Establishment of Cancer Gene Therapy

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This book is dedicated to Annie Descamps who passed in 1975, the best French immunology student decorated with the CNRS Medal. She predicted that cancer therapies would evolve into immunotherapies.

Invented in 1993, cancer gene therapy, which has evolved to cancer immuno-gene therapy, is described in this book.

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INTRODUCTION

´´The supreme achievement would be to see that stating a fact is starting a theory"
(Johan Wolfgang von Goethe)

Treatment of malignant tumors by surgery, radiation and hormones, and chemotherapy, has been limited in terms of efficacy. Since 2016, immunotherapy, including immuno-gene therapy, has become one of the most relevant and revolutionary cancer treatments, through the help of the Cancer Moonshot Initiative. Historically, the development of a gene therapy approach by Anderson (Anderson et al., 1990), and the "creation" of cancer gene therapy or cancer immuno-gene (CIG) therapy by Trojan, (Trojan et al., 1993) was largely followed by others (i.e. Habib, 2002; You, 2011; Libutti, 2019; Giamas, 2020). The CIG therapy was successfully introduced in clinical trial (Trojan et al., 2007) in parallel with cancer immunotherapy "created" by Townsend (Townsend and Allison, 1993), and then largely expanded in basic and clinical research (i.e. Guo et al., 1994; Sampson et al., 2009; Dietrich et al., 2010; Baumeister et al., 2016; Wolchok et al., 2017).

The presented book focuses on the description of cancer gene therapy of malignant tumors targeting oncoprotein Insulin–like growth factor 1 (IGF-I), which plays a key role in neoplastic differentiation (Daughaday, 1972; Pollak et al., 2004). The anti-*IGF-gene* technology, uses either an antisense (AS) approach (Rubenstein et al., 1984; Weintraub et al., 1985), or a triple helix (TH) approach (Dervan, 1992; Helene, 1994; Anthony et al., 1998; Kasprzak et al., 2006). The anti-IGF-I, AS and TH approaches, produce cell immunogenicity (MHC-1 and B7) accompanied by apoptosis (Ly et al., 2000), both phenomena mediated by a signal transduction pathway of IGF-I (IGF-I receptor/PI3K/PKC / RAS/ MAPK/Bcl2/GSK) (Hakuno and Takahashi, 2018). The cell anti-IGF-I vaccines induce *in vivo* an immune anti-tumour response mediated by T CD8 (Lafarge –Frayssinet et al., 1997; Laidlaw et al., 2016).

To increase the immunogenicity of cancer gene therapy (the *sine qua non* condition for anti-tumor vaccine efficiency), plant phytochemicals inducing the transduction pathway signal complementary to that of IGF-I, were applied (Deyhim et al, 2005; Wu et al., 2007; Hernandez-Ramos et al., 2019; Salehi et al., 2019; Castillo-Maldonado et al., 2020). The cancer gene biotherapy (anti–gene technology/phytochemicals) sheds light on its clinical effectiveness in cancer treatment, particularly of brain tumor *glioblastoma multiforme* (GBM) (Perez-Larraya and Delattre, 2014; Cimino and Holland, 2019; Sampson et al., 2020), if compared to different cancer therapy strategies targeting oncoproteins/growth factors (alpha-fetoprotein, IGF-I, VEGF, EGF, TGF- β, and their receptors), as well as related signal transduction elements (i.e. Beckener et al., 2005; Moro-Sibilot et al., 2010; Lo, 2010; Hau et al., 2011; Yang et al., 2011; Schlingesiepen et al, 2011; Costa et al., 2013; Zhang et al, 2013; Kalman et al., 2013; Zhao et al., 2015; Qu et al., 2017; An et al., 2017; Sun et al., 2018; Rinaldi and Wood, 2018; Maldonado et al., 2018; Trojan. 2019).

Clinical cancer gene therapies

When applying the anti IGF-I cancer gene therapy (antisense anti IGF-I) in hepatocarcinoma patients (Second Military Hospital of Shanghai), survival reached five years; and two years following the treatment, there was a marked reduction in tumor recurrence – from 20 to 86 % (Liu et al., 2000a and 2000b). In the case of glioblastoma therapy (Hospitals of CWRU University, Cleveland, and Nicolaus Copernic University, Torun) survival reached 18 months (Trojan et al., 2007 and 2020). Strategies for GBM treatment using IGF-I-Receptor AS (IGF-I-R AS) instead of IGF-I AS were discontinued, due to weak immunogenicity (Andrews et al., 2001; Pollak et al, 2004). In parallel to IGF-I AS and IGF-I-R AS therapies, the TGF- β AS therapy, using either the TGF-β AS expression vector or the direct application of TGF-β oligodeoxynucleotides, was applied (Hau et al., 2007, Schlingensiepen et al. 2011). These promising clinical results could be much more important if the stimulation of an immune anti–tumor response (similar to the one achieved in the IGF-I-R AS therapy) was more efficient. The clinical results when treating different cancers and especially GBM using anti-TGF-β as well as anti-EGFR strategies, were close to those

obtained with IGF-I AS therapy, our first strategy of cancer gene therapy (Sampson et al., 2009; Schlingensiepen et al., 2011).

Following the establishment of cancer gene therapy, different theses related to this domain were presented in European universities and more recently, South American universities (Colombia) (http://www.theses.fr/, 2018; Castillo, 2016)

CHAPTER 1

COMMON GOAL IN CANCEROLOGY / NEUROLOGY: INCURABLE BRAIN TUMORS

Malformations and Tumors

Congenital malformations continue to be a major cause of infant death in the Western world, and among the most common are malformations involving the central nervous system (CNS). Primary malformations are defined as morphological defects resulting from an intrinsically abnormal developmental process, as opposed to a disruption, where breakdown occurred in a previously normal developing tissue. Circumstantial clinical evidence and experimental manipulations indicate that the same anomaly can be produced by several different causes, both genetic and environmental [1,2]. The neurohistogenesis of malformations is closely related to embryonal tumors. They can be merged under the term primitive neuroectodermal tumors as follows [1-4]: Central neuroblastoma, Ependymoblastoma, Medulloblastoma, Supratentorial PNETs, Medulloepithelioma and atypical teratoid/rhabdoid tumor.

Malformations - Etiology

Over 50 per cent of cases have unknown etiology, and genetic factors may contribute to about a third of all malformations. Teratogens are also responsible for malformations in many births [1,2].

***IGF-I*:** The increase of intracytoplasmic IGF-1 is often associated with CNS malformations such as macrocephaly or tumors [5,6]. IGF-I in parallel to used markers such as alfa-fetoprotein has become useful in the diagnostics of neonatal malformations and tumoral diseases [5-8].

Cytomegalovirus and other organisms: Cytomegalovirus can lead to hydrocephalus with widespread evidence of inflammation and periventricular calcifications. Herpes simplex infection was associated with chorioretinitis, microcephaly, hydranencephaly and microphthalmia. Toxoplasmosis causes a necrotizing meningoencephalitis which results in hydrocephalus and widespread calcification.

Maternal infections: The malignant effects of rubella have been amply confirmed. The most common abnormalities were microcephaly, hydrocephalus, absent corpus callosum and anomalies of the cerebral cortex.

Genetic factors: Malformations are being reported in association with an increasing number of inborn metabolism errors.

Cytogenetic abnormalities: The most important anomalies are the trisomies. There is a poverty of secondary sulci. A conspicuous smallness of the cerebellum as a whole is also a common feature. Morphometry studies have shown a marked reduction in neuron numbers, up to 50 per cent compared with controls.

Fragile X syndrome: The second most frequent chromosomal disorder associated with developmental disability. Dendritic spine abnormalities associated with synaptic immaturity have been found.

Irradiation: Deep x-ray therapy to the pelvis during the first four months of pregnancy has produced many cases of microcephaly. Various anomalies of the eyes, cerebral hemispheres and thalamus were present.

Malformations - Pathology

The described lesions exemplify the uncertain dividing line between true and “primary” malformations/defects which result from an intrinsically abnormal developmental process, disruption, or “secondary” malformation.

Neuronal migration defects - Cerebral cortical dysplasia: Trojan *et al,* using a new AFP marker, have demonstrated that the peak of the nervous tissue differentiation in the developing rat CNS is situated 18-19 days post coitum [9,10]. Neuroblasts are generated in the ventricular zone and migrate

in successive waves to the expanding cortical zone guided by radial glial processes which stretch from the vertical to the cortical surface. In man there are two main waves of neuroblast migration and therefore manifestations either of arrested or impaired migration or of faulty postmigrational organization.

Neural tube defects – ***dysraphism's disorders,*** and ***Craniorachischisis***: Two theories have been proposed - reopening of a previously closed neural tube or failure of the neural walls to close. Classical descriptions concern abnormal neural cleft formations, called neuroschisis, and fluid-filled blebs raising the ectoderm and subsequently rupturing. Most of the information on dysraphic disorders has been derived from experimental studies. Craniorachischisis is the most severe form of dysraphism. The brain and spinal cord are exposed to the surrounding amniotic fluid, resulting in necrosis, degeneration or angioma-like formations.

Early prenatal detection of neural tube defects is possible by the estimation of alpha-fetoprotein (AFP) in amniotic fluid. This protein [11] synthesized by fetal liver and excreted in the urine, increases in the amniotic fluid in various malformations, mainly those that involve the neural tube. AFP has been determined in maternal serum. The test is likely to be replaced by measurement of acetylcholinesterase in the amniotic fluid. This enzyme passes into the amniotic fluid only in cases with defects of the neural tube. In our studies of neoplastic development of the CNS using the model of mouse teratocarcinoma, we have found the following successive cytoplasmic appearance of the oncoproteins: IGF-I, AChE, alpha-fetoprotein [Trojan et al., non-published data].

Spina bifida, spinal meningocele, myelomeningocele *and* ***myelocele***: Spina bifida occulta is the mildest lesion with no external cystic swelling; spina bifida cystica includes meningocele and myelomeningocele; rachischisis is an extensive open defect usually in association with anencephaly.

Exencephaly and **anencephaly**: Both lesions are different stages of the same developmental anomaly. Exencephaly has been rarely described in

human fetal pathology probably because of the rapid necrosis of the brain tissue exposed to amniotic fluid, leading to anencephaly.

Holoprosencephaly, ***Agenesis of the corpus callosum*** and ***Anomalies of the septum pellucidum:*** Disorder of forebrain induction - Holoprosencephaly may represent an extreme hypoplasia of the neocortex. Alobar holoprosencephaly, the severest form, denotes a very small brain with a monoventricular forebrain, a "holosphere" undivided into hemispheres or lobes. Well recognized etiological factors include chromosomal anomalies - the most frequent is trisomy [12-14]. There is a notable risk of holoprosencephaly in the offspring of maternal diabetics yet maternal toxoplasmosis, syphilis, rubella and fetal alcohol syndrome have also been implicated. Agenesis of the corpus callosum may form part of a holoprosencephaly, or the callosum may be totally or partially absent or hypoplastic in an otherwise normal brain. The absence of the septum may be the only abnormality in a brain or it may be associated with holoprosencephaly or with callosal agenesis.

Agyria, Pachygyria *and* ***Polymicrogyria:*** These terms denote an abnormal surface configuration of the brain. Agyria implies absence of gyri, and pachygyria implies reduced numbers of broadened gyri.

The term of polymicrogyria denotes multiple miniature malformed convolutions; it should not be confused with polygyria (excessive superficial sulcation). Widespread involvement of both hemispheres usually accompanies microcephaly and profound psychomotor retardation. In polymicrogyria the cortical ribbon is abnormally thin and laminated, excessively folded and shows fusion of the adjacent gyri. The commonest arrangement is a two layered cortex-molecular layer and neuronal layer forming a thin undulating ribbon. Many cases of polymicrogyria have been associated with intrauterine infection, notably cytomegalovirus, but also toxoplasmosis, syphilis and varicella-zoster.

Microcephaly: Microcephaly does not refer to a particular etiology; the fetus is particularly vulnerable to rubella virus, cytomegalovirus and the herpes virus. Herpes virus contamination usually occurs during birth, although microcephaly may develop after birth. The offspring of women

with phenylketonuria are often microcephalic. Microcephaly is also a feature of the fetal alcohol syndrome.

Megalocephaly: defined as a brain weight 2.5 standard deviations above the mean for the age and sex. Primary megalencephaly may be linked to endocrine disorders, or it may be familial. Secondary megalencephaly is due to genetic disorders, such as the sphingolipidoses.

Hydranencephaly: Most of the cerebral mantle is replaced by a thin and partly translucent membrane without surface convolutional pattern. Survival is rarely more than a few weeks [2,6].

Tumors - Pathology

The following tumors will be considered [2,4,6,12,15]:

Medulloblastoma: This malignant lesion, an invasive embryonal tumor of the cerebellum, corresponds to WHO Grade IV. As far as its histogenesis is considered, this distinct clinicopathological entity is a derivation from medulloblasts, i.e., undifferentiated, proliferating embryonal cells with the capacity to differentiate into spongioblasts and neuroblasts. The prognostic significance of cellular differentiation is still controversial; the expression of GFAP, glial fibrillary acidic protein, appears to be associated with a poor prognosis; the expression of retinal S-antigen and rhodopsin [1,6] as well as of tyrosine kinase C (TrkC) [1,6] have been found to be associated with a better prognosis.

Medulloepithelioma: This highly malignant neoplasm corresponds histologically to WHO grade IV, and is characterized by pseudostratified neuroepithelium similar to that of the embryonic neural tube, often in association with a spectrum of neuroectodermal differentiation. Its histogenesis is probably a result of abnormal reexpression of genes in early neural tube determinants.

Ependymoblastoma: The highly malignant primitive neuroectodermal tumor corresponds histologically to WHO grade IV, and is characterized by distinctive ependymoblastoma rosettes lying in fields of monotonous

undifferentiated cell. The incompletely differentiated ependymal cells show immature characteristics, such as dense chromatin and mitotic activity.

Central neuroblastoma and ganglioneuroblastoma: Central neuroblastoma, a malignant embryonal neoplasm, corresponds histologically to WHO grade IV, and is composed of primitive cells with a limited capacity for neuronal differentiation. The ganglioneuroblastoma is characterized by its additional ability to form ganglion cell.

Glioblastoma (see the last paragraph)

Experimental Teratology

Malformations

Different theories were proposed as follows [1,2,5,15]:

Infection: The rat viruses and the feline panleucopenia virus can cause cerebellar hypoplasia in their respective species

Tissue antibodies: Malformations can be induced in fetal rats by injections of heterologous antibodies against the rat yolk sac. The most common malformation caused by the apparent yolk sac dysfunction is a non-obstructive hydrocephalus.

Vitamin deficiencies and Hypervitaminosis A: Exencephaly and eye anomalies can be caused by pantothenic acid deficiency. Other malformations are induced by a combined vitamin B12 and folic acid deficiency producing hydrocephalus. Exencephaly was also produced by hypervitaminosis A, with a high degree of consistency in rats.

Hypoxia: Hypoxia in chick embryos has been shown to lead to anencephaly, cyclopia and forking of the aqueduct. The neuroblast is much more resistant to anoxia than is the mature neuron.

Radiation: It is probable that the teratogenic activity of x-rays depends on the balance of destruction and repair processes. The exact mechanism is still controversial.

Tumors

The model that "reproduces" the development of the normal CNS, mouse teratocarcinoma derived of PCC3 and PCC4 embryonal carcinoma cell lines, was performed to understand the details of the CNS neoplastic development morphology; after examining almost 12,000 photos of histologic and electron microscopy sections (Figures 1-2), we established the different stages of abnormal nervous tissue histogenesis [13,14,16,17]. The histogenesis of the neuroectoblast starts as a structured embryonal carcinoma, followed by vesicles which mimic a developing neural tube. These conspicuous structures eventually dislocate into solid diffuse patterns which retrace the cytogenesis of the differentiating layer. We have demonstrated that neuroblastic structures are composed of different stages:

1. Undifferentiated carcino-embryonic structures
2. Medulloepithelial structures (composed of a mixture of ectoblastic and neuroectoblastic components).
3. Neuroblastic structures
4. Neuroepithelial structures

The observed structures were trabecular, tubular and vesicular, and also included rosettes and various components. The basic patterns were either diffuse growths of neuroblastic stem cells or a mixture of neuronal and glial precursors with a marginal zone which finally transformed into a neuropile. The final differentiation was the encephaloid tissue. These results were confirmed studying the localization of oncoproteins as alpha-fetoprotein (AFP), serum-albumin (SA) and IGF-I, directly included in normal and neoplastic histogenesis, the last using the teratocarcinoma model [8,16,18-20].

The model of mouse teratocarcinoma containing neuroglial structures [1,12,16,18] described in the first studies of Stevens and then by his followers during almost forty years of investigations [21-30], should be useful for understanding human embryonic tumors of the CNS which are able to differentiate into both neuronal and glial lineages [2,4,12,13,15], The mouse teratocarcinoma, containing neuronal and glial cells, will also be

useful for studies of future gene therapies targeting CNS tumors, and more specifically, glioblastoma [31-34].

Experimental tumor therapy

Neoplastic brain related antigens: The processes involve the expression of oncoproteins (such as alpha-fetoprotein and serum albumin) [18], growth factors and their respective receptors (i.e., IGF-I, EGF, FGF, VEGF, and TGF-alpha and -beta) [35]. Their downstream proteins and glycogen signaling elements including glycogen synthase (GS), are also involved [7,36]. IGF-I and GS reappear during the development of the brain tumor, *glioblastoma multiforme*, GBM [7,37] (Figure 3).

Glioblastoma: Using radiotherapy and chemotherapy, the mortality of the most aggressive malignant brain tumor – GBM, remains close to 100% and the median survival, using conventional therapy, is 10-14 months. Current pharmacology increases the survival to 15 months yet rarely to 18 [38]. The etiology of glioblastoma is still being investigated using molecular biology techniques [39]. New or proposed therapies are based either on immune treatment or on immuno-gene strategies [40,41]

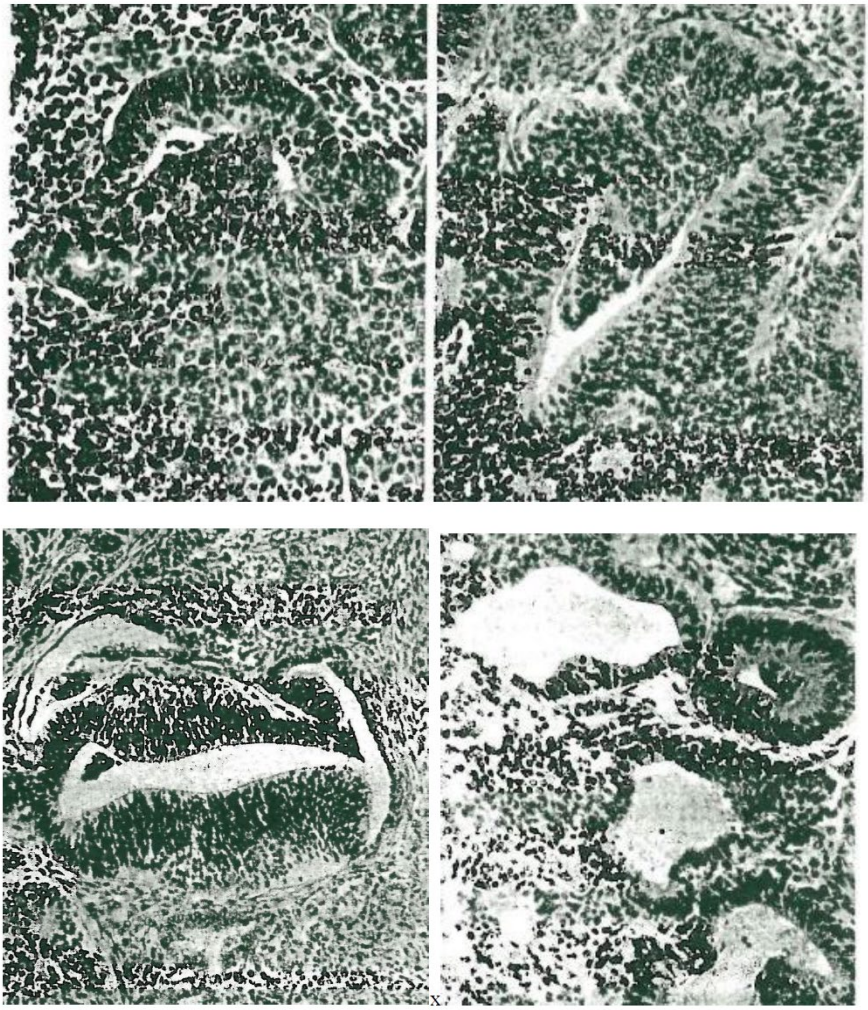

Figure 1. Neoplastic histogenesis of nervous system. Model of mouse teratocarcinoma, containing neuroglial tissue, derivative of PCC4 embryonal carcinoma cell line. Formation of vesicle: (left up) Beginning of the border formation; (right up) Structured vesicle (resembling to neural tube). HE staining (x150); (left down) Well differentiated vesicle - similar to normal neural tube, bordered by neuroepithelium. HE staining (x80); {right down) Proliferative aspect showing formation of neuroepithelial rosettes (cystes). HE staining (x120).

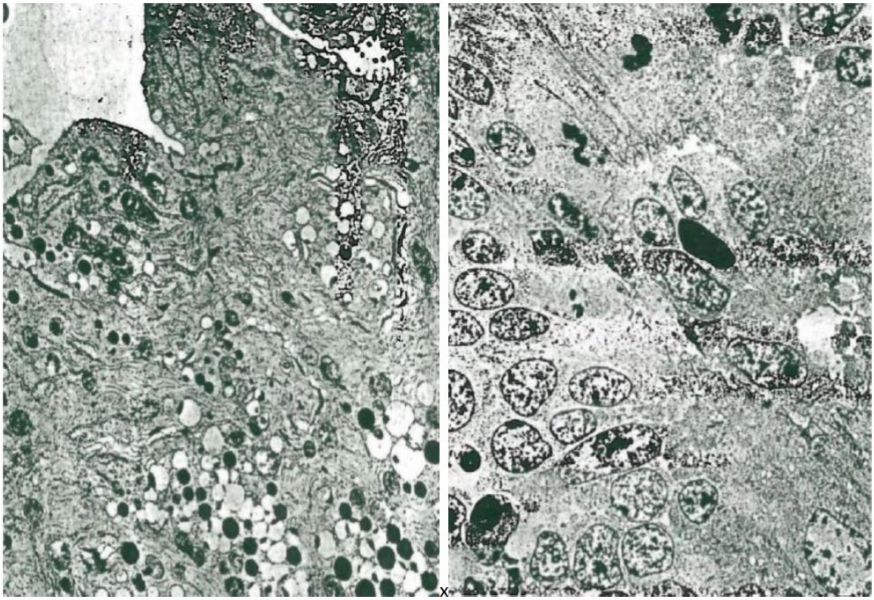

Figure 2. Neoplastic histogenesis of nervous system. Model of mouse teratocarcinoma, containing neuroglial tissue, derivatived of PCC4 embryonal carcinoma cell line. (Left) Electron microscopy of well differentiated vesicule - similar to normal neural tube, shown in fig. 2 - left down) (x3000); (right) Electron microscopy of neuroepithelial rosette, shown in Fig. 2 – right down. Note the terminal bars delimiting the cavity; neuroepithelial cells in the center, and also the cells in mitotic cycle (upper part) (x1000).

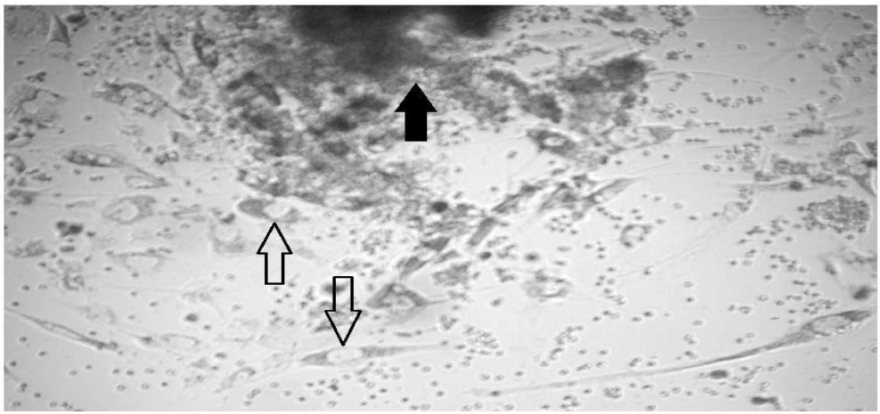

Figure 3. One week of culture established from human glioblastoma biopsy. Note the cells (empty arrows) proliferating from compact tissue of biopsy (black arrow). The tissue and cells are stained for IGF-1 using anti IGF-1 antibodies applied in immunoperoxidase technique: note dark cytoplasm (x200).

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CHAPTER 2

THERAPY OF BRAIN TUMORS TARGETING ONCOPROTEINS

Introduction

In 1963, Abelev and his coworkers discovered the reappearance of the fetal globulin, alpha-fetoprotein (AFP), in the sera of mice bearing primary hepatomas [1]. AFP is an oncoprotein present in different neoplastic tissues [1] and more specifically in the nervous system. AFP is also synthetized during a normal development coming from the visceral endoderm cells around the embryonic region of the egg cylinder up to the yolk sac and the fetal liver [2-4]. A number of fetal structures that do not synthetize AFP have nevertheless a high affinity for this protein. In 1978/79, Benno and Williams [5] and Trojan and Uriel [6] drew attention to the presence of AFP in the developing rat brain, and demonstrated the presence of the protein in several embryonic and fetal tissues in the developing rat, mouse, and monkey as well as in the chicken embryo [2]. The presence of AFP seems to be related to the stage of tissue differentiation. AFP is absent from either undifferentiated or fully differentiated cells [2].

The localization of AFP and its mRNAs was investigated in primitive neuroectoblastic structures of rat and mouse embryos, and of the teratocarcinomas presenting similar neoplastic structures. AFP-mRNA was observed only in differentiating structures [7]. In teratocarcinoma-bearing mice injected intraperitoneally with J-125 radiolabeled AFP, and comparatively with another protein – serum albumin, SA, significant accumulations of both proteins were demonstrated in the tumors (SA being about three-fold higher than that of AFP after normalization to quantity of uptake in liver). In the case of another tumor studied - neuroblastoma, presenting only neuroblastic components (different from teratocarcinoma containing both neuroectoblastic and neuroblastic elements), the accumulation of radiolabeled

SA and AFP showed a 1:1 relationship; the last observations were useful for differential diagnosis of tumors [7].

In 1992, Trojan and his co-workers, using teratocarcinoma model, demonstrated that another oncoprotein, an insulin like-growth factor, IGF-1 [8-12], is present in glioma cells but absent in neuroblastoma cells, the last expressing IGF-II [13,14]. These observations allowed to study separately glial and neural tumors [8-11,15-20] using IGF-I and IGF-II as the oncoprotein markers.

AFP and IGF-II, fetal onconeural antigens are expressed by both neuroblastoma and fetal neural cells [13,21,22-24]. Several *in vitro* and *in vivo* studies support the conclusion that the presence of AFP, and perhaps of SA, results from protein uptake as opposed to *in situ* synthesis [25-27]. AFP, common in many tissues during ontogenesis may reappear in neoplastic cells [28]. We have tested two models: the C-1300 neuroblastoma cell line, and derivative solid tumor of neuroblastoma, and comparatively PCC4 embryonal carcinoma cell line, and derivative solid tumor of teratocarcinoma [14,29] for their potential to internalize AFP and SA, both *in vitro* and *in vivo* for diagnosis purpose.

Material and methods

Protein preparations: Mouse AFP was isolated from a PBS-homogenate of 17-day old mouse fetuses as previously described [30]. The rat serum albumin was from Nordic (the Netherlands) and ovalbumin from Sigma (USA). The mouse AFP, rat SA and OA were conjugated to fluorescein isothiocyanate (FITC) following the technique described previously [28]. A fluorescein-lysine conjugate (FITC-lys) was prepared by coupling 1 ml of 0.2 M L-lysine with 0.4 mg of FITC and used as a control. The nuclei were counterstained with p-phenylenediamine [31]. The proteins (20 pg) were labelled with 1 mCi of either ^{125}J or ^{131}J by the chloramine T method [32]. Specific activities ranged from 2 to 15 pCi $pg^{-}{}'$ of protein.

Cell culture: The C-1300 and PCC4 cell lines were maintained in Eagle's medium (MEM enriched with non-essential amino acids; Seromed, West Germany) containing 10% fetal calf serum (FCS) inactivated at 56°C for 30

min, penicillin and streptomycin (100 U/100 pg $m1^{-1}$). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures were trypsinized before attaining confluency and replated in plastic tissue culture dishes (35 mm; Falcon) at a density of 7 x 10^4 cells per dish in 1.5 ml of growth medium and cultured for 48 h. After incubation for 48 h, the medium was removed and the plates incubated for 1 h in serum-free medium to deplete cells of endogenous bovine AFP. Then, 1 ml per plate of fresh medium containing 100 pg of fluorescein conjugates of mouse AFP (FITC-AFP), rat serum albumin (FITC-SA) or ovalbumin (FITC-OA) was added. The cells were incubated in this medium for 4 h at 37°C. They were fixed in acid ethanol (ethanol 70% in PBS, acetic acid 1%) at room temperature, mounted in 30% glycerol phosphate buffer 0.05 M pH 7.6 and examined with a microscope equipped with fluorescein optics and epi-illumination. Alternatively, cultures were processed for immunocytochemical labelling.

Immunocytochemistry: Vectastain ABC kit was purchased from Vector Lab., USA. The antibodies, anti-mouse AFP were previously described, and antisera to rat SA and to ovalbumin (OA) were obtained from Nordic (the Netherlands). Experimental and control dishes were treated with either rabbit anti-AFP, anti-SA or anti-OA (1/200 v/v) for 45 min at room temperature and then processed by the ABC immunoperoxidase technique [6,33,34].

Tumors: Male 129 SV mice were inoculated s.c. in the scapular region with 0.5 ml of a suspension containing 10^6 viable tumor cells. Mice that were injected with neuroblastoma cells as well as PCC4 embryonal carcinoma cells developed neuroblastoma and teratocarcinoma tumors, respectively, within 15-20 days after injection. When the tumors measured 9 mm in diameter, 3 pg each of ^{125}J-SA or ^{125}J-AFP or ^{125}J 0A were injected i.p. Three to four days after injection, the mice were anaesthetized with ether and perfused at 37°C through the left ventricle with 50-60 ml of 10 mM K-phosphate, 150 mM NaC1 and 1 mM EDTA buffer, pH 7.4. Perfusion was carried out with a peristaltic pump after dissection of the jugular vein before perfusion was started. The tumors and other organs were rapidly dissected, washed in PBS, weighed and measured for radioactivity in a y-counter. Fragments of tissues were fixed for 3 days in cold ethanol/acetic acid (98/2; v/v) or Bouin's fixative, embedded in paraffin and sectioned at 3-4 pm for a

hematoxylin-eosin observation or autoradiography. Blood, liver and tumor samples were homogenized with PBS (1/2; w/v) and precipitated with trichloroacetic acid (TCA, 10% final concentration). Concentration values in nCi g^{-1} of tissue were estimated, and tumor to liver ratios were calculated by dividing nCi g^{-1} values in the tumor by those in the liver. For a comparison of ^{125}J-AFP, ^{125}J-SA and ^{125}J-0A distribution in mice specificity indices were obtained by dividing individual nCi g^{-1} values for AFP or SA by those obtained for OA. To test the possibility of tumor localization of radiolabeled AFP by external photo scanning, mice were injected i.p withp ^{131}J-AFP (20-40 pCi, i.e. 0.5-1.0µg AFP) or with ^{131}J-0A (40 pCi; 4 pg-OA). Images were obtained 3-6 days after injection with a standard y-camera linked to a computer with data display. During photo scanning, mice were anaesthetized with sodium pentobarbital.

Results

Morphology: The majority of neuroblastoma cells in culture had round or ovoid bodies of 15-30 pm in diameter, with a single nucleus of 12-20 pm (Figure 1). Tumors of neuroblastoma (Figure 1) consisted of masses of round cells separated by small quantities of intercellular substance. The undifferentiated tumor cell typically displayed a high nuclear: cytoplasmic ratio. The PCC4 cells and derivative tumors were described earlier [29,35]. After a 4 h incubation with FITC-conjugates of AFP, SA or OA, specific fluorescence for AFP and SA could be observed in a large number of cells: the fluorescence appeared to be intracytoplasmic and often extended into the pseudo neuronal processes. No positive labelling could be observed for the FITC conjugated OA. Control cultures containing the FITC-lysine also appeared negative. AFP positive cells revealed with antibodies to AFP are shown in Figure l. AFP staining was indistinguishably positive in the whole population. Cell nuclei were systematically AFP negative (Figure 1). The same localization was observed in cells incubated with SA and revealed with anti-SA antibodies. No significant staining was revealed in cultures treated with OA.

Autoradiographs: The tissue distribution of ^{125}J-AFP after injection into tumor bearing animals is shown in Table 1. Radioactivity concentration (mean value+ s.e.) in the tumor was the highest among all solid tissues