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N-Nitroso-N-ethylurea-induced Hamster Melanoma: A New Method for Efficient Induction and Schwannian Differentiation of Melanoma

Tetsuo Nemoto,[1,3] Takuro Nakamura,[2] Kenichi Ohashi[1] and Tsutomu Kasuga[1]

[1]*Department of Pathology, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113 and* [2]*Department of Pathology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113*

Melanocytic tumors as well as multiple neurofibromas were induced in 35 of 88 Syrian golden hamsters by a single s.c. administration of 100 mg/kg of N-nitroso-N-ethylurea applied 48 h after birth. The lesions were all observed proliferating in the dermis and demonstrated melanosomes and premelanosomes. High cellularity, nuclear atypia and transplantability of the tumors in outbred hamsters suggested a malignant nature. Some of the melanomas were morphologically heterogenous and contained Schwann-like cells as minor components. In addition, transplantation of the melanomas resulted in increased schwannian differentiation even for primary tumors which did not contain any Schwann-like cell foci. One of the transplanted melanomas mimicked malignant peripheral nervous tumor. Schwannian differentiation was also proved by the fact that glial fibrillary acidic protein was positive in 22.2% of the cases. The present results suggest that the induced hamster melanomas originate from neural crest-derived cells which are able to differentiate into both melanocytes and Schwann cells.

Key words: Melanoma — N-Nitroso-N-ethylurea — Neural crest — Schwannian differentiation

Malignant melanoma is a highly malignant skin neoplasm which has a high metastatic potential even in its early stage and is resistant to chemotherapy and radiotherapy. It has been suggested that malignant melanoma not only might develop from mature melanocytes but also could be derived from premature neural crest stem cells.[1)]

An appropriate animal model is needed to facilitate further understanding of the histogenesis and potential for differentiation of malignant melanoma. In one approach, melanomas were induced by transplacental administration of N-nitroso-N-ethylurea (ENU) to the Syrian golden hamster, arising together with neurofibromas.[2)] Although the melanoma frequency was not very high, this animal model could be of interest for studying aspects of differentiation since some of the neurofibromas contained foci of melanocytic proliferation.[2)]

In an attempt to increase the frequency of melanomas in this system, newborn animals received a single administration of ENU. In this report we document effective induction of hamster melanoma and schwannian differentiation in this tumor, with emphasis on the nature and histogenesis of neural crest-derived neoplasms.

MATERIALS AND METHODS

Animals and induction of the tumors Pregnant female Syrian golden hamsters were purchased from Clean Experimental Animal Center Co. (Saitama). Eighty-eight offspring received a single subcutaneous injection of 100 mg/kg of ENU (Nakarai Chemical Co., Kyoto) at 48 h after birth. The solution was freshly prepared by dissolving ENU at 10 mg/ml in physiologic saline. A total of 50 female and 38 male treated hamsters were weaned at 28 days of age, separated by sex, and maintained on basal diet CE-2 (Clea Japan Inc., Tokyo) with tap water *ad libitum*.

Autopsies were performed on dead or moribund animals, and all survivors were killed and subjected to complete autopsy by the 61st week. Tissues were fixed with 10% neutral buffered formaldehyde for light microscopy and 2.5% glutaraldehyde for electron microscopy, followed by postfixation with 1% osmium tetroxide.

Immunohistochemistry Rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP) (Dakopatts, Glostrup, Denmark), laminin (HEYL, Berlin, Germany), S-100 (Dakopatts), and mouse monoclonal antibodies against vimentin (Dakopatts) and myelin basic protein (MBP) (Innogenetics, S.A., Antwerp, Belgium) were used for immunohistochemical studies. After deparaffinization, tissue sections were incubated with primary antibodies overnight at 4°C, and then, after washing, were incubated with biotinylated anti-rabbit antibody (Vector Laboratories Inc., Burlingame, CA) or biotinylated anti-mouse antibody (Vector) as the secondary antibody. Subsequent to incubation with avidin-biotin-peroxidase complex (Vector), the sections were stained with diaminobenzidine.

[3] To whom correspondence and reprint requests should be addressed.

Melanoma transplantation Nine primary hamster melanomas induced by ENU were transplanted into non-treated golden hamsters. The freshly prepared primary tumor tissues were aseptically cut into small fragments 1 mm in diameter with scissors and transplanted subcutaneously into the backs of recipient animals.

RESULTS

Frequency and types of tumors At the 61st week, there were 7 survivors (survival rate was 7.95%). The mean survival time was 47.1 weeks. The histologic types of the induced tumors are summarized in Table I. Melanomas were present in 35 of 88 hamsters (39.8%), and the total number of lesions was 42. Six hamsters had two or more melanomas. The frequency of melanomas in females was 38.0% and that in males was 42.1% (difference not significant). All melanomas originated in the skin with the most common site being the back region (36 tumors). Some melanomas were observed at the base of the ear (2 tumors), head region (2 tumors) and extremities (2 tumors). No melanoma arose in the cost-vertebral spot. Only one tumor showed multiple lung metastases; the others did not show any metastases.

Two hundred and sixty-one peripheral nerve tumors were found in 81 of the 88 hamsters. The frequency in the females (49 of 50 hamsters, 98.0%) was significantly higher than that in the males (32 of 38 hamsters, 84.2%) ($P<0.02$). An average of 4.2 tumors per hamster were found in females and 1.3 tumors per hamster in males (difference statistically significant, $P<0.01$). The most common site of peripheral nervous tumor development was subcutaneous, followed by the abdominal or thoracic cavities. A small number of the tumors arose from the trigeminal nerves and spinal nerve roots.

Wilms' tumors (8%), and pheochromocytomas (2.3%) were also observed. There was no apparent relationship between the site of ENU injection and the location of tumor development.

Morphological findings for melanomas Macroscopically, the melanomas varied in size from 2 to 25 mm in largest diameter. They formed cutaneous nodules and ulceration were observed in the larger tumors (Fig. 1). Cut surfaces demonstrated various levels of pigmentation from grayish white to dark brown or black, some showing an uneven distribution of pigment.

Histologically, the tumors were observed as discrete units proliferating in the dermis, often around hair follicles. The bulk of each tumor was present within intradermal sites, even in the 18 melanomas with attachment to the epidermis. Some lesions contained hair follicles in their central regions. Melanomas showed tightly packed cellular growth with medullary or alveolar patterns (Fig. 2A). The shapes of the tumor cells varied from tumor to tumor, i.e., individual tumors could be divided into small cell, round cell, epithelioid cell or spindle cell types. The tumor cells of all types had atypical and hyperchromatic nuclei and their nuclear:cyto-

Fig. 1. Hamster with melanoma arising at the base of the ear.

Table I. Histologic Types of the Tumors Induced by ENU in the Golden Hamster

	Total (88)		Females (50)		Males (38)	
	No. of animals (%)	Average No. of tumors	No. of animals (%)	Average No. of tumors	No. of animals (%)	Average No. of tumors
Melanoma	35 (39.8)	1.2	19 (38.0)	1.3	16 (42.1)	1.1
Neurofibroma	81 (92.0)	3.2	49 (98.0)	4.3	32 (84.2)	1.6
Wilms' tumor	7 (8.0)	1.1	3 (6.0)	1.0	4 (10.5)	1.3
Others						
Sarcoma	3 (3.4)	1.0	1 (2.0)	1.0	2 (5.2)	1.0
Pheochromocytoma	2 (2.3)	1.0	1 (2.0)	1.0	1 (2.0)	1.0
Papilloma (skin)	1 (1.1)	1.0	1 (2.0)	1.0	0 (0)	0
Hemangioma	1 (1.1)	1.0	1 (2.2)	1.0	0 (0)	0

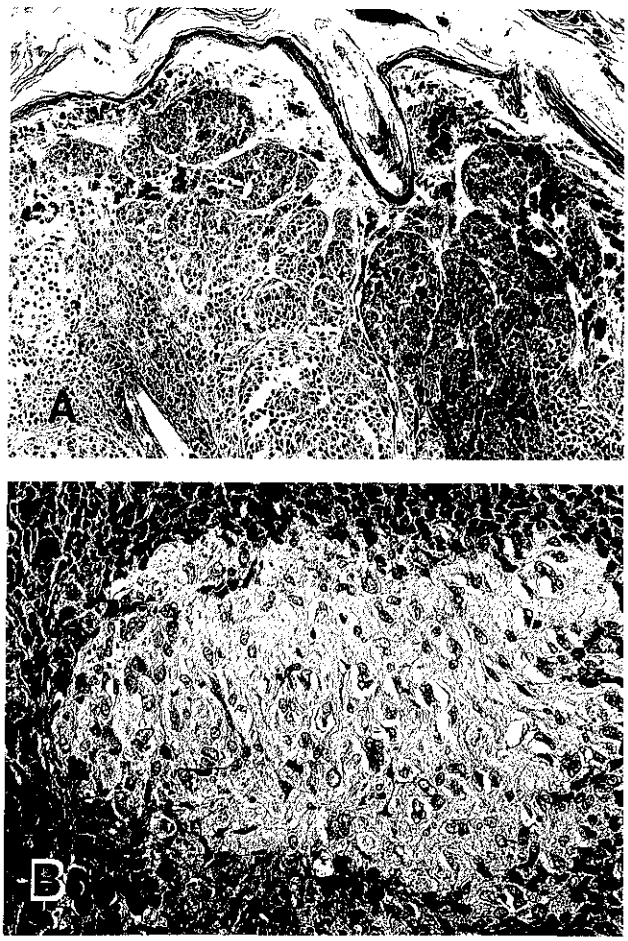


Fig. 2. A. Melanoma showing tightly packed growth in the dermis. Clumps of epithelioid cells are evident proliferating in alveolar patterns (H & E, ×130). B. Proliferative focus of Schwann-like cells showing Meissner's corpuscle-like structures (H & E, ×260).

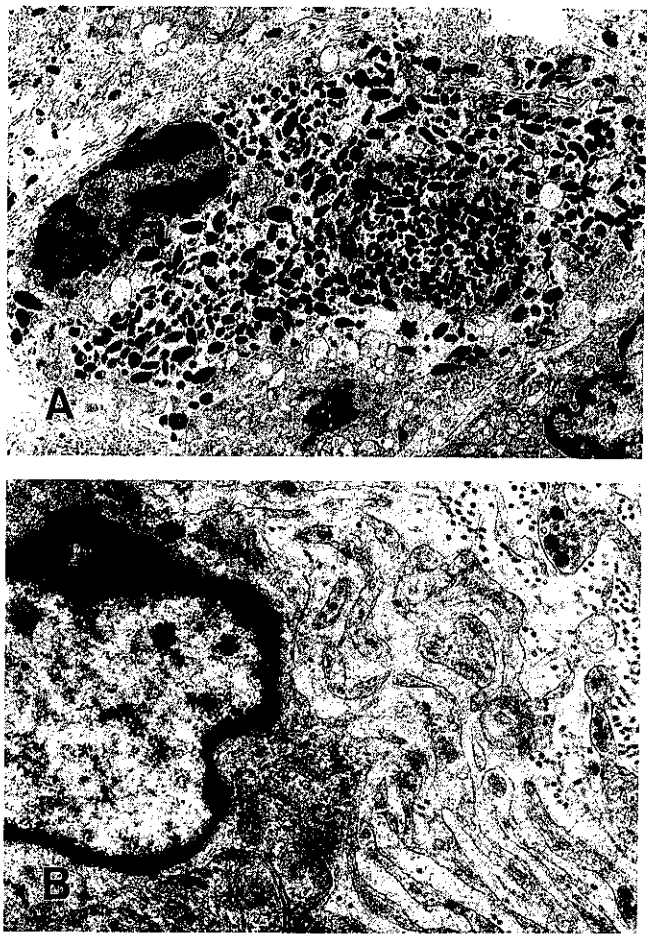


Fig. 3. A. A melanoma cell demonstrating abundant premelanosomes and melanosomes (×7,000). B. A Schwann-like cell with interdigitating cytoplasmic process and only a few scattered melanosomes (×23,000).

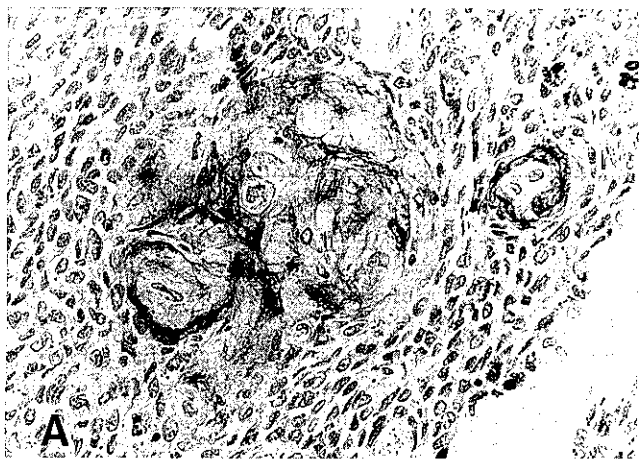
plasm ratios were high. The melanoma cells produced to various extents melanin pigment, which was positive for Fontana-Masson staining. In a few tumors, the cells were completely filled with fine melanin granules. In 4 of 42 melanomas (9.5%), small proliferative foci of Schwann-like cells with abundant cytoplasm and small nuclei were observed, these forming Meissner's corpuscle-like structures (Fig. 2B).

Electron microscopic findings Typical melanoma cells had ovoid nuclei and relatively abundant cytoplasm with well developed rough endoplasmic reticulum, Golgi apparatus, and large numbers of premelanosomes and melanosomes (Fig. 3A) measuring 150–550 nm, with lamellar membrane structures.

Table II. Schwannian Differentiation in the Primary and Transplanted Melanomas

	Total	GFAP(+)	Meissner-like corpuscle
Primary melanoma	23.8%	22.2%	9.5%
(n=42)	(10/42)	(8/36)	(4/42)
Transplanted melanoma	44.4%	44.4%	22.2%
(n=9)	(4/9)	(4/9)	(2/9)

Schwann-like cells in the melanomas demonstrated interdigitating cytoplasmic processes, in typically Schwann cell patterns (Fig. 3B). A few scattered melanosomes were observed in their cytoplasm.



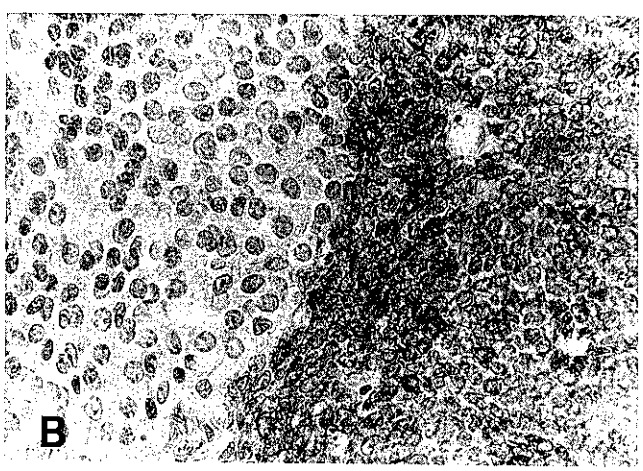
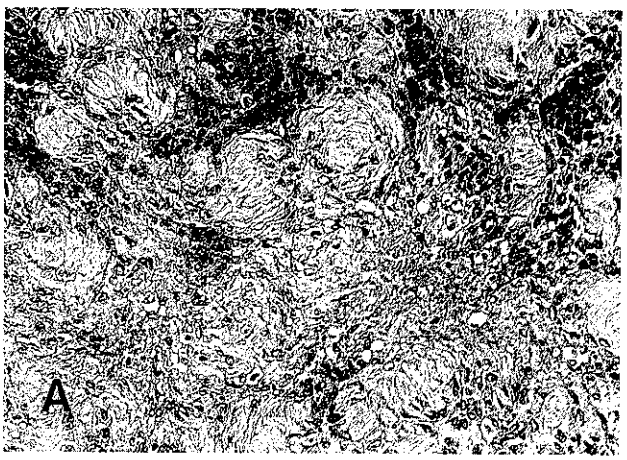


Fig. 4. A. Cytoplasmic GFAP in Schwann-like cells (×400). B. GFAP-positive round cells forming a nest within a melanoma (×400). C. GFAP-positive cells in a transitional area between round cells and spindle cells (×200).



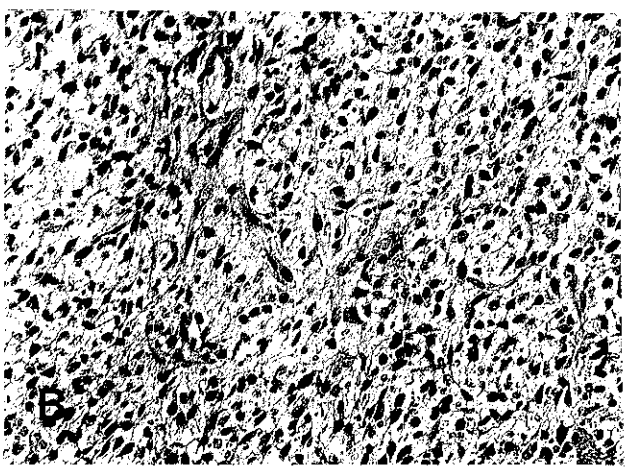


Fig. 5. A. A melanoma after transplantation with prominent schwannian differentiation (H & E, ×260). B. After the second transplantation, the melanoma consists of spindle tumor cells and myxoid stroma mimicking a malignant peripheral nervous tumor (H & E, ×330).

Immunohistochemistry GFAP staining was positive in 8 of 36 melanomas investigated (22.2%, Table II). Positive binding of antibody was observed not only in Schwann-like cells (Fig. 4A) but also in small round cells (Fig. 4B). GFAP-positive cells usually formed small nests within melanomas. Some of them were located in transitional regions between round cell and spindle cell areas (Fig. 4C), and others were found in the perivascular areas. GFAP was particularly strongly expressed in the peripheral parts of the cytoplasm of melanoma cells. The normal Schwann cells of the hamster peripheral nerves were positive for GFAP, whereas normal hamster melanocytes were negative.

Laminin staining was positive in all schwannian differentiated foci in melanomas studied but negative in mela-

noma cells without schwannian differentiation. Vimentin was positive in all 6 of 6 GFAP-positive melanomas studied. More than half of the GFAP-positive melanoma cells were also positive for vimentin. The S-100 protein was found to be diffusely positive in all 8 melanomas. MBP was negative in all of 15 melanomas investigated.

Transplantation of the melanoma All nine melanomas selected could be successfully transplanted into normal outbred golden hamsters. Twenty of 22 transplanted tumors (91.8%) formed multiple distant metastases within one year. Mean survival time of the animals with metastases was 41 weeks and metastasis of melanoma was observed from 22 weeks after transplantation. Schwannian differentiation of the tumors became more prominent in four in nine (44.4%) of the transplanted tumors, with multiple foci of Schwann-like cells forming Meissner's corpuscle-like structures (Fig. 5A) and/or GFAP-positive tumor cells. Four tumors were further transplanted to the second recipient animals. One of 4 transplanted tumors consisted of spindle tumor cells and myxoid stroma mimicking malignant peripheral nervous tumors (Fig. 5B). The tumors retained the same histological features even after the 5th transplantation more than one year after the first transplantation.

DISCUSSION

In the present study, we succeeded in inducing melanoma efficiently with an incidence of up to 39.8% (38.0% in females and 42.1% in males) by a single administration of ENU to hamsters at the most susceptible time, 48 h after birth.

There have been numerous reports concerning the induction of melanomas by chemical carcinogens, such as dimethylbenzanthracene[3] and urethan.[4] Among the chemical carcinogens, ENU is unique in acting as a potent carcinogen not only for melanocytes but for neural crest-derived cells in this system. With regard to melanoma induction by ENU, this is the most efficient induction so far reported. Pelfrene and Love[5] induced melanoma in only 15% of Syrian hamsters using a combination of transplacental administration and topical application of carcinogen. In our previous ENU hamster neurofibromatosis model, melanomas were induced in 10% of the animals.[2] In our present study, the incidence of melanoma was over twice that previously obtained.

Invasive growth, central ulceration, atypical nuclei, increased cellularity and frequent mitotic figures strongly suggested malignant nature of the tumors. Their malignant character was also proven by the facts that the primary tumors were transplantable and that one primary and 20 of 22 transplanted tumors showed distant metastases.

In addition to the ENU-induced primary melanomas which contained Schwann-like cell components, transplanted melanomas showed prominent proliferative foci of Schwann-like cells resembling Meissner's corpuscles. Moreover, 22.2% of the melanomas were immunohistochemically positive for GFAP, which is an intermediate filament protein widely expressed in normal and neoplastic glias[6] and is also positive in non-myelin-forming Schwann cells.[7–9] Therefore GFAP positivity was considered to be an important indicator of schwannian differentiation. Schwannian differentiation was also confirmed by positivity for laminin. S-100 protein was positive for all melanomas; however, being also positive in the neurofibromas, S-100 protein was not a useful marker to detect schwannian differentiation in the melanomas.

Both the Schwann cell and the melanocyte are derived from the neural crest.[10] The human malignant melanoma is believed to originate from epidermal melanocytes and nevus cells,[11–14] the latter also being of neural crest origin. Cramer suggested that the precursor of the melanocyte may be a pluripotential cell, and the axon-investing Schwann cell or another nerve sheath cell must be the closest relative of the epidermal melanocyte.[1] The schwannian differentiation of melanomas documented here suggests a potential for bi-directional differentiation of the neural crest-derived cell. The results indicate that some subtypes of melanomas arise from non-epidermal melanocytes which are more closely related to Schwann cells.

Histological heterogeneity including schwannian or neuronal differentiation is one character of malignant melanomas in man.[15–18] There have been several reports of pluripotential differentiation of chemically induced neural crest-derived tumors. The cellular blue nevus-like tumor of the hamster induced by topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA) showed neurofibroma-like structures and/or small nerve fascicles.[19] Kanno *et al.* further demonstrated melanin-producing activity in Schwann cells after topical application of DMBA and 12-O-tetradecanoylphorbol-13-acetate to BDF1 mice.[20]

Further studies on the clonality of melanomas and factors which regulate the differentiation of the component tumor cells are needed to understand the histogenesis and other aspects of carcinogenesis involving neural crest-derived cells.

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