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Kazuhiko Hayashi*

*Okayama University,

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TISSUE CULTURE OF TRIGEMINAL NERVES FROM RATS ADMINISTERED TRANSPLACENTALLY WITH ETHYLNITROSOUREA

Kazuhiko HAYASHI

*Second Department of Pathology, Okayama University Medical School, Okayama
700, Japan (Director : Prof. K. Ogawa)*

Received March 8, 1983

Abstract. The morphological and biological changes in long term culture cells of normal-appearing trigeminal nerves from 2, 8, and 30-day-old S-D rats administered transplacentally with 75 mg ENU/kg were examined. After a marked degeneration of cells, crisscross multiple proliferative foci of transformed spindle cells appeared at the 3rd passage culture from 2 and 8-day-old rats, but not from 30-day-old rats. The transformed cells with S-100 protein and basal lamina had Schwann cell characteristics. Transformed spindle cells continued to form a crisscross pattern more than 700 days and some transformed spindle cells became round in shape 3-6 months after the primary culture. These transformed cells were transplantable to newborn S-D rats and the transplanted tumors were histologically similar to those of malignant Schwannoma of trigeminal nerves induced by ENU. Round-shaped transformed cells were more malignant than spindle-shaped cells and produced rapidly growing transplanted tumors. Spontaneous transformation with multinucleated giant cells occurred in one of the control cultures. These results indicate that the sequential changes of ENU-treated trigeminal nerves *in vitro* were corresponded to developmental changes of malignant Schwannoma *in vivo* induced by ENU. This system will be useful for analysis of ENU-carcinogenesis.

Key words : experimental malignant Schwannoma, trigeminal nerves of rat, ENU,
in vitro transformation, spontaneous transformation.

Ethylnitrosourea (ENU) is a highly selective carcinogen for the nervous system of rats (1). When a single dose of 20-70 mg ENU/kg body weight is given to a rat late in pregnancy, various types of glioma of the central nervous system and malignant Schwannoma of the peripheral nervous system frequently occur in the offspring (2-5). The trigeminal nerve is a common site for peripheral nerve tumors. Histological studies on the early stage of development of rat trigeminal nerve tumors induced by ENU have been recently performed by Swenberg (6) and Yanagisawa (4). Degenerative Schwann cells in the trigeminal nerves appeared 6 h after transplacental administration of ENU and reached a peak after 24 h (4). Twenty days after exposure to ENU, hypercellularity with hyperchromatism and some mitoses of Schwann cells occurred in the proximal end of the trigeminal nerve, including the central nervous system-peripheral nervous system

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(CNS-PNS) junction, and 2-6 months later macroscopic trigeminal nerve tumors developed (6).   Furthermore it has been reported (2, 7-10) that culture cells of trigeminal nerve tumors were morphologically similar to those of culture cells from human acoustic neurinoma (11) and induced intracerebral or subcutaneous tumors when transplanted to syngeneic hosts.

However, carcinogenesis induced by ENU has not been clarified enough at the cellular level though an *in vitro* transformation model of the central nervous system was established which provided a useful analysis of carcinogenesis induced by ENU (12).   But an *in vitro* transformation system of peripheral nerve tumors induced by ENU has not been reported.   The purpose of this experiment was to describe the morphological and biological changes observed during long-term culture of normal-appearing trigeminal nerve cells from 2, 8, and 30-day-old rats administered ENU transplacentally and to compare their changes with developmental changes in ENU-induced malignant Schwannoma *in vivo*.

MATERIALS AND METHODS

Carcinogen pulse . ENU (Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan) was dissolved in saline : citrate-phosphate buffer (pH. 6.0) shortly before use, and a single 75 mg/kg dose was given intravenously to ten pregnant Sprague-Dawley rats (Japanese Charles River Inc., Kanagawa) in the 18th day of gestation.   Five normal pregnant rats were used as controls. All rats not used for the *in vitro* experiments were examined histologically on the 30th day and after 150 days, and the incidence of trigeminal nerve tumors was estimated.

Tissue culture.   As shown in the Fig. 1. the trigeminal nerves from each of 6 offspring of both ENU-treated and untreated rats were removed aseptically at 2, 8, and 30 days after birth.
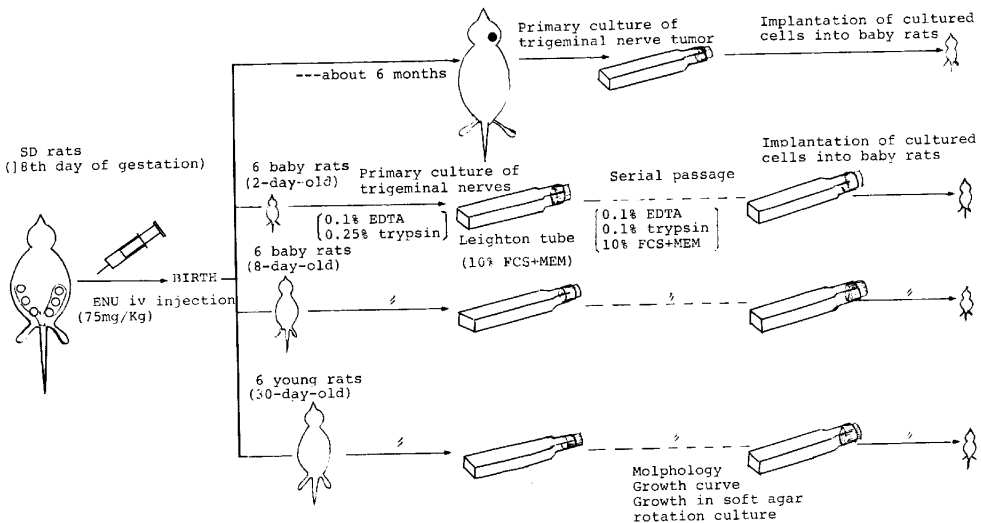

Fig. 1.   Diagrammatic representation of the *in vivo-in vitro* system for carcinogenesis induced by ENU in the rat trigeminal nerves.

The nerves were freed of any adhering tissue and meninges, cut into small pieces with scissors, digested with a 1 : 1 mixture of 0.25 % trypsin and 0.1 % EDTA (ethylenediaminetetraacetic acid) for 1 h at 37 ℃, and dissociated by pipetting. The dispersed trigeminal nerve cells were centrifuged at 1,000 rpm and suspended in Eagle's minimum essential medium supplemented with 10 % fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. These cells were cultured on cover slips in Leighton's tubes containing 2 ml of medium and kept at 37 ℃. The medium was renewed twice a week. After the cells reached confluence, they were sub-cultured by harvesting with trypsin and diluting 1 : 3 or 1 : 5. For serial passages, the same procedures were used. The same experiment of the culture of ENU-treated trigeminal nerves from 2-day-old rats was repeated (2E [2]).

Light microscopy. At different stages of cell growth, cells on cover slips were fixed in 10-20 % buffered formalin and stained with hematoxylin-eosin (H. & E.) and if necessary, with phosphotungstic acid-hematoxylin (PTAH). At some passages, S-100 protein in the culture cells was examined by an immunohistochemical method according to Taylor (13). Anti-bovine S-100 protein rabbit serum, a protein specific for the nervous system of all vertebrates, was kindly supplied by Dr. T. Nakajima (14). Pathology Division, National Cancer Center Research Institute, Tokyo, Japan.

Transmission electron microscopy. Pellets of culture cells were fixed for 1 h in 2.5 % phosphate-buffered glutaraldehyde at room temperature. They were postfixed at 4 ℃ for 60 min in 1 % phosphate buffered OsO_4, serially dehydrated in ethanol, and embeded in Epon 812. Ultra-thin sections cut with a Reichert-Jung ultramicrotome were stained with uranyl acetate and lead-citrate and examined under an electron microscope (Akashi LEM).

Growth curves. The *in vitro* growth curves of culture cells at the 3rd, 7th, and 23rd passages were determined. The growth curve of culture cells from ENU-treated trigeminal nerves of 2-day-old rats at the 30th passage (2E-30P) was compared with that of culture cells from the ENU-induced trigeminal nerve tumor at the 5th passage (T-5-5P).

Rotation culture. Culture cells from ENU-treated trigeminal nerves and ENU-induced trigeminal nerve tumors were cultured and gyratory shaker (Ikemoto Co.) at 60 rpm and 37 ℃ for 7 days.

Colony formation in semisolid agar medium. To observe colony formation, 10_3-10_4 culture cells were explanted to a 5 cm dish containing 3 % soft agar (Difco Labo.) and MEM with 20 % fetal calf serum and were kept at 37 ℃ under a humidified atmosphere of 5 % CO_2 in air.

Chromosome number of culture cells. The chromosome numbers of 50 culture cells in metaphase were caluculated after treatment with hypotonic solution and stained with Giemsa.

Transplantation of culture cells. At different passages, 0.5-5 $\times$ 10_6 cells of each cell line were injected subcutaneously into new-born S-D rats. Enlarged tumors were excised and prepared for light and electron microscopy.

RESULTS

Distinct differences were evident at 30 days of age between control and ENU-exposed trigeminal nerves. Thirteen of 15 nerves from 9 treated rats exhibited hypercellularity and hyperchromatism. These changes were always in the pre-ganglionic portein of the nerve at the CNS-PNS junction (Photo. 1.) But 10 nerves from 5 control rats exhibited no such changes. Malignant Schwannomas of trigeminal nerves were detected in 12 of 56 ENU-treated rats (21 %) after a

median latency period of 192 days.   Hypercellularity of the nerves occurred in five of 56 treated rats (9 %) at more than 150 days of age.

Morphological appearance of cells in culture.   Primary monolayer cultures of both control and ENU-treated nerves of all groups contained bipolar long spindle cells (Schwann cells) and a few neurons on the seats of fibroblast-like cells.   S-100 protein was detected in neurons and bipolar long spindle cells (Photo. 2).

During the 2nd passage of both control and ENU-treated culture cells, neurons disappeared and bipolar long spindle cells became shorter and more plump.

During the 3rd passage, culture cells of trigeminal nerves from 2 and 8 day-old rats administered transplacentally with ENU (2E-3P and 8E-3P at 15 and 23 days after the primary culture, respectively) decreased significantly in number compared with control culture cells due to degeneration and detachment, but gradually recovered in a few weeks (Photos. 3, 4, Fig. 2).   Thirty days after primary culture multiple proliferative foci of bipolar long spindle cells, which were piled on the seats of fibroblast-like cells in a crisscross pattern, appeared in 8 of 15 Leighton's tube cultures (2E-3P) (Photo. 5).

Similar proliferative foci were observed in 5 of 15 tubes at the 4th passage of ENU-treated nerve culture cells from 8 day-old rats (8E-4P) about 60 days after the primary culture.   Crisscrossed proliferative cells had short bulging nuclei with bipolar slender cytoplasmic processes, were positive for S-100 protein (Photo. 6a.) and stained nonspecifically with PTAH.
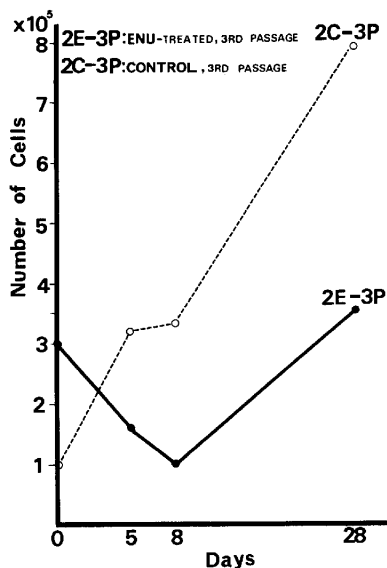

Fig. 2.   Growth curve at the 3rd passage of 2-day-old rat trigeminal nerves exposed in utero to ENU or untreated and cultured *in vitro.*

Such proliferative foci appeared in the culture cells of neither control nor ENU-treated nerves of 30-day-old rats, the culture cells of which were fibroblast-like or rounded spindle cells and which was negative for S-100 protein (Photo. 6b).

After serial passage, proliferative cells in 2E and 8E were crisscrossed, S-100 protein-positive spindle cells with a few fibroblast-like cells. A few multinucleated giant cells appeared after the 16th passage (6 months) in 2E, and the 18th passage (12 months) in 8E culture (Photo. 7), and increased in number during long term culture. The 2E and 8E cultures continued to proliferate at the 55th passage (more than 700 days) and the 51st passage (more than 700 days), respectively (Table 1.). Both control and ENU-treated cultures from 30-day-old rats (30C and 30E) continued to proliferate at the 46th passage (more than 700 days). But control cultures from 8 and 2 day-old rats did not survive the 8th passage (133 days) and 24th passage (379 days), respectively (Table 1.).

Electronmicroscopically the 8th passage culture cells of untreated nerves from

TABLE 1. MORPHOLOGICAL AND BIOLOGICAL CHARACTERS OF EACH CELL LINE FROM TRIGEMINAL NERVES DURING LONG TERM CULTURE.

Cell line[a]	Passage no.	Morphological change	Transformation[b]	S-100	Basal lamina	Transplantability		S-100
2E	55 (>700)*	Spindle cell + giant cell	TE1+ST	+	+→−	+	8/32[c]	+ or −
2E [2][d]	8 (>700)*	Spindle cell → round cell	TE1→TE2	+		+++	3/3	+
2C	24 (379)*[e]	Fibroblast-like cell	−	−	−	−	0/3	
8E	51 (>700)*	Spindle cell + giant cell	TE1+ST	+		−	0/21	
8C	8 (133)*[e]	Fibroblast-like cell	−	−		−	0/3	
30E	46 (>700)*	Fibroblast-like + giant cell	ST	−	−	+	2/10	−
30C	46 (>700)*	Fibroblast-like + giant cell	ST	−	−	+	3/10	−

* Numbers in parentheses indicate observation days *in vitro*.

a; 2E (cell line from ENU-treated nerves of 2-day-old rats), 2C (cell line from untreated nerves of 2-day-old rats)

b; TE1 (1st step transformation of Schwann cells treated with ENU), TE2 (2nd step transformation of Schwann cells treated with ENU), ST (spontaneous transformation of fibroblasts)

c; eight transplanted tumors were composed mainly of spindle cells (TE1) or of fibroblastic tumor cells (ST).

d; 2E [2] (cell line of 2nd experiment)

e; no further passages were possble.

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2 day-old rats (2C-8P) were composed of round cells with large clear ovoid nuclei and well developed cytoplasm which had no basal lamina.

However, 2E-8P were chiefly long spindle cells with irregular nuclei rich in chromatin and moderately developed polar cytoplasmic processes which had many short fine processes (Photo. 8a), and a few cells were surrounded by basal lamina (Photo. 8b).   After long term culture, the cells in 2E-45P were composed of short spindle cells which had lost definite basal laminas.

Marked degeneration and piled foci in the 2E-3P cultures were reconfirmed in a second experiment in which some proliferative cells cultured from ENU-treated 2-day-old rats (2E [2]) became shorter or tripolar in shape a month after the 5th passage (3 months after the primary culture), and more than half of them became round or elliptical in shape 3 months after the 5th passage (Photo. 9).   These round cells were easily detached and floated in the medium when the Leighton's tubes were shaken slowly.   Electronmicroscopically, the 8th passage culture cells from ENU-treated 2-day-old rats in the second experiment (2E[2]-8P) were composed of almost round or elliptical cells with a high N/C ratio and poorly developed cytoplasm with many fine processes and no basal lamina (Photo. 10).
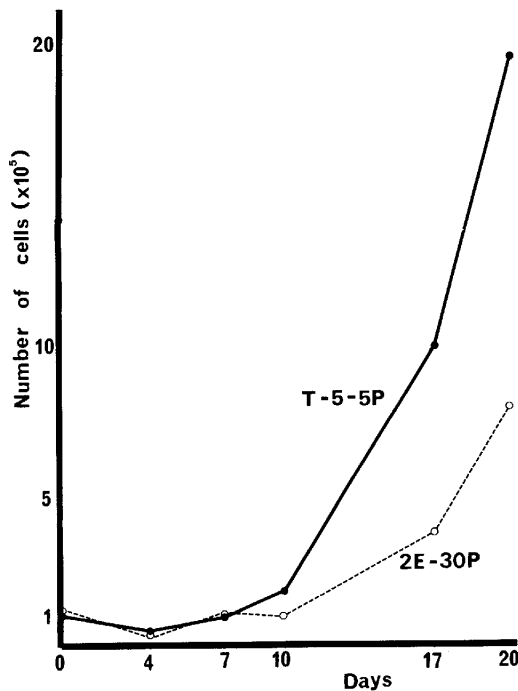

Fig. 3.   Growth curve at the 30th passage of 2-day-old rat trigeminal nerves exposed to ENU in utero and cultured *in vitro*, and growth curve at the 5th passage of trigeminal nerve tumor induced by ENU and cultured *in vitro*.

On the contrary, the primary culture from ENU-induced trigeminal nerve tumors had monolayers of short spindle or polygonal cells and crisscrossed piled foci of bipolar long spindle cells which were nonspecifically stained by PTAH and were positive for S-100 protein (Photo. 11). After serial passages, short spindle and polygonal cells predominated in this culture, and some of them became round cells which were easily detached from the cover slip.

Growth curves. The growth rate of cells in 2E-3P decreased drastically during the first 8 days and proliferated slowly thereafter. The growth of control cultures at the 3rd passage (3C-3P) increased steadily (Fig. 2). There was more proliferative activity in 2E-7P, 8E-7P and 2E-23P cells than in control cells (2C-7P, 8C-7P, and 2C-23P). The growth rates of both 30C-6Pand 30E-6P cells were similarily low.

The growth rate of cells from ENU-induced trigeminal nerve tumors at the 5th passage (T-5-5P) showed higher proliferative activity than that of the transformed cells in the first experiment (2E-30P) (Fig. 3).

Colony formation in semisolid agar medium. No colony formation was observed in the cultures of 2E-8P, 2E-13P, and 2E-50P and even in the cultures of the second experiment at the 8th passage (2E [2]-8P) and in the cultures of ENU-induced trigeminal nerve tumors at the 4th passage.

Rotation culture. The cells in 2E-9P, 8E-8P and the 3rd passage culture of ENU-induced trigeminal nerve tumors did not develop aggregates.

Transplantation of culture cells. The results of transplantation studies are given in Tables 1 and 2. The proliferating cells from the cultures of ENU-treated 2-day-old rats (2E) in both experiments, from the cultures of control and ENU-treated 30-day-old rats and from the cultures of ENU-induced trigeminal nerve tumors produced subcutaneous tumors (Photo. 12). The cells from 2E in the second experiment and from ENU-induced trigeminal nerve tumors frequently produced large tumors within 7-14 days, but the cells in 2E in the first experiment, 30E and 30C produced only a few small tumors within 3-12 months.

Histologically these tumors were divided into 2 groups. The first group of tumors was produced by transplantation of the 15th passage culture cells from ENU-treated 2-day-old rats (2E-15P) and of the culture cells from control and ENU-treated 30-day-old rats. The tumors consisted of many pleomorphic polygonal cells with very rich cytoplasm and round or elliptical nuclei with 1 or 2 nucleoli, coarse chromatin and abnormal mitoses, and were proliferated with many multinucleated giant cells (Photo. 13, 14a). Secondary bone formation was observed in some areas (Photo. 14b). A part of the subcutaneous tumor induced with 30E-35P showed a dense area with spindle cells forming concentric circles around vessels (Photo. 15). Individual cells of tumors were circumscribed by reticulum fibers. Marginal infiltration with many lymphocytes and neutrophils was seen. The second group of tumors were produced by transplantation, after more than 25 passages, of cultures from ENU-treated nerves of 2-day-old rats

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TABLE 2. SUBCUTANEOUS TUMORS INDUCED WITH CULTURE CELLS OF RAT TRIGEMINAL NERVES.

Cell line	Passage no.	No. of transplanted cells ($\times 10^5$)	Observation period(days)	Size of tumors (cm)	Histology of transplanted tumors
2E	15	15	385	1 (Diameter)	A marked pleomorphism with many giant cells, mitosis and secondary bone formation.
2E	25	10	217	$3 \times 2 \times 1.5$	A dense area with short spindle cells forming streams and whorls.
2E	26	10	154	$1.5 \times 1 \times 1$	Pleomorphism with giant cells
2E	26	10	164	$3 \times 2 \times 2$	A large dense area with short spindle cells and large necrotic areas. S-100(+)
2E	30	10	207	$2.5 \times 2 \times 2$	A dense area with short spindle cells.
2E	30	10	207	1 (Diameter)	Pleomorphism with giant cells and mitoses.
2E	45	20	100	0.3 (Diameter)	Pleomorphism with giant cells.
2E	45	20	100	0.5 (Diameter)	
2E [2]	8	10	12	$4 \times 3 \times 3$	A dense area with short spindle cells and a few multinucleated cells, a loose area with spindle or more round cells, and many cystic degenerations. S100(+)
2E [2]	8	10	23	$4 \times 3 \times 2.5$	
2E [2]	8	5	14	$3.5 \times 2 \times 2$	
30C	17	10	221	0.8 (Diameter)	A marked pleomorphism with bizarre giant cells, mitosis, and cystic degeneration. S-100(−)
30C	30	15	130	0.5 (Diameter)	Moderate pleomorphism with fibrosis and secondary bone formation.
30C	35	15	92	1 (Diameter)	Pleomorphism with bizzar giant cells and secondary bone formation.
30E	35	20	96	1 (Diameter)	A pleomorphic area with bizarre giant cells, more round cells and cystic degeneration, a dense area with spindle cells forming concentric circles around vessels. S-100(−)
30E	35	20	100	$2 \times 1 \times 1$	

in the first experiment, of cultures from ENU-treated nerves of 2-day-old rats in the second experiment, and of cultures from ENU-induced trigeminal nerve tumors. The tumors consisted of large dense areas with many short spindle cells with a high N/C ratio forming streams and incomplete whorls (Photo. 16), and had large central necrotic areas surrounded by extreamly loose edematous areas

with spindle cells and some small elliptical cells with cytoplasmic processes. The tumors induced by transplantation of the cells from 2E in the second experiment (2E [2]) and from ENU-induced trigeminal nerve tumors had essentially the same findings (Photo. 17, 18a).

S-100 protein was detected in cytoplasm and nuclei of almost all tumor cells of the large subcutaneous tumors induecd with 2E-26P or 2E [2]-8P (Photo. 18b), and also detected in some tumor cells of the small subcutaneous tumors induced with 2E-26P. But S-100 protein was not present in the tumor cells of the tumors induced with 30C-17P, 30E-35P, or culture cells derived from ENU-induced trigeminal nerve tumors.

Electronmicroscopically, the large subcutaneous tumors induced with 2E-26P were composed of many short spindle or elliptical cells which had a relatively well developed cytoplasm with many short processes and irregular nuclei with 2-3
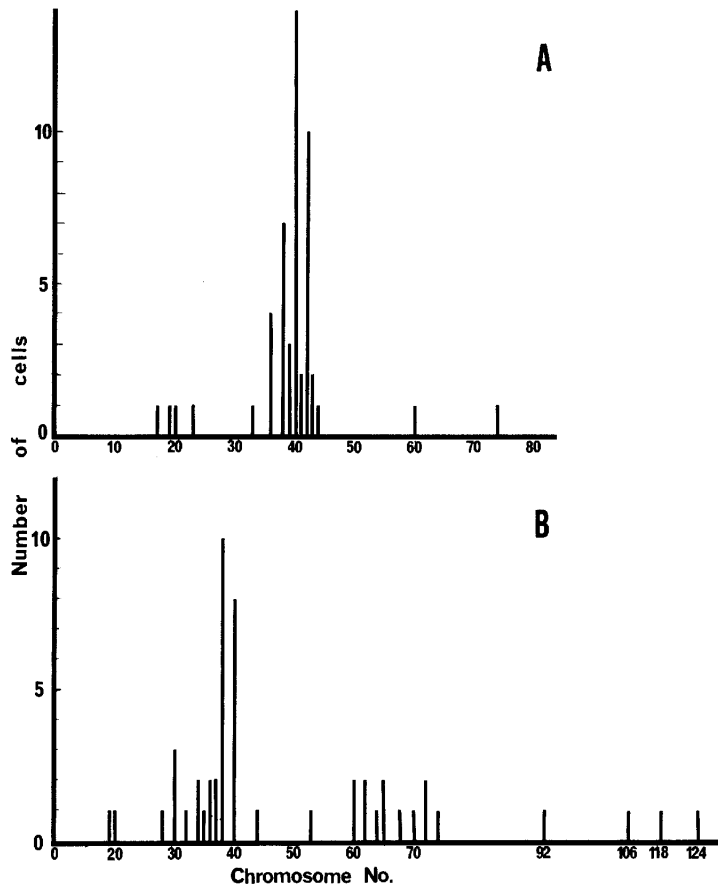

Fig. 4 (A, B). Distribution of chromosome numbers in the cell lines of 2E [2]-8P (A) and 30C-31P (B).

nucleoli. The subcutaneous tumors induecd with 2E [2]-8P showed many short spindle cells with no basal lamina which were surrounded by amorphous material.

Chromosome number of cell lines. Distribution of chromosome numbers of cell lines is presented in Fig. 4 (A, B). The mode of the chromosome numbers was 38, 40 or 42, *i.e.*, hypodiploid or diploid, but the chromosome numbers were quite variable.

DISCUSSION

It is known that the *in vivo* treatment with ENU yields an electrophilic ethyl cation via nonenzymatic, heterolytic decomposition which results in the appearance of $O6$-ethylguanine in DNA. An insufficient capacity of rat brain cells to completely eliminate $O6$-ethylguanine from DNA might be a determinat in the nervous system-specific neoplastic transformation by ENU, *e.g.*, by enhancing the probability of the "fixation" of structural alterations in DNA during subsequent replication (15). Transformation of brain cells by ENU has only been established by transfering brain cells of rats given ENU transplacentally to long-term *in vitro* culture (12). Since direct application of nitrosocompounds *in vitro* is not efficient for neoplastic transformation of cells, trigeminal nerve tissues cultured from rats transplacentally administered with ENU were used in this study to elucidate the serial changes of trigeminal nerve tumor development at the cellular level.

Because it is very difficult to remove the trigeminal nerves from fetal rats, the nerves from six 2,8, and 30 day-old rats were transferred to long-term culture with serial passages. The primary cultures of the nerve tissues were composed of fibroblast-like cells (S-100 protein negative), neurons (S-100 protein positive), and bipolar long spindle cells (S-100 protein positive) which were considered to be Schwann cells (8, 16). During the 2nd to the 8th day of the 3rd passage a significant degeneration and decrease in cultured cells were observed 15 days after the begining of the primary culture in the 2E-3P cultures and 23 days in the 8E-3P cultures. What kind of cells went into such a degeneration was obscure since the degeneration occurred before the cells had differentiated morphologically. Crisscrossed, multiple proliferative foci of bipolar spindle cells piled on the seats of fibroblast-like cells appeared among 2E-3P and 8E-4P, 30 and 60 days after the primary culture, respectively. These proliferative foci were morphologically consistent with the criteria of *in vitro* neoplastic transformation described by Kuroki (17), and seemed to correspond to the cellular hypercellularity in trigeminal nerves observed 20 days after exposure to ENU (6). The crisscrossed bipolar long spindle cells were thought to be derived from Schwann cells because S-100 protein was detected (16) and a basal lamina was demonstrated by electron microscopy (7).

No transformation was observed for 12 months in control and ENU-treated nerve cultures of 30-day-old rats.

In the second experiment, cultures of ENU-treated nerves of 2-day-old rats had crisscrossed bipolar long spindle cells after a period of degeneration. Also

in this second experiment, during serial passage from the 3rd to the 6th month after the primary culture, the bipolar long spindle cells became short-spindle or elliptical in shape and later became small round cells which were easily detached from cover slips and lost their piling characteristic.

However in the first experiment, cultures from ENU-treated nerves of 2 day-old rats continued to form crisscross patterns even at the 55th passage (more than 700 days), and, additionally, multinucleated giant cells appeared after the 16th passage (6 months).

The transplantability of transformed cells and the histology of transplanted tumors were variable (Table 2, 3). According to Kakunaga (18) and Kuroki (19), there are several processes in the *in vitro* transformation mechanism which must occur before the cell acquires transplantability. Kuroki (19) classified malignancy into four degrees : M0, the lowest degree of malignancy, in which no transplantation occurs : Ml in which transplanted tumors disappear ; M2 in which death of the hosts occurred long after regression of the transplanted tumor, and M3 in which transplanted tumors grow without regression until death of the hosts.

The cultures in this study may be divided into the following 5 kinds of cell lines according to the *in vitro* characteristics, Kuroki's (19) degree of malignancy and the histology of the transplanted tumors : a) the 2C and 8C cultures which showed neither transformation nor transplantability ; b) the 8E cell line which consisted of crisscrossed, long spindle cells revealing morphological transformation but no transplantability, and corresponding to the malignant degree M0 ; c) the 2E [2] cell line in which the cells changed from long spindle to small round, from which large transplanted tumors developed in a short 7-14 day period, and which resembled histologically *in vivo* ENU-induced malignant Schwannoma,corresponding to the degree M3 ; d) the 30C and 30E cell line which spontaneously transformed and produced small transplanted tumors having marked pleomorphic,bizarre giant cells and which corresponded to M1-M2 in malignancy, and, finally, e) the 2E cell line consisting of long spindle cells which transformed in early passages and some bizarre giant cells which seemed to be spontaneously transformed from fibroblast-like cells in later passages, in which transplanted tumors were composed of both cell types and corresponded to M1-M2. Large transplanted tumors from the 2E cell line were chiefly composed of short spindle cells, whereas the small ones were chiefly composed of pleomorphic polygonal cells. Furthermore, as the number of passages increased, the latency period was shortened (2E-15P, 12 months ; 2E-26P, 5 months, and 2E-45P, 3 months).

The cultures in the second experiment transforming to the M3 degree of malignancy may be considered a good *in vitro* model of chemical carcinogenesis of the rat trigeminal nerve, because from them transplanted tumors were produced which were essentially the same as those induced with the culture of *in vivo* ENU-induced malignant Schwannoma of the trigeminal nerve.

On the contrary, cultures from ENU-treated nerves of 2-day-old rats in the

first experiment not only did not transformed from crisscrossed, long spindle cells to small round cells after more than 700 days, but also needed a very long 3-12 months period for small subcutaneous tumors to develop, compared with 7-14 days in the case of cultures in the second experiment (2E [2]). This suggests that the transformed cells in the first experiment stayed at a lower level of malignancy than those in the second experiment.

The culture of ENU-treated nerves of 8-day-old rats (8E) did not produce transplanted tumors, and, thus, probably was at the lowest level of malignancy or the character of its surface antigens changed.

Although it is uncertain what factors induced the different levels of malignancy in cultures after the first crisscross pattern transformation, the level of malignancy of *in vivo* ENU-induced tumors also showed remarkable variability after the early neoplastic change of hypercellularity of trigeminal nerves which occurred in practically 100 % of 30-day-old rats. For example ENU-induced trigeminal nerve tumors developed in only 12 of 56 rats more than 150 days old (21 %) and hypercellularity of trigeminal nerves which stayed in a low level of malignancy was found in 5 of 56 rats (9 %).

Both control and ENU-treated nerve cultures of 30-day-old rats underwent spontaneous transformation. About 12 months after the primary culture, both cell cultures became more pleomorphic with fibroblastic polygonal cells and multinucleated giant cells. Some of the cultures produced small transplanted tumors, about 1 cm in diameter, after 3-6 months.

It is known that normal tissue after serial cultivation may spontaneously transform into neoplastic cells (19). It is more difficult to induce spontaneous transformation in rat tissue than in mouse or hamster tissue (20). The spontaneous transformation of rat fibroblasts usually occurs more than one year after the primary culture (21).

That the cultures of ENU-treated nerves of 30-day-old rats did not have crisscrossed long spindle cells in some what curious but may be due to exposure of the nerves to an insufficient amount of ENU or difficulty to cultivate Schwann cells in rats more than 30 days old than fibroblasts.

The bone formation observed in the transplanted tumors induced with cultures 2E-15P, 30C-30P and 30C-35P was probably due to metaplasia of transplanted cells.

Transplanted tumors induced with 2E-26P or 2E [2]-8P are considered to be derived from transformed Schwann cells because of their morphological appearance and being positive for S-100 protein. On the contrary, subcutaneous tumors induced with 30C-17P or 30E-35P are thought to be derived from non-neuroectodermal cells, probably fibroblast cells, because the fibroblast-like tumor cells were negative for S-100 protein and produced reticulum fibers.

It is well known that the normal chromosome number of the rat is $2n=42$ (23). As shown in Fig. 4 (A, B), the modes of chromosome numbers in the

cultures of this experiment were hypodiploid (40 or 38) or diploid (42) in the case of 8E-27P.   Though some chromosome numbers over 100 were observed in cultures with multinucleated giant cells (2E-31P, 8E-27P, and 30C-31P), the majority of chromosome numbers in cultures of the M3 degree of transplantability (2E [2]-8P, T-5-6P) were distributed near diploid (42).   Mennel *et al.*(9), reported that the chromosomal numbers of cultures of trigeminal nerve tumors induced by ENU inclined toward smaller numbers.   In this experiment, a similar tendency was observed.   Since the number of chromosomes was variable in each cell line, no conculsion could be drawn regarding the relationship between the distribution of chromosome numbers and transplantability.

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LEGENDS FOR PHOTOGRAPHS

Photo. 1. ENU-exposed trigeminal nerve (the PNS-CNS junction) of a 30-day-old rat shows hypercellularity and hyperchromatism. H. & E. × 225

Photo.2. Primary culture from trigeminal nerves of an untreated 8-day-old rat with S-100 protein staining. Note S-100 protein positive neurons (→) and Schwann cells (▶). PAP × 300

Photo. 3. Trigeminal nerve of an untreated 2-day-old rat (4 days in culture, 3rd passage). Note many fibroblast-like cells. Phase-contrast × 300

Photo. 4. Trigeminal nerve of 2-day-old rat administered ENU transplacentally (4 days in culture, 3rd passage). Note the degeneration of cultured cells with relatively more long spindle cells remaining. Phase-contrast ×300

Photo. 5. Trigeminal nerve of a 2-day-old rat administered ENU transplacentally (77 days in culture, 3rd passage). Note crisscross formation of bipolar long spindle cells on the fibroblast-like cells. PTAH × 200

Photo. 6a & b. a; Trigeminal nerve of a 2-day-old rat administered ENU transplacentally (35 days in culture, 10th passage). Crisscrossed bipolar spindle cells have intracytoplasmic S-100 protein. b; All control cultured cells have no S-100 protein. PAP a & b; ×600

Photo. 7.   Trigeminal nerve of an 8-day-old rat administered ENU transpracentally (27 days in culture, 18th passage).   Note a multinucleated giant cell with rich cytoplasm among crisscrossed spindle cells. H. & E.   × 100

Photo. 8a & b.   Ultrastructure of cultured cells from trigeminal nerves of a 2-day-old rat administered ENU transplacentally (90 days in culture, 8th passage).   a ; Note bipolar spindle cells with indented nuclei and cytoplasmic processes. b ; Some of the cultured cells are surrounded by basal lamia.   a ; × 4,000   b ; × 16,000

Photo. 9.   Trigeminal nerves of a 2-day-old rat administered ENU transplacentally (2nd experiment, 6 months in culture, 8th passage). Small round or elliptical cells predominate over bipolar or tripolar spindle cells.   Phase-contrast   × 100

Photo. 10.   Ultrastructure of cultured cells from a trigeminal nerve of a 2-day-old rat administered ENU transplacentally (2nd experiment, 15 months after primary culture, 8th passage).   Note round cells which show a high N/C ratio and prominent cytoplasmic processes but no basal lamina.   × 3,000

Photo. 11.   Trigeminal nerve tumor of rat administered ENU transplacentally (75 days in culture, primary culture).   Bipolar spindle cells and giant spindle cells are crisscrossed.   PTAH   × 450

Photo .12.   Subcutaneously transplanted tumor induced 217 days after inoculation of a newborn rat with 25th passage culture cells of trigeminal nerves of a 2-day-old rat administered ENU transplacentally.

Photo. 13.   Histology of transplanted tumor from 15th passage culture cells of trigeminal nerves of a 2-day-old rat administered ENU transplacentally (2E-15P).   Tumor is mainly composed of pleomorphic fibroblastic tumor cells showing pathologlcal mitoses. H. &E.   × 600

Photo. 14a &b.   Histology of transplanted tumor from 17th passage culture cells of trigeminal nerves of a 30-day-old untreated rat. Tumor shows marked pleomorphism with bizarre giant cells (a) and some fibrosis and bone formation (b). H. &E.   × 300

Photo. 15.   Histology of transplanted tumor from 35th passage culture cells of trigeminal nerves of a 30-day-old rat administered ENU transplacentally.   Tumor shows a dense area with spindle cells forming concentric circles arround vessels. H. & E.   × 300

Photo. 16.   Histlogy of transplanted tumor from 26th passage culture cells of trigeminal nerves of a 2-day-old rat administered ENU transplacentally (2E-26P).   Tumor is mainly composed of S-100 positive spindle cells forming streams. H. & E.   × 600

Photh. 17.   Histology of transplanted tumor from 6th passage culture cells of trigeminal nerve tumor induced in vivo with ENU.   The tumor shows a dense area, a loose area, and cystic degeneration. H. & E.   × 300

Photo. 18a & b.   Histology of transplanted tumor from 8th passage culture cells of trigeminal nerves of a 2-day-old rat administered ENU transplacentally in the 2nd experiment (2E[2]).   a : The tumor has a dense area with spindle cells (A), a loose area with spindle and more round cells (B), and cystic degeneration (C) as in Photo. 17. H. & E.   × 600   b ; S-100 protein was detected in the cytoplasm and nuclei of these tumor cells.   PAP   × 1,200

316   K. HAYASHI

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