———	52.60	7.56

TableIV. PCNA and AgNOR in malignant melanoma and nevi.

TYPE	No. of cases	% PCNA	AgNOR
Intradermal nevi	5	0.64	1.88
Junctional nevi	4	0.85	2.63
Compound nevi	4	1.13	3.16
Spitz nevi	4	7.11	3.61
Dysplastic nevi	6	2.88	2.24
Melanoma, primary	47	34.21	7.37
Melanoma, metastasis			
LN	36	49.72	8.47
Skin	27	67.42	10.55

DISCUSSION

The identification of the proteins involved in the control of cell proliferation may contribute to the understanding of the molecular mechanisms underlying malignant transformation and carcinogenesis [2].

Monoclonal antibody PC10 has been used by many investigators to detect PCNA protein in paraffin embedded tissue. There are several publications concerning PCNA expression in many kinds of tumors and normal tissues [2, 9, 10, 11, 14, 21, 22, 24, 25]. Takahashi has studied the expression of PCNA in nevi and malignant melanoma [20]. He observed 100 cells of each case and found that PCNA positive cells increase in number and staining intensity according to the following: common melanocytic nevi, dysplastic nevi, primary melanomas and metastatic melanomas. By observing at least 1,000 cells in each case we obtained results supporting Takahashi's findings. We also found that the PCNA index increased according to the progression from benign to malignant tumor cells: intradermal nevi, junctional nevi, compound nevi, dysplastic nevi, Spitz nevi, primary melanomas, lymphnode metastatic melanomas and skin metastatic melanomas. Surprisingly the Spitz nevi showed a higher index of cell proliferation than dysplastic nevi, which are considered clinically to be precursor to melanocytic malignancy, although it is postulated that the dysplastic nevus is a variant of common nevi. However, the PCNA index of the Spitz nevi is much lower than that of malignant melanomas. It seems likely that the PCNA index of Spitz nevi correlates with histological characteristics which are sometimes similar to melanoma.

The technique of silver staining of nucleolar organizer region-associated protein (AgNOR) has been shown to be of value in detecting nucleolar structure and variation in nucleolar activities. This technique has several additional advantages over other published staining protocols [4,19]. Some investigators have used this AgNOR method for malignant melanoma and/or cutaneous melanocytic leison and the results are contradictory [6,7]. Crocker and Skilbeck found 7.9 AgNOR per nucleus in malignant melanoma and 1.20 in nevocelluler nevi and suggested that this method can be used as semi-quantitative method to differentiate benign melanotic lesions from malignant melanoma [6]. Mackie et al also stated that this technique may be a useful adjunct in separating true melanomas from borderline melanocytic nevi although there was no difference among the nevi [16]. However, Fallowfield and Cook, and Hourst et al, stated that the differential diagnosis of borderline melanocytic lesions is still difficult using the AgNOR technique. They also mentioned the difficulty of distinguishing Spitz nevi from malignant melanomas [8,13]. Orrell et al and Evans et al stressed that in order to use the AgNOR technique for differentiating benign from malignant melanocytic lesions optimized and standardized AgNOR techniques and defined diagnostic criteria are prerequisites [4,18].

We showed the following:

1. The increase in the cell proliferation index generally correlated with the increase in nucleolar activities in benign and malignant melanocytic tumors.
2. Nevi of benign lesions generally showed the lowest cell

proliferation and nucleolar activities. These two cellular characteristics began to increase after junctional nevi, correlating with the stage of malignancy.

3. Dysplastic nevi did not show significant differences in cell poliferation indices and nucleolar activities compared with other nevi.
   This might suggest that dysplastic nevi are not necessarily predisposing conditions for malignancy.
4. Spitz nevi showed a significantly higher PCNA index than other nevi but still lower than that of primary malignant melnomas. PCNA staining was useful to differentiate Spitz nevi from malignant melanomas or other nevi.
5. Primary malignant melanoma and metastatic malignant melanoma lesions had significantly higher cell proliferation indices and nucleolar activity.
   Nodular melanomas had the highest index.
6. Metastatic malignant melanoma lesions either in lymphnode or skin showed higher PCNA index and AgNORs than primary lesions.

Our results indicate that the combined use of both techniques may be useful for distinguishing benign melanocytic tumors from malignant melanomas.

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