Tumour induction by ethylnitrosourea in the central nervous system

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TUMOUR INDUCTION BY ETHYLNITROSOUREA IN THE CENTRAL NERVOUS SYSTEM

Summary. Introduction. *Experimental central nervous system (CNS) tumours have been proposed as a useful model for the study of oncogenesis, epiphenomena related to cancer and for the design of new therapeutic strategies.* Development. *The administration of chemical substances is one of the most commonly-used methods to induce CNS neoplasms. N-ethyl-N-nitrosourea (ENU) belongs to the nitrosourea family, a wide group of alkylating agents that are able to induce brain tumours in litters after transplacentary administration at the 15th day of pregnancy. This nitrogenous urea compound has a high mutation inducibility affecting the expression of oncogenes such as* p53, neu/erbB-2 *and* Ras. *Prenatal exposition of Sprague Dawley rats to ENU induces intra-axial tumours of glial lineage and extra-axial malignant schwannomas. Although the precise mechanism of tumour induction is unclear, it is known to affect cell differentiation of primitive neuroepithelium from the subventricular plate generating oligodendrogliomas, astrocytomas, mixed gliomas or ependimomas.* Conclusion. *The transplacentary administration of ENU induces the development of gliomas and schwannomas that are similar to those found in humans. Animal models are necessary and useful for further studies to get an early diagnosis and to establish correct therapeutic indications. [REV NEUROL 2006; 43: 733-8]*

Key words. *Brain tumour. Ethylnitrosourea. Glioma. Neurooncology. MPNST. Schwannoma.*

INTRODUCTION

A profound knowledge of the genetic and molecular changes that occur when a cell acquires a tumoural phenotype, and its biological behaviour during the development of the tumour, would be of great help in early diagnosis and in establishing the precise therapeutic approach. This field is especially suitable for translational research, in which both basic and clinical research are necessary and complementary in order to achieve significant progress.

The need for experimental models of brain tumours as similar as possible to human tumours has prompted researchers to attempt various approaches. Animals such as dogs, cats and rodents have been used to model tumour induction in the central nervous system (CNS) [1-3].

Certain rat strains, such as Wistar, Sprague Dawley or Fischer varieties, are among the animals most used in research. This is due to several reasons; their high rate of reproduction, a gestation period of 21 days, and average litter of 10 young and their hardiness, which allows surgical operations and therapeutic treatments.

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In memoriam Prof. Escalona, colleague and friend of many researchers on this field. Without his impulse, to inquire into the biopathology of brain tumours will be now a very harder task.

DEVELOPMENT

Over the years, different methods have been employed to induce tumours in the central nervous system of rats [4]:

- *Exposure to radiation, either ionising or non-ionising:* non-ionising radiations, such as ultraviolet rays, provoke localised mutations in cells that can be repaired by the DNA repair system. Ionising radiation, such as X-rays, generate changes in the DNA that are not repaired, producing mutations that are expressed at a cellular level. This is not a productive model, due to the low incidence and the long delay before the tumour appears. Moreover, a source of X-rays is required and authorised installations where it can be used.
- *Inoculation of viri with carcinogenic capacity:* Avian Sarcoma Virus (ASV), Simian Sarcoma Virus (SSV), etc. This is a productive model, with a short development time and a high incidence. The main problem is the risk associated with manipulation of this kind of material. Neonatal inoculation of F344 rats with avian sarcoma virus generates the RT-2 line of glial cells [5].
- *Xenografts of tumour cell lines in athymic nude rats and mice:* this is a widely-used and highly productive model with which much important data has been obtained regarding the interaction between the tumour and the surrounding tissue [2,6,7]. This model allows human tumour development to be studied *in vivo*, since the tumoural tissue is developing within a healthy environment.
- *Administration of chemical substances:* this is one of the most-used methods for inducing tumours. The delay for the tumour to appear is long and the incidence is high. One problem that must be taken into account when using this method is the non-organ-specific mutagenic effect of the majority of the chemical substances employed. This can lead to lesions appearing in unexpected organs.

Several glial tumour cell lines have been obtained from tumours produced using the procedures described above. Some of these cell lines are commonly used for *in vitro* research, such as [8]:

– *9L gliosarcoma*: a cell line generated from gliomas induced in Fischer rats. The gliomas are induced by weekly intravenous injections of methylnitrosourea (MNU) during 26 consecutive weeks [9-11].
– *C6 glioma*: a cell line obtained from tumours produced by injecting MNU in adult Wistar rats during 8 months [10,11].
– *F98 and RG-2:* cell lines obtained from tumours induced by a single injection of ethylnitrosourea (ENU) on the 20th day of gestation in Fischer rats [12].
– *CNS-1*: a cell line from tumours induced by repeated injections of MNU in Levis rats during 7 months [13].

These cell lines quickly become malignant and dedifferentiated during the first stages of culture. Consequently, they are not suitable for studying the evolution of a tumour during the first stages of tumoural growth.

Nitrosoureas

The description of the carcinogenic activity of nitrous compounds in 1956, and their use to induce tumours in the central nervous system, provided experimental neuro-oncology with a useful research tool [8,14].

Nitrous compounds have a common basic molecular structure with a 'guide' radical on which their organotropic effects apparently depend. For example, compounds in the methyl group (DMNA) cause hepatic tumours; the urethane group (MNUT) causes pulmonary tumours and the urea group (MNU and ENU) induces tumours in the central nervous system [15].

About 65 nitrous compounds have been described with a high capacity for CNS tumour induction. The ones that are most used are nitrogenous urea compounds, N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea (ENU). These nitrosamides are N-alkyl N-nitrosourea compounds with a high carcinogenic power. They are non-selective carcinogens, i.e., they cause genetic modifications not only on the central nervous system, but also in organs such as the breasts, the salivary glands, the liver and the oesophagus [16].

Koestner [17] carried out a study of the neurocarcinogenic activity of nitrosoureas, and observed that this varied, depending on the animal used, the animal age, the administration route used and the dose. Even within the same species, such as rats, using identical doses and administration routes, some rats developed tumours while others did not. The age at which these substances are administered is critical, and a fundamental factor is whether the animal is an adult, a fetus or an embryo, since the mutagenic activity will occur at different levels, depending on the stage of development of the animal.

The experimental model

Rats are one of the species most susceptible to developing CNS tumours after ENU administration [18]. A single dose of ENU administered to gestating rats induces neurological neoplasia in the offspring [19]. This model has allowed *in vivo* studies of tumour behaviour and possible treatments [20-23]. The first studies indicated that the major incidence of CNS tumours induced by ENU was obtained by intravenous injection of a dose between 40 and 80 mg/kg b.w. during the gestation period [15]. It has been found that administration of ENU to adult rodents did not induce tumours. However, transplacentary administration during the last week of gestation and the first week of post-natal development induced tumours in more than half of the offspring [24,25].

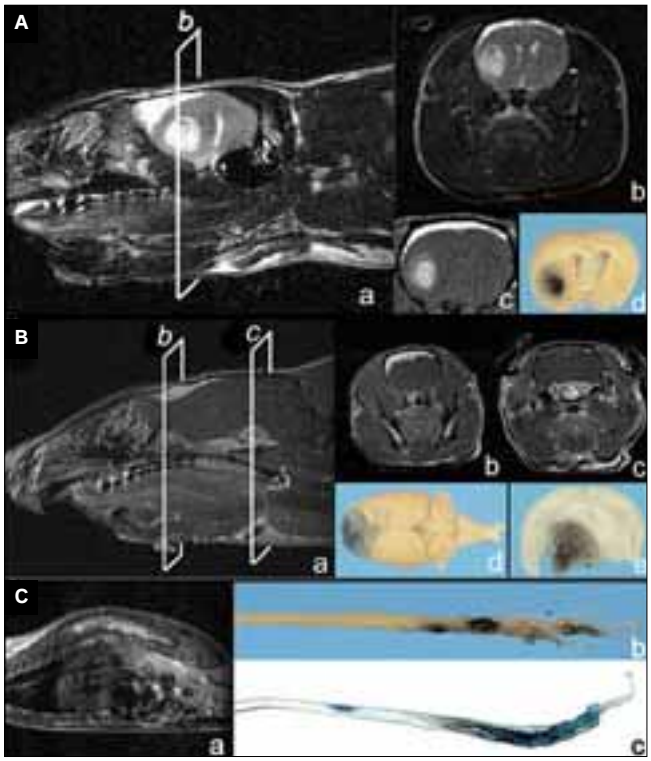

Figure 1. Macroscopic images of tumours in the central nervous system induced by ENU. A) Anaplastic glioma representation: a) Magnetic resonance T_2 image of a rat head sagittal slice; b) T_2 coronal section image; c) The same image on T_1 after gadolinium injection: the glioma shows a hyperintense signal both for T_2 and T_1; d) Coronal section of the rat brain after autopsy: the blue region shows the location of the tumour, the colour is due to extravasation of Evans blue, after half an hour intravenous injection. B) Magnetic resonance T_1 images of a malignant schwannomas after gadolinium administration: a) Rat head sagittal section showing the planes in which two tumours are located; b) Extra-axial tumour in the convexity; c) Basal location associated with the 5th cranial nerve; d and e) Coronal brain sections with Evans blue corresponding to those previously described in b and c. C) Tumours located in the spinal cord: a) Magnetic resonance T_2 images show hyperintense signal of the glioma; b) The spinal cord following injection of Evans blue with glial tumours in the sacrolumbar region; c) Schwannomas in the spinal nerves.

We have used this method to induce CNS tumours in Sprague Dawley rats, using prenatal exposure to a dose of ENU. Gestating rats were given an intraperitoneal injection of 80 mg ENU/kg body weight (10 mg/mL in 0.9% NaCl) on the 15th pregnancy day. After their sixth month, the offspring began to exhibit clinical symptoms such as: exophthalmia, lateralisation of the head, ataxia, dyspnea, loss of weight, uncared-for appearance, lethargy, apathy and paralysis of one or both rear limbs [26]. By correlating the most significant clinical symptoms and the appearance of tumours, we conclude that:

– Paralysis of rear limbs corresponds to the presence of one or more intra-axial tumours (gliomas) in the spinal cord.
– Dyspnea corresponds to the presence of an extra-axial tumour (schwannoma) in the basal region of the brain, associated with the sheath of Gasser's ganglion, or extending along the meningeal sheaths covering the convexity, the falx cerebrum and penetrating into the Virchow-Robin spaces.
– Apathy (somnolence, immobility) corresponds to the existence of an intra-axial tumour (glioma) with significant incidence on a cerebral hemisphere.

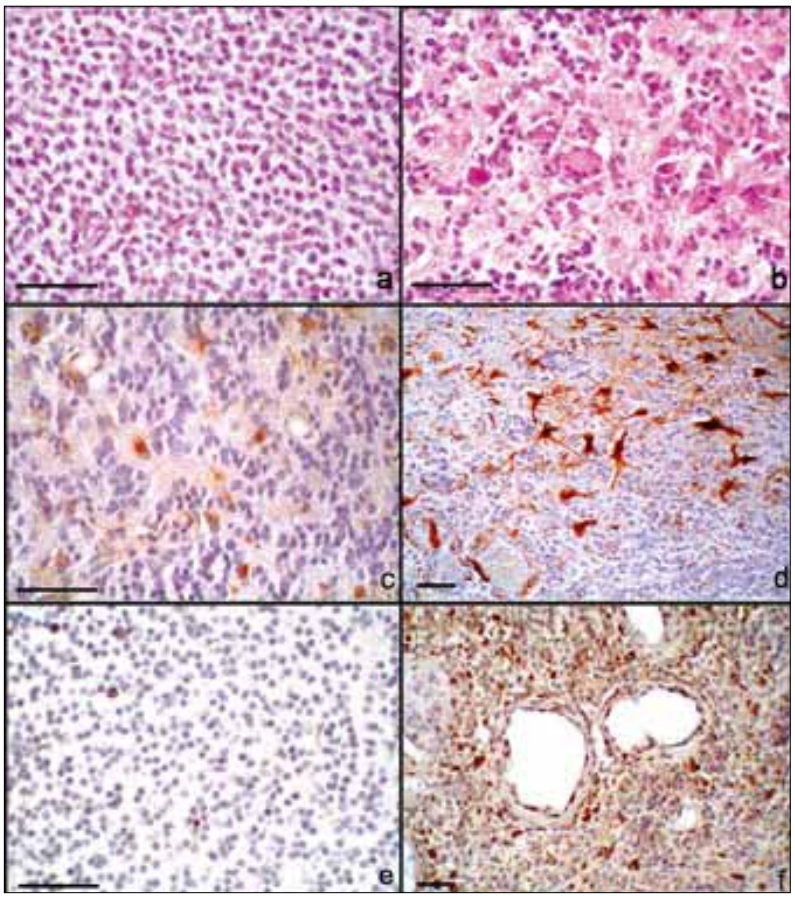

Figure 2. Histological images of non-anaplastic (a, c, e) and anaplastic gliomas (b, d, e) induced by ENU: a and b) Haematoxylin-eosin stain showing the isomorphous (a) histological pattern characteristic of a classic oligodendroglioma, and the polymorphous (b) growth with macrocysts and necrotic areas of the anaplastic glioma; c and d) GFAP-positive cells in the tumour core (c), and on the marginal rib (d); e and f) Immunoexpression for Ki-67 in early stages (e), and in advanced stages (f) of a tumour. Bar: 50 µm.

Table. GFAP, NF, S-100, Ki-67 and Olig-2 antibodies positivity of tumours induced by ENU (number of positive cells counted in a 400×-magnified field).

	Glioma		Schwannoma
	No anaplastic	Anaplastic	
NF	–	–	–
Olig-2	±	±	–
S-100	–	–	++
GFAP	–	+	–
Ki-67	*	**	**

–: no positive cell; ±: no. of cells under 5; +: positive cells from 5 to 10; ++: positive cells over 10; *: positive nucleus for Ki-67 under 10%; **: positive nucleus for Ki-67 over 10%.

Experimental tumours *in vivo* are located using nuclear magnetic resonance [27-32]. This non-invasive technique has the great advantage of detecting even small tumours, which allows the early stages of tumour development to be studied. The tumours are observed in enhanced images using two relaxation time constants, T_1 and T_2, both before and after injection of gadolinium (Gd-DTPA) [28,33,34].

To locate and detect tumours *ex vivo*, Evans blue intravital stain is injected before the autopsy. Evans blue is an acidic dye with a high affinity for serum proteins, and its distribution provides data on the vascular permeability for proteins, which aids in the macroscopic location of small tumours. In control rats, it was observed that the brain and the spinal cord were not stained with this dye, while structures without blood brain barrier, such as the pineal gland, were stained blue. Once the material had been dried and examined on a macroscopic scale, a histological and immunological study was undertaken to determine the development of the tumours. In order to relate the findings to human pathology, an immunocharacterization has been made (Table):

- *GFAP* (polyclonal, Dako Z334, 1:200): acidic fibrilar glial protein, a constituent of the intermediate filaments of the astrocytes cytoskeleton.
- *S-100* (polyclonal, Dako Z311, 1:2000): a calcium-binding protein expressed in Schwann cells.
- *NF* (polyclonal, Chemicon International, 1:200): protein constituent of the neurofilaments of neuronal cytoskeleton.
- Oligodendrocyte Ab-2 (Olig-2) (monoclonal, Neomarkers 1:200): a protein specific to myelin and oligodendrocytes.
- *Ki-67/MIB-5* (monoclonal, Dako 1:75): cell-proliferation antigen.

Intra-axial tumours

Histological identification of intra-axial tumours corresponds to tumours of glial lineage [35,36] namely: oligodendrogliomas [37,38], astrocytomas [39], mixed gliomas or ependimomas [40]. Their growth is mainly associated with the white matter, both in the spinal cord and in the brain (Figs. 1A and 1C).

These experimental gliomas in the early stages of tumour development consist of cellular proliferation that, as it grows, constitutes a nodular tumour that, in later stages, may infiltrate an entire cerebral hemisphere.

The early stages correspond to isomorphous tumours consisting of round cells with a homogeneous nucleus and clear cytoplasm. Overall, the cell has a characteristic 'fried egg' appearance, and the interstices acquire the honeycomb structure typical of oligodendroglioma (Fig. 2a). These are gliomas with a low proliferation rate, with an average proliferation index for Ki-67 close to 6% (Fig. 2e). These tumours do not react to antibodies for Olig-2, NF or S-100. Some cells in the interior of the tumour were positive for GFAP. These cells have been called 'mini-gemistocytes' or glial fibrillary oligodendrocytes, and considered as transition cells between oligodendrocyte and astrocyte [41,42] (Fig. 2c).

The advanced stages of development display a polymorphic histological pattern with anaplasia and occasional cellular pleomorphism with polynucleated cells. Atypical mitoses are observed and histopathological signs of malignity, such as hemorrhages, cysts and necrosis (Fig. 2b). These are aggressive, infiltrating tumours with a high proliferation index with an average value

of about 14% (Fig. 2f). On the basis of their histopathological pattern, they can be diagnosed as anaplastic oligodendrogliomas [41,43] that in the final stages become glioblastomas. These tumours present some cells with a slight cytoplasmic reaction to the Olig-2 antibody. A significant number of cells are found that have reacted to the GFAP antibody, which can make it difficult to diagnose an oligodendroglioma, an astrocytoma or a mixed glioma (Fig. 2d).

Extra-axial tumours

These appear with certain frequency, and start their development at the fifth cranial nerve. As they develop, they progressively occupy the entire base of the cranium, enclosing both nerves. In the course of their development, they tend to wrap around the brain, extending across the cavity, occupying the sub-aracnoid space and penetrating into the Virchow-Robin spaces, both basally and laterally (Fig. 1B). These tumours also appear in the spinal nerves in the sacrolumbar region (Fig. 1C). According to histopathological analysis, they are malign schwannomas (MPNST), positive for anti-S-100 antibody, and negative for GFAP and NF. The expression of the S-100 antigen varies according to the degree of malignity, more marked cells when the dedifferentiation is higher (Fig. 3).

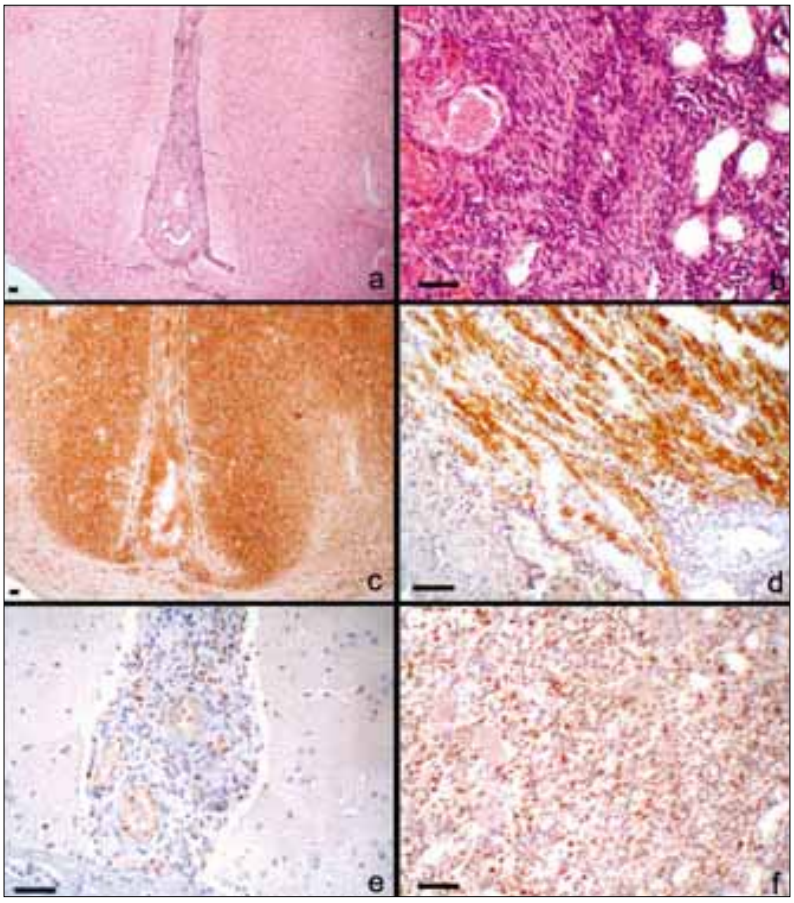

Figure 3. Malign schwannoma spreading up to the interhemispheric fissure (a, c, e) and associated with the fifth cranial nerve (b, d, f): a and b) Haematoxylin-eosin stain; c and d) Cellular cluster positives for S-100; e and f) High density of Ki-67-positive cells in both schwannomas. Bar: 50 µm.

The mechanisms of ENU activity

The exact mechanism by which ENU induces tumours in the CNS is as yet unknown. It is known to have an alkylating effect on the O_6 of guanine (G) and the O_2 of thymine (T), provoking an alkyl-lesion in the DNA. Guanine alkylated during the replication phase links to a thymine instead of to a cytosine (C). This thymine will then link to an adenine (A) in the next replication, causing a mutation called a 'GC-AT transition'. On the other hand, alkylated thymine links to another thymine in the first replication, instead of to an adenine. As a result, in the second replication, the non-alkylated thymine links to an adenine, producing an 'TA-AT transversion'.

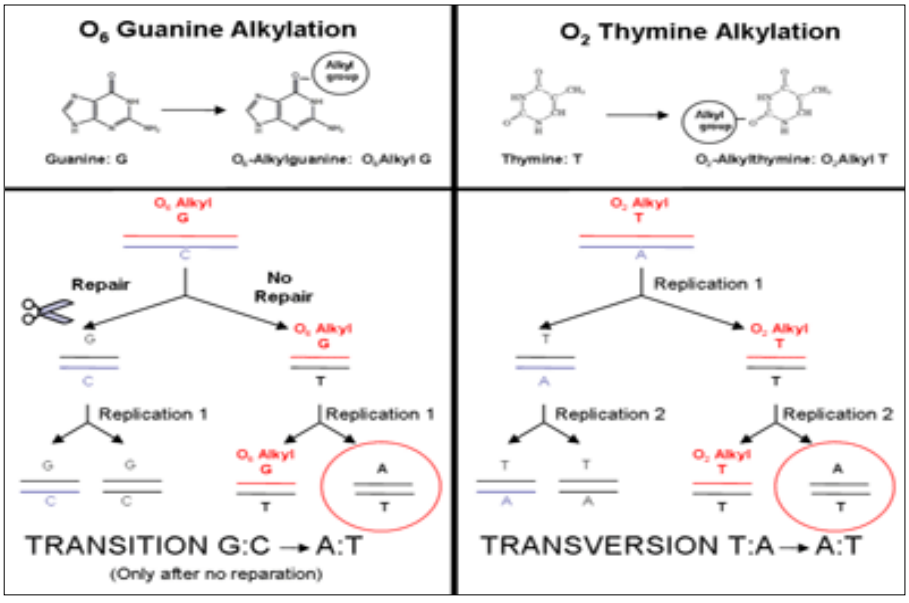

Figure 4. Diagram of the possible mechanism for tumours induced by ENU.

Affected cells inhibit the replication process, activating repair enzymes such as O_6-alkylguanine-DNA-alkyltransferase (AGT), which eliminates the O_6-alkylguanine. However, no repair enzyme has been detected for the O_2-alkylthymine, which implies the continuous generation of transversions [18,44,45]. This repair enzyme acts at a slower speed in the brain than in other organs. It is thought that this prolonged presence of O_6-alkylguanine in the brain could be, amongst other factors, the cause of the appearance of tumours [17].

Studies using ENU marked with ^{14}C have revealed that the DNA mutation in newborn rats persists in the brain, since the O_6-alkylguanine-DNA-alkyltransferase has not been repaired.

Katayama et al [46,47] observed that in the fetal CNS, immediately after administering ENU to gestating mothers, apoptosis was induced and the cellular cycle was arrested in neuroepithelial cells. It is for this reason that ENU is a known teratogenic agent that induces congenital abnormalities, especially in the central nervous system [16,48]

The successive cycles of DNA replication that take place during post-natal development of the rats up to adult age produce a cumulus of mutations that affect the expression of certain oncogenes such as *neu/erbB-2*, *Ras*, *p53* and genes that code for caspase-9 [49-52]. The mutation in *p53* affects the expression of the Id proteins that are involved in the development of the brain and in the differentiation of the neuroepithelial cells. Deregulation of the expression of the *Id* genes has been described in cell lines from the lung, the colon, the pancreas and in neuronal and astrocytic lines of CNS tumours [18,53]. This could be one of the causes for the appearance of intracerebral tumours of glial lineage following ENU administration (Fig 4).

CONCLUSIONS

In short, our conclusion is that administration of the ENU carcinogen to Sprague Dawley rats allows us to obtain malign gliomas and schwannomas similar to those found in human beings.

There is a general consensus regarding identification of extra-axial tumours as malign tumours that have their origin in Schwann cells, and of intra-axial tumours as tumours of glial origin. These glial-derived tumours are derived from primitive neuroepithelial cells from the subventricular plate, which can then generate oligodendrogliomas, astrocytomas, mixed gliomas and ependimomas.

It is both useful and necessary to have animal models for cerebral tumours available that allow studies to be carried out in different stages of the growth of a tumour, especially in the early stages that are difficult to observe in clinical practice.

This experimental model of CNS tumours *in vivo* allows us to study the behaviour of a tumour submitted to different therapeutic treatments, using imaging, genetic and molecular techniques. It also allows evaluation of the response to chemotherapy, radiotherapy, immunotherapy and genetic therapy.

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INDUCCIÓN DE TUMORES EN EL SISTEMA NERVIOSO CENTRAL MEDIANTE ETILNITROSOUREA

Resumen. Introducción. *La utilización de modelos experimentales* in vivo *de tumores en el sistema nerviosos central (SNC) ha traído importantes avances a la neurooncología. Estos modelos animales han permitido el estudio de procesos de oncogénesis, de sus epifenómenos y del diseño de nuevas estrategias terapéuticas.* Desarrollo. *Existen varios métodos de inducción de neoplasias en el SNC, de los cuales la administración de sustancias químicas es una de las modalidades más utilizadas. La N-etil-N-nitrosourea (ENU) es un agente alquilante capaz de inducir tumores cerebrales en la descendencia de ratas gestantes tras su administración transplacentaria. Se tata de un compuesto nitrogenado de la urea con alto poder mutagénico que afecta a la expresión de ciertos oncogenes como* p53, neu/erbB-2 *y* Ras. *Mediante la exposición prenatal a ratas Sprague Dawley del carcinógeno ENU se inducen tumores intraaxiales de estirpe glial y tumores extraaxiales como los schwannomas malignos. Aunque se desconoce el mecanismo preciso de inducción de los tumores gliales, se sabe que afecta a la diferenciación de las células neuroepiteliales primitivas de la placa subventricular, lo que genera oligodendrogliomas, astrocitomas, gliomas mixtos o ependimomas.* Conclusión. *La administración transplacentaria de ENU permite obtener gliomas y schwannomas malignos similares a los encontrados en los humanos. Esto puede ayudar al estudio en profundidad de dichos tumores para llegar a realizar un diagnóstico precoz y asentar unas indicaciones terapéuticas precisas. [REV NEUROL 2006; 43: 733-8]*

Palabras clave. *Etilnitrosourea. Glioma. MPNST. Neurooncología. Schwannoma. Tumor cerebral.*

INDUÇÃO DE TUMORES NO SISTEMA NERVOSO CENTRAL COM ETILNITROSOUREIA

Resumo. Introdução. *A utilização de modelos experimentais in vivo de tumores no sistema nervoso central (SNC) trouxe importantes avanços à neuro-oncologia. Estes modelos animais permitiram o estudo de processos de oncogénese, dos seus epifenómenos e do desenho de novas estratégias terapêuticas.* Desenvolvimento. *Existem vários métodos de indução de neoplasias no SNC, dos quais a administração de substâncias químicas é uma das modalidades mais utilizadas. A N-etil-N-nitrosoureia (ENU) é um agente alquilante capaz de induzir tumores cerebrais na descendência de ratos gestantes após administração transplacentária. Trata-se de um derivado nitrogenado da ureia com alto poder mutagénico que afecta a expressão de certos oncogenes como* p53, neu/erbB-2 *e* Ras. *Com a exposição pré-natal de ratos Sprague Dawley ao carcinógeno ENU induzem-se tumores intra-axiais de estirpe glial e tumores extra-axiais como os schwannomas malignos. Ainda que se desconheça o mecanismo preciso de indução dos tumores gliais, sabe-se que afecta a diferenciação das células neuroepiteliais primitivas da placa subventricular, contribuindo para o desenvolvimento de oligodendrogliomas, astrocitomas, gliomas mistos ou ependimomas.* Conclusão. *A administração transplacentária de ENU permite obter gliomas e schwannomas malignos similares aos encontrados nos humanos, favorecendo desta forma o estudo de tais tumores, no sentido de permitir um diagnóstico cada vez mais precoce e a definição de indicações terapêuticas precisas. [REV NEUROL 2006; 43: 733-8]*

Palavras chave. *Etilnitrosoureia. Glioma. MPNST. Neurooncologia. Schwannoma. Tumor cerebral.*