RADIOBIOLOGICAL STUDIES OF GLIAL STEM CELLS

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The cover illustration shows 'Portrait of a Doctor as a Young Man' by W.J. van der Maazen.

RADIOBIOLOGICAL STUDIES OF GLIAL STEM CELLS

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Medische Wetenschappen.

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never judge a book by the cover

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CHAPTER 1

RADIATION INJURY TO THE HUMAN CENTRAL NERVOUS SYSTEM

Introduction.

Radiotherapy is still the treatment of choice for malignant and some benign tumors of the Central Nervous System (CNS). X-rays, however, can not be delivered in such a way that the normal tissue is spared and the severity of the damage to the CNS limits the use of ionizing radiation in the treatment of these diseases. The same limitation applies to the treatment of malignant tumors in the vicinity of the CNS (head and neck tumors, lung tumors or paravertebral tumors). The probability of developing side effects and their severity depends on the total dose, fractionation parameters and the time between the treatment and onset of symptoms. Because there is a clear relationship between the prognosis and the time of onset, the radiation-induced syndromes are divided according to the time sequence of occurrence. The syndromes that develop after irradiation of the human brain and spinal cord will be discussed on the basis of some review articles (Sheline 1980, Leibel 1987, Leibel 1991).

Before discussing the different syndromes it should be emphasized that it is sometimes very difficult to discriminate between treatment-related and disease-related symptoms. Especially in the treatment of primary malignant CNS tumors the treatment results are poor and recurrences are likely to develop. The symptoms caused by a recurrent tumor or by white matter necrosis may look similar. Further investigations are necessary to develop methods that can discriminate unambiguously between these different possibilities. Until now the only reliable method to discriminate between recurrence and white matter necrosis has been histological examination. Hopefully, new diagnostic techniques will be developed and refined [e.g. magnetic resonance imaging and spectroscopy, and single-photon emission computed tomography (Schwartz 1992)] that allow the distinction between white matter necrosis and recurrence without surgical intervention.

Acute and Late Reactions.

Acute reactions develop during or immediately after the radiation treatment. If the *brain* is irradiated, non-specific symptoms such as headache, nausea, vomiting, and increased temperature may develop. These symptoms are thought to be caused by radiation-induced edema leading to an increased intracranial pressure. The symptoms are only temporary and disappear completely within several weeks. General symptoms as nausea and vomiting are not seen after

irradiation of the *spinal cord*. Edema, however, may deteriorate the neurological signs caused by a primary spinal cord tumor or an epidural metastasis. Symptoms caused by edema may benefit from corticosteroid therapy.

Early delayed reactions are seen in the period following radiation treatment up to 3 months. The most commonly seen syndrome after irradiation of the *brain* is characterized by somnolence, lethargy, irritability, anorexia, and sometimes an increase of the tumor related symptoms. These symptoms are independent of the disease status. In approximately 50% of the patients no signs of recurrence or progression of the tumor could be demonstrated. There are no evident focal neurological deficits. This syndrome is transient and will generally disappear completely within 6 weeks. Occasionally, however, this syndrome does not resolve and will lead to the death of the patient.

Within 6 months after irradiation of the *spinal cord* a syndrome may develop that is characterized by transient, electrical shock-like paresthesias or numbness radiating from the neck to the extremities. These symptoms can be evoked by neck flexion (= Sign of Lhermitte). Apart from these signs, there are no associated abnormal neurological findings. The syndrome usually disappears completely within several months, but it may persist for years.

A late delayed reaction can be seen from approximately 6 months after the completion of radiotherapy and the CNS stays prone to the development of late side effects for years. The onset of symptoms can be acute but most frequently will take several weeks. The clinical picture is highly variable after irradiation of the *brain* because it depends on the anatomical localization of the induced damage. If the damage is limited to a relatively well circumscribed region, the symptoms may be minimal or merely caused by the associated edema. The patient might benefit from a surgical resection. If the damage is more extensive the patient dies from gross neurological deficits (paralysis, convulsions, coma). The syndrome is irreversible.

Another irreversible reaction of the *brain* after radiotherapy has been recognized recently by computerized tomography (Asai 1989). The syndrome is characterized by brain atrophy with mental and neurological deterioration (disturbances in mental status and consciousness, akinesia, tremor-like involuntary movements). Although this syndrome was seen relatively short after the radiation treatment (within a few months), it is categorized under "late delayed reactions" because of its irreversibility and progressiveness.

After irradiation of the *spinal cord* the neurological symptoms depend on the level of involvement. If there is a gradual development of neurological deficits

paresthesias may be followed by sensory and motor dysfunction, bowel and bladder dysfunction and paraplegia or quadraplegia. If the level of injury is localized high in the cervical spinal cord a respiratory arrest may cause death. A typical onset of late delayed myelopathy is a Brown-Sequard's syndrome, which is characterized by a homolateral motor dysfunction and a contralateral sensory deficit. Occasionally a quick onset of neurological deficits has been observed in laboratory animals and appears to be caused by a sudden vascular accident (bleeding). Such a quick onset of symptoms is rare in man. The motor and sensory deficits may be partial, but they may progress to total motor inability and sensory dysfunction. The symptoms are irreversible.

Modifying factors.

The effect of radiotherapy can be modified by the use of intravenously (i.v.) or intrathecally (i.t.) administered drugs (DeAngelis 1991). The toxicity of systemically administered drugs depends on their ability to cross the blood brain barrier. Drugs that have shown to increase the toxicity of cranial irradiation are: methotrexate, nitrosoureas, and cytosine arabinoside. Best documented is the increased neurotoxicity seen after prophylactic cranial irradiation combined with i.v. or i.t. methotrexate in children treated for acute lymphocytic leukemia (ALL). Two specific syndromes can be distinguished in these children: necrotizing leukoencephalopathy and mineralizing microangiopathy. These syndromes develop after 4-6 months and should be considered as late delayed reactions.

Necrotizing leukoencephalopathy is characterized by dementia, ataxia and paralysis. This syndrome can also occur after chemotherapy alone, but the incidence is higher (2-15%) after combination therapy. Some of the patients that are affected by this syndrome will recover although some neurological impairment is expected to be permanent. In other patients the symptoms are progressive and lead to death. Pathologically leukoencephalopathy is restricted to the white matter and is characterized by multiple areas of demyelination and necrosis.

Mineralizing microangiopathy has relatively mild neurological signs which disappear completely. The signs can be headache, focal seizures, behavior disorders and perceptual motor disability. It affects the gray matter of the CNS and a deposition of calcium is seen in the wall of small blood vessels resulting in occlusion of these vessels. Dystrophic calcification of the basal ganglia and subcortical zones can be visualized on CT-scans.

Leukoencephalopathy and leukomyelopathy can also been seen after a combined treatment of cranial irradiation with nitrosoureas or cranial irradiation combined with cytosine arabinoside. Combined therapy seems to affect mainly the white matter without signs of increased damage to the vasculature. The probability

to develop CNS injury increases with higher doses of both radiation and chemotherapy.

In addition to chemotherapy other factors have been addressed as potential modifiers of the radiation response of the CNS: the age of the patient; vascular disease (associated with e.g., diabetes mellitus, Cushing's disease or acromegaly); extent of the surgical procedure; and infection of the CNS. There is a clear effect of age on CNS tolerance to radiation and will be discussed later (chapter 2). The other factors are difficult to examine and their influence remains unclear.

Some general remarks.

It is difficult to estimate the true incidence of certain syndromes on the basis of the literature. Most authors report patients with side effects without mentioning the total number of patients that did receive an identical treatment. Furthermore, the survival of patients with malignant CNS tumors is limited and patients may die before the expression of radiation injury, which would have developed if the patient had lived longer.

On the other hand, improvements in treatment strategies have led to prolonged survival of specific patient categories that received irradiation of the CNS. For example: children treated for ALL (chemotherapy and prophylactic CNS irradiation), children treated for medulloblastomas (high dose radiation therapy, with or without chemotherapy), patients with small cell lung cancer (chemotherapy and prophylactic brain irradiation). In these patients, however, the combination of radiotherapy with drugs, that are (potentially) neurotoxic, complicates the evaluation of radiation-induced damage. Careful evaluation and follow-up of all patients irradiated on the CNS should provide better information about the adverse effects of radiation. Not only the effect on the tumor but also changes in the normal CNS should be determined. New imaging techniques (MRI, MRS, SPECT) might help to discriminate between recurrence, necrosis and physiological changes in normal brain and spinal cord. Intelligence and neuropsycological tests can give information about changes in mood, feeling and intelligence associated with the tumor or treatment. All these aspects become more important if the treatment results become better and consequently patients survive longer.

Pathogenesis of Radiation-Induced Syndromes.

Theories about the pathogenesis of radiation-induced syndromes are mainly based on histological examinations of irradiated human and animal CNS tissue. Several cellular mechanisms have been proposed to explain the different

pathological findings and the importance of these factors in the development of radiation-induced syndromes will be discussed on the basis of a tentative model of radiation injury that relates pathological entities to cellular dysfunction or depletion (Figure 1.1; adapted from Van der Kogel 1991).

The differences in the clinical syndromes that develop after irradiation of the brain and spinal cord are attributed to differences in tissue organization and not to fundamentally different responses to irradiation. The underlying cellular mechanisms, that determine the expression of radiation damage, are thought to be the same for all regions of the CNS.

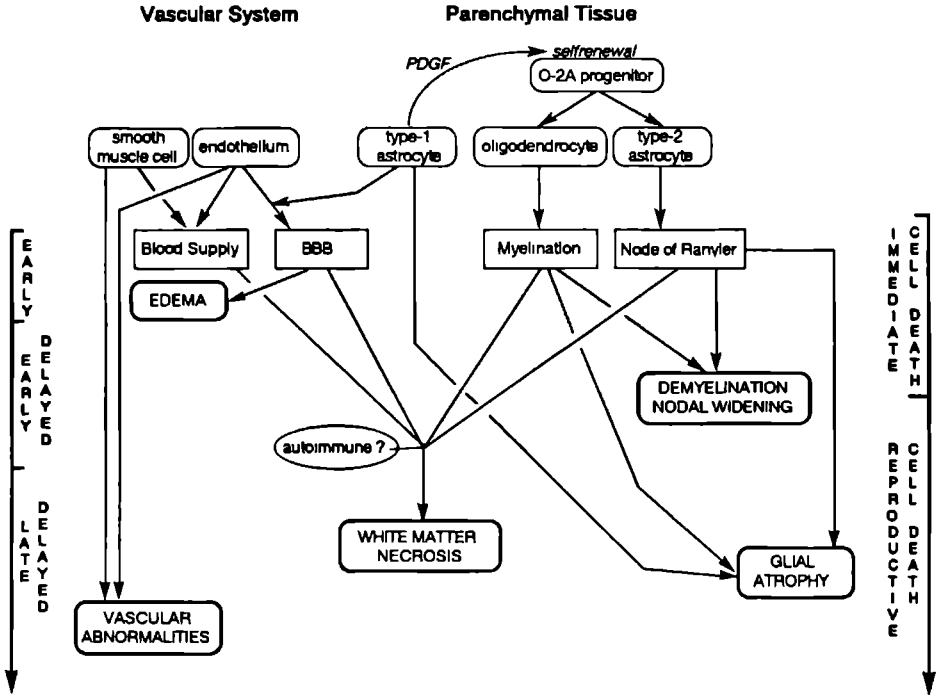

Figure 1.1 Tentative model to explain the different radiation-induced syndromes in the CNS.

Acute reaction.

The blood brain barrier (BBB) is a physical barrier located at the level of endothelial cells of almost all intracerebral vessels. It selectively regulates the passage of molecules across the capillaries of the brain and spinal cord. The barrier is characterized by tight junctions between endothelial cells and appears

to be induced by type-1 astrocytes (see chapter 3). Radiation damage to this barrier has been noticed within hours after very high single doses of X-rays (960 Gy) but also after irradiation of the rat brain with relatively moderate single doses (8 Gy) (Trnovec 1990). The subsequent development of edema is thought to be responsible for the acute symptoms seen after irradiation of the human CNS. Improvement of clinical symptoms after administration of corticosteroids supports this theory, although it is realized that corticosteroids may have many other effects. There seems to be a relation between the fraction size and the probability to develop acute symptoms in the clinical situation. A daily fraction size of 2.0 Gy, with a total dose not exceeding 70.0 Gy, is thought to be without severe acute complications. Acute deaths, however, have been reported after treatment of cerebral metastases with high fraction doses (2 x 7.5 Gy or 1 x 10 Gy)(Sheline 1987).

Acute reactions after irradiation of the human brain have also been correlated with the single dose to the vomiting center (area postrema) in patients treated with stereotactic radiosurgery (Loeffler 1990). A median dose of 6.18 Gy (range 2.75-12.57 Gy) delivered to the area postrema caused nausea and vomiting in this patient group.

Early delayed reaction.

The morphological changes that emerge after irradiation of the rat spinal cord have been studied by means of single fiber analysis (Mastaglia 1976). Two weeks after exposure of the cord with doses ranging from 5.0 to 60.0 Gy, degenerative changes were found which consisted of breakdown of paranodal myelin and widening of the nodes of Ranvier. Three months after irradiation thinly myelinated fibres appeared as a sign of remyelination. The authors suggested that the early delayed syndrome occurring after irradiation of the human CNS is probably due to these changes in myelination. The observed remyelination at the time that the clinical symptoms disappear fits into the proposed model of pathogenesis. In addition to the changes in the myelination of axons, Wallerian-type degeneration of fibres of all calibers were detected in the spinal white matter (Mastaglia 1976). The relevance of these findings is not clear but the authors assume that the degeneration of fibres is a forerunner of white matter necrosis.

Myelination of axons and formation of nodes of Ranvier is the task of specialized cells in the CNS generated by O-2A progenitor cells: oligodendrocytes and type-2 astrocytes (see chapter 3). Radiation damage to either of both these cell types or their precursors could lead to the development of paranodal widening and demyelination. The relatively short period between irradiation and the observed morphological changes favors the hypothesis that the

functional glial cell types are directly damaged by radiation. The observed remyelination of axons by thin myelin sheaths is likely to have occurred by newly formed oligodendrocytes.

Late delayed reaction.

The late delayed reactions can be divided in several different entities. Before the different forms are discussed it should be clear that the terminology, used by different authors is not always consistent. On the basis of dose response studies Van der Kogel (1979) divided the late complications occurring after irradiation of the rat spinal cord in early delayed and late delayed. The early delayed reaction is associated with white matter necrosis and the late delayed reaction with vascular abnormalities. This does not correspond with the terminology discussed above.

After irradiation of the rat spinal cord two different pathological entities could be discriminated (Van der Kogel 1979). The first occurred after a latent period of approximately 4-6 months; was mainly restricted to the white matter; and was characterized by demyelination, followed by loss of axons, focal necrosis, and ultimately liquefactive necrosis. The clinical symptoms associated with this syndrome developed within several days to weeks. If this syndrome did not develop, the spinal cord remained susceptible to the later development of vascular changes in the gray as well as the white matter. These vascular changes consisted of hyaline thickening of the vessel walls; various degrees of telangiectasia; fibrinoid necrosis; vascular occlusion; focal hemorrhage; and hemorrhagic necrosis. Symptoms developed gradually but sometimes severe neurological symptoms were seen within hours due to the sudden onset of bleeding. Vascular changes in the rat spinal cord occurred later than white matter necrosis and were most of the time seen from 1 year after irradiation.

The histopathology of the late delayed syndromes is not identical in all species. Knowles (1981), for example, did not observe vascular lesions in the spinal cord of guinea pigs, but found instead of these vascular lesions diffuse demyelination associated with characteristic vacuolar spaces. This diffuse demyelination was not associated with white matter necrosis and is in contrast with the rat spinal cord where demyelination is only seen in combination with white matter necrosis. An exception is the demyelination of the CNS seen after combined treatment with radiotherapy and chemotherapy. Ara-C has been shown to reduce the radiation tolerance of the rat spinal cord with a factor of 1.2 - 1.3. There is also clinical evidence that methotrexate (MTX) reduces the tolerance of the CNS, but experimental studies could not confirm this observation and even indicate MTX to be slightly protective. The histology of affected CNS showed

mainly demyelination without extensive signs of necrosis or vascular involvement (Van der Kogel 1991).

Schultheiss et al. (1988) reviewed the literature and analyzed the histopathology of 56 human cases of radiation myelopathy. A close resemblance between the histology of human and rat spinal cord abnormalities was found. White matter necrosis and vascular damage could be distinguished and developed according to the same time schedule: "Early" development of white matter necrosis was followed by "late" appearance of vascular abnormalities. The bimodal distribution of late delayed syndromes led to the hypothesis that two different cascades of cellular events are involved in the development of the two different syndromes (Van der Kogel 1979; Hopewell 1979). Late vascular abnormalities are generally thought to be the result of a defective vascular system caused by the depletion of endothelial cells or smooth muscle cells. The pathological changes leading to white matter necrosis are still not clear, but possible mechanisms will be discussed.

In 1964 Zeman et al. commented on the pathogenesis of white matter necrosis and reviewed some older literature: Scholz (1934) assumed that radiation injury to blood vessels constituted the major pathogenic factor of delayed radionecrosis. His assumption was based on the occurrence of morphological changes in vessel walls and of extravasations in areas of delayed radionecrosis. Bailey and Brunschwig (1938), however, suggested that radionecrosis was due to direct radiogenic cell death of parenchymal cells. A third hypothesis proposed that an immune reaction resulted in delayed radionecrosis (Kindt, 1953; Lampert et al. 1959).

If more recent literature is reviewed it appears that the three mechanisms which might explain white matter necrosis are still supported. The role of autoimmunity in the development of white matter necrosis, however, is thought to be small but it may play a minor part. Tissue damage after irradiation of the CNS may lead to the release of autoantigens (e.g., myelin basic protein), which initiate an autoimmune response by activating T-lymphocytes. The disruption of the BBB plays an important role in this concept, because after irradiation changes in the BBB allow contact between lymphocytes and the autoantigen. Or, alternatively, astrocytes are thought to serve as antigen-presenting cells. After the initiation of the immune response, the disruption of the BBB at the sites of irradiation allows lymphocytes to get access to the CNS, where they are thought to destroy normal structures.

The role of glial cell depletion or vascular abnormalities as the principal causative factor in the development of white matter necrosis is still a matter of discussion. The clear differences of the histological and clinical features between

white matter necrosis and late vascular changes have focussed the attention on the myelinating cells of the CNS. The myelinating cells of the CNS (oligodendrocytes) are constantly renewed (Schultze 1981) and depletion of these cells and their precursors (O-2A progenitor cells; chapter 3) may play a major part in the development of white matter necrosis. Depletion of these glial stem cells will interfere with the normal replacement of aging oligodendrocytes. The slow turnover of oligodendrocytes and the activation of compensatory mechanisms may prevent that the initial cell loss leads to clinically detectable deficits. If, however, the cell loss proceeds, the functional cell number falls below the number necessary for normal tissue function or integrity and neurological problems become evident. Quantitative determination of glial cell numbers after irradiation of different parts of the CNS has provided evidence for this hypothesis (see chapter 4).

The "vascular" theory to explain white matter necrosis is based on the same principle but with respect to endothelial cells. Depletion of endothelial cells, which also have a slow turn-over rate (Schultze 1981), will lead to defective blood vessels and results in an inappropriate blood supply of the parenchymal tissue. Differences in vascular supply and cell density make specific parts of the CNS more susceptible for the development of ischemic necrosis: not the whole CNS is affected homogeneously. Quantitative studies, indeed, confirm the loss of endothelial cells and vessel density before the development of white matter necrosis (Ljubimova 1991).

The problem with all models is that they are unable to explain all the aspects of white matter necrosis. White matter necrosis probably develops along a complex chain of events and the contribution of the different factors is likely to change with differences in treatment variables (total dose, fraction size). Schultheiss et al. (1990) classified late reactions in three different categories: Type-1 lesions: predominantly white matter parenchymal lesions of malacia and demyelination without significant vascular alterations. Type-2 lesions: predominantly vascular lesions of sufficient severity to cause clinically evident damage to the cord. And type-3 lesions: combination of both type-1 and type-2 lesions. In 11 of 13 symptomatic rhesus monkeys irradiated on the cervical spinal cord type-3 lesions were found and this confirms the idea that not the loss of a single target cell population is contributing to white matter necrosis. Calvo et al. (1988) also realized the multi-event mechanism contributing to white matter necrosis and proposed a scoring system based on histological changes in blood vessels, endothelial cells and glial cells. The severity and extent of the radiation-induced changes in blood vessel dilation, blood vessel wall thickening, endothelial cell nuclear enlargement and the hypertrophy of perivascular

astrocytes could be combined and appeared to represent a "unit of tissue injury". Changes in this "unit of tissue injury" increased with time after irradiation and predicted the development of white matter necrosis.

The clear difference and dose dependency between white matter necrosis and late vascular abnormalities were first identified in the rat spinal cord model (Van der Kogel 1979). These findings re-enforced the dispute between the advocates and opponents of the "parenchymal" theory. But close examination of the histopathological changes of irradiated rat spinal cords, revealed in addition to overt white matter necrosis the presence of vascular damage (dilated blood vessels, small hemorrhages and perivascular cuffing, Van der Kogel 1979) (fibrinoid necrosis and trombosis, Delattre et al. 1988). These findings further stress the complexity of the problem.

In addition to the above discussed histological changes, functional changes as a result of irradiation of the CNS have also been reported. In the acute phase after irradiation there is a transient impairment in the BBB. After an initial episode in which the BBB is restored, a second wave of impairment is observed which is clearly progressive and converts to white matter necrosis. Godwin-Austen et al. (1975) emphasized the importance of disruption of the BBB in the development of white matter necrosis after irradiation of the human spinal cord. They found swelling of the spinal cord on myelography and at necropsy. Histologically, hyaline proteinous lakes were found not only in but also outside the irradiated spinal cord regions. Impairment of the BBB was assumed to be the underlying disorder of proteinous edema fluid spreading through the white matter and into the arteriolar walls narrowing these vessels and causing local ischemia and infarction. Impairment of the BBB was confirmed after irradiation of the canine brain by sequential quantitative CT and MR measurements (Fike 1991). Changes related to the BBB breakdown and resultant edema appeared to precede the development of white matter necrosis.

Decreased regional blood flow, which might be related to changes in the BBB, may also contribute to the development of white matter necrosis. Decreased regional blood flow results in ischemia of the afflicted CNS region which may precipitate or even cause white matter necrosis. Changes in regional blood flow were observed after irradiation of the monkey brain (Tanaka 1975) and rabbit brain (Lo 1991).

In addition to white matter necrosis and late vascular abnormalities, decrease in the volume of the CNS has been described as another delayed reaction of the CNS to radiation. This decreased volume seems to be related to specific loss of glial cells without affecting the vasculature and without signs of

necrosis. The syndrome has been observed after irradiation of the canine brain (Fike 1991), rat brain (Keyeux 1987) and human brain (Asai 1989).

CHAPTER 2

THE INFLUENCE OF RADIOBIOLOGICAL FACTORS ON THE TOLERANCE OF THE CENTRAL NERVOUS SYSTEM

Introduction.

The interpretation of the influence of changes in treatment parameters on the incidence of radiation-induced damage is facilitated when a well defined endpoint or response-level is chosen. In animal studies the spinal cord with its unequivocal presentation of late delayed damage is therefore preferred to the brain. Dependent on the anatomical level of irradiated spinal cord, the fore- or hind-limbs become paralyzed. In addition other symptoms, such as incontinence, may develop. Several animal-models (guinea pig, rabbit, monkey) were developed to study the influence of radiobiological variables on the tolerance of the CNS. Most investigations, however, were performed on the rat spinal cord because the rat is a convenient laboratory animal and neurological deficits are easy to score.

Before discussing the radiobiological variables and their influence on the tolerance of the CNS, it should be realized that the term tolerance is a subjective interpretation of radiation effects. A temporary hair loss might be intolerable for one patient whilst another accepts paralysis as a possible side effect of a treatment to obtain analgesia. Therefore, it is more accurate to express the effect of a specific treatment regimen as the (expected) percentage of individuals that will develop a well-defined radiation-induced effect. For example: approximately 5% of the patients that are irradiated on the cervical spinal cord with a total dose of 60.0 Gy (given in daily fractions of 2.0 Gy; 5 times a week) will be susceptible to develop white matter necrosis. This can also be written as ED_5 = 60.0 Gy (ED = effective dose).

Total Dose and Fraction Size.

Of the different syndromes that may develop after irradiation of the CNS, the late delayed reactions are clinically the most relevant. The effect of total dose and fraction size on the probability of inducing white matter necrosis or late vascular abnormalities will be discussed first. The CNS, together with other so-called late-responding tissues, is highly susceptible to the number of fractions in which a specific total dose is given. For example: a single dose of approximately 20 Gy of γ- or X-rays on the adult rat cervical spinal cord will lead to white matter necrosis in 50% of the irradiated animals ($ED_{50} \approx 20$ Gy). To achieve the same level of response a total dose of at least 120 Gy has to be given in daily fractions of 2 Gy.

This increased tolerance for fractionated irradiation is due to the repair of sublethal damage (SLD). Analysis of the fractionation effects according to the linear-quadratic (LQ-) model allows the determination of a value (the α/β-ratio) which is a measure of the fractionation sensitivity of a specific tissue/organ. A low α/β-ratio ($\approx$2.0 Gy) is characteristic for late-responding tissues such as lung, kidney, liver and CNS. Acute-responding tissues, such as bone marrow, skin and intestines, are characterized by high α/β-ratio's of $\approx$10.0 Gy. If the fractionation sensitivity of a specific tissue is known, it becomes possible to calculate equivalent treatment schedules and to estimate the probability of side effects to occur (Also see appendix 1 for mathematical models of cell survival).

The number of studies dealing with the radiosensitivity of the human spinal cord are limited but based on these reports Schultheiss (1990) estimated an ED_5 of 57.0 to 61.0 Gy and an ED_{50} of 68.0 to 73.0 Gy (daily fraction sizes of 2.0 Gy; 5 times a week) for the development of late delayed reactions of the CNS. Fifty Gy in daily 2.0 Gy fractions is usually recommended as an acceptable spinal cord dose, but this recommendation is probably a conservative one. Schultheiss states that the frequently quoted ED_5 of 45.0 Gy given in daily fractions of 1.8 to 2.0 Gy is clearly incorrect. Although it seems that the LQ-model is able to predict accurately the consequences of changes in the fraction size and total dose, drastic changes in treatment protocols should be viewed with great caution. Factors normally thought not to influence treatment outcome may become very important. The high incidence of myelopathies in the CHART protocol is a recent example: Dische and Saunders (1989) treated patients with advanced head and neck, and bronchial carcinomas with continuous hyperfractionated accelerated radiotherapy (CHART). Instead of a single fraction per day, 5 days a week for a period of 5 to 7 weeks, these patients were treated 3 times a day for a continuous period of 12 days with a 6-h interval between fractions. Radiobiological and clinical data had suggested that a better tumor control could be achieved if the overall treatment time was shortened. In addition, hyperfractionation would further spare late-responding tissues such as the CNS. Indeed a better tumor control was observed, but the number of myelopathies, observed after a dose considered to be safe, exceeded the number predicted on the basis of the LQ-model. The radical change in overall treatment time and the number of fractions per day revealed the importance of the duration of repair processes in the CNS.

Latent Period.

The effect of dose on latent time and type of radiation damage has been studied most extensively in the adult rat spinal cord (Van der Kogel 1979). White matter necrosis developed after irradiation of the cervical and thoracic spinal cord

after single doses of 20.0 Gy or more. The latent period was inversely related to the total dose and could be reduced from 240 to 140 days after increasing single doses from 20 to 40 Gy, respectively. In the rat spinal cord white matter necrosis always developed within 7 months. Animals not affected by white matter necrosis and animals irradiated with single doses between 17 and 20 Gy were susceptible, after a latent period of more than one year, for the development of vascular damage. The same bimodal distribution for the development of the different histological lesions could be observed after analysis of clinical data (Schultheiss 1988).

Van der Kogel (1979) also studied radiation damage in the rat lumbosacral cord and cauda equina. A clear difference was noticed between this part of the spinal cord and the higher region. Two dose related pathological entities were distinguished: progressive necrotizing radiculopathy and chronic nerve root degeneration with hypertrophic neuropathy. After single doses of 19.0 to 60.0 Gy and an identical latent period as observed for white matter necrosis in higher cord regions, paralysis of the hindlegs developed due to demyelination and necrosis of the nerve roots. It was striking that even after single doses of 60.0 Gy the white matter of the irradiated lumbosacral spinal cord showed no demyelination or necrosis, while the nerve roots were totally necrotic. Doses below the threshold for the induction of necrotizing radiculopathy led in a number of irradiated animals to degenerative and proliferative changes in the nerve roots.

Time factor.

Repair of radiation damage occurs at different levels and its extent will determine the additional dose in fractionated treatments that can be delivered to obtain the same level of radiation-response as obtained for single doses. Enzymatic repair mechanisms are responsible for the relatively quick repair of sublethal intracellular (=DNA) damage. In contrast, proliferation and migration are thought to be processes that counteract in the long-term the loss of stem cells. For the CNS, both short- and long-term repair processes are of importance and their extent can be studied either by fractionation or retreatment experiments.

To study the effect of decreasing fraction size below 2.0 Gy, Van der Schueren et al. (1988) irradiated the rat spinal cord with fractions of down to 1.0 Gy, but in order to keep the overall treatment time constant 2 fractions per day were given with an interval of 4 h. An increased tolerance as predicted by the LQ-model was not observed and a likely explanation of this phenomenon was incomplete repair of sublethal damage.

Until that time it was thought that 6 to 8 h were sufficient for the repair of SLD in the CNS *in vivo*. However, when this concept was applied in the clinical

situation of accelerated hyperfractionated irradiation of head and neck carcinomas a number of myelopathies was recorded that exceeded the number predicted by the linear-quadratic model (Disch and Saunders 1989). Radiotherapy was given 3 times a day for a continuous period of 12 days with a 6-hour interval between fractions. Incomplete repair of sublethal damage has been proposed as a possible reason for the unexpected number of late myelopathies and indeed Ang et al. (1992), in a detailed study of the repair kinetics of sublethal lesions in the rat spinal cord, found that the experimental data were best fitted by a bi-exponential repair model with repair half times of 0.7 and 4.0 h for a fast and a slow component, respectively. [Repair proccesses are considered to occur according to exponential kinetics and the rate of repair is expressed as the time needed to repair half the radiation damage (= half time of repair).] The contribution of the slow component was considerable: 65% of the total repairable damage.

When the CNS was irradiated with one fraction per day, the overall treatment time had minor influence on the development of late delayed injuries (Sheline 1980; Van der Kogel 1979). Long-term recovery, however, was observed if the treatment time exceeded a period of 6 to 8 weeks. In the adult rat spinal cord (Van der Kogel 1991) long-term repair occurred predominantly between 2 and 6 months after the initial treatment. White and Hornsey (1980) observed that an increase in radiation tolerance took place earlier: between 15 and 60 days. Long-term recovery is attributed to an increased number of target cells either due to proliferation in the irradiated CNS region or due to migration into the irradiated area from adjacent non-irradiated CNS. The difference in the onset of long-term recovery between Van der Kogel and White and Hornsey could be due to strain differences and differences in experimental design.

After irradiation of the rat cervical spinal cord, the extent of recovery appeared to be mainly determined by the level of injury caused by the initial radiation treatment: A dose of 95% of the ED_{50}-value for the development of white matter necrosis showed a partial recovery after 210 days and allowed a retreatment dose of only 40% of the ED_{50}-value (Van der Kogel 1991). An initial dose of 50% of the ED_{50}-value led to an almost full recovery. Re-irradiation of the primate spinal cord two years after 44 Gy (≈63 % of the ED_{50} value) led to an identical observation: the majority of occult injuries was recovered within two years (Ang 1993). These findings were based on only a few re-irradiated monkeys but stress the importance of such studies because confirmation of almost complete recovery of the CNS after an initial treatment of 44 Gy in 20 to 22 daily fractions would have a major impact on treatment options for previously irradiated patients. Histological examination of the spinal cords of the two symptomatic re-irradiated

monkeys showed severe vascular lesions and may indicate that recovery of vascular injury is less efficient than the recovery of white matter damage.

Volume.

The influence of the volume of irradiated CNS on the development of late delayed reactions has been studied in the rat spinal cord by Hopewell et al. (1987) and by Van der Kogel (1991). For the development of white matter necrosis there seemed to be a clear influence of the field size, albeit at relatively small sizes. A decrease of the field size from 16 mm to 4 mm resulted in an increase in the ED_{50}-value for white matter necrosis from 21.5 Gy to 51.0 Gy. For vascular damage the field size dependence was less obvious: an ED_{50} of 20 Gy for a field size of 16 mm and an ED_{50} of 26 Gy for a field size of 4 mm (Hopewell 1987). Of interest was the observation that the clinical picture of white matter necrosis depended on the field size (and consequently on the total dose)(Hopewell 1987): White matter necrosis which developed after irradiation of 16 mm of cervical rat spinal cord (dose ≈ 21.5 Gy) resulted in paralysis of only the forelimbs. After irradiation with 8 and 4 mm fields (dose up to ≈ 51 Gy) paralysis developed in both fore- and hind-limbs. In addition, the histological presentation differed also with the size (and related dose) of the irradiated spinal cord. White matter necrosis was the only observed lesion in 16 mm fields after a relative low dose of ≈ 21.5 Gy whilst additional nerve root necrosis and gray matter necrosis was observed in 8 and 4 mm fields after much higher doses (up to ≈ 51 Gy).

In the histological specimens of animals, that did not develop white matter necrosis at a dose level that would have induced white matter necrosis in larger fields, groups of small, dark, round nuclei were seen in the neighborhood of foci of demyelination which suggested the repair of demyelination by oligodendroglia (Hopewell 1987). These cells might come from unirradiated adjacent areas of the CNS and would explain the field size dependence of white matter necrosis. If the distance between radiation-induced demyelination and viable non-irradiated migratory cells, with myelinating capacities, is not too large (2 to 4 mm), replacement of lost cells may increase the tolerance of the CNS. Further evidence that the radiation tolerance of the spinal cord is determined by the presence of migratory target cells which enter the irradiated region from adjacent non-irradiated CNS regions is provided by experiments of Van der Kogel (unpublished): An ED_{50}-value for white matter necrosis of approximately 45 Gy was found after irradiation of a 4 mm part of the rat cervical spinal cord. When the same 4 mm of cervical spinal cord were irradiated within a region that had been pretreated with a single dose of 18.0 Gy the ED_{50} declined to 27.5 Gy. A

substantial depletion of migratory target cells which could have rescued a small part of irradiated CNS is a likely explanation for this observation.

Few clinical studies have been published dealing with the tolerance dependence of the CNS on field size. Abbatucci et al. (1978) analyzed the influence of the length of irradiated human cervical spinal cord on the development of radiation myelopathy and found a positive relation between the incidence of radiation-induced paralysis and the number of irradiated vertebrae (and included cervical spinal cord). Firm conclusions, however, were difficult to draw due to the restricted number of patients. Marcus and Million (1990) could not detect any correlation between the volume of irradiated spinal cord and the probability to develop white matter necosis.

Age.

Gilmore (1963) evaluated the effect of 44.3 Gy of soft X-rays (15 kV) on 5 mm of lumbosacral spinal cord of 3-day-old rats. Radiation did not interfere with the maturation of neurons of the ventral horn but a marked and consistent decrease in neuroglia was observed in the first 9 to 11 days after irradiation. At this time ≈65% of the animals exhibited severe neurological deficits. Oligodendrocytes seemed more susceptible for radiation damage than astrocytes and this resulted in disturbed myelination: dysmyelination (not demyelination). In addition to the dysmyelination small petechial hemorrhages in both gray and white matter and swelling of the endothelium were occasionally observed.

Beal and Hall (1974) irradiated the spinal cord from the midthoracic region to beyond the caudal extent of 3-day-old rats. Seven of 80 rats exposed to a dose of 20.0 Gy (250 kV) showed signs of weakness or paralysis of the hindlimbs. The latent period between irradiation and development of neurological signs was short (9 to 11 days) and is comparable with the latent period normally found for acute-responding tissues. Morphological evaluation showed destruction of oligodendroglia; inhibition of proliferation of the surviving glia and an inhibition of myelination. Some of the animals with neurological deficits were kept alive and completely recovered normal locomotion 20 days after irradiation. Histologically, the return of neuroglia appeared to occur according to a cephalo-caudal gradient and it was hypothesized that migration of glial cells from the adjacent unirradiated CNS region was at least partially responsible for the tissue recovery.

Single dose response curves were obtained for 1-day-old; 30-day-old and 1-year-old guinea pigs (Knowles 1983). Neonatal guinea pigs were more susceptible for the development of paralysis than older animals (ED_{50} of 14.75 Gy and 20.0 Gy, respectively; 250 kV X-rays). A latent period, however, of approximately 90 days for neonatal guinea pigs was considerably longer than the

9 to 11 days observed by Gilmore (1963) and Beal & Hall (1974) for the 3-day-old rat.

Recently, Ruifrok et al. (1992) investigated the radiation tolerance and fractionation sensitivity of the developing rat CNS. At the age of 1 week the cervical spinal cord was irradiated with 4 MV photons. A short latent period of 10 to 15 days was observed and confirmed the findings of Gilmore (1963) and Beal and Hall (1974). The short latent period indicated that there was an "acute" need for newly formed functional cells (oliogodendrocytes) and that the developing CNS may be considered as an early-responding tissue. The observed relatively high α/β-ratio of 4.5 Gy points in the same direction. This contrasts with the adult CNS which has an α/β-ratio of 2.0 Gy. Also the tolerance of the developing rat spinal cord differed significantly from the adult CNS: ED_{50} of 19.5 Gy for 1-week-old rats as compared with an ED_{50} of 21.5 for adult rats.

Ruifrok et al. (1992) also investigated the effects of radiation on the 3-week-old rat spinal cord and found that the radiation tolerance expressed as the ED_{50}-value for white matter necrosis was identical to that of the adult spinal cord, but that the mean latent period between irradiation and expression of damage was still shorter for the 3-week-old rats (90 days as compared with ≈180 days for adult rats). Retreatment experiments also confirmed the different kinetics of the target cells in the immature CNS: Long-term recovery was complete within 1 month after irradiation. In the adult rat CNS long-term recovery occurred between 2 and 6 months.

These studies show that irradiation interferes with the normal process of myelination in the developing CNS. The oligodendroglia seems more susceptible to radiation-induced damage than the vasculature although vascular changes were also observed. The sensitivity of the developing CNS is higher than the adult CNS and is probably dependent on the grade of immaturity of the CNS. A short latent period between irradiation and the expression of damage found by most authors indicates that in the developing CNS the turnover of the critical cells is much faster than in the adult CNS. At the moment there are insuffient clinical data to estimate the influence of age on the radiation tolerance of the CNS and although a clear effect of age has been observed in experimental studies, the extrapolation of these data to the human situation remains difficult.

CHAPTER 3

THE O-2A PROGENITOR CELL STORY

Introduction.

Irradiation of the central nervous system (CNS) leads to changes which can be examined by histological examination, functional tests and cell kinetic studies. The underlying cellular mechanisms, however, are still not clear although several hypotheses exist to explain the development of specific syndromes. The irreversible syndromes, which are clinically the most relevant, are thought to be caused by the reproductive cell death of either endothelial cells or glial cells. In this thesis we have restricted our investigations to the possible role of glial stem cells in the development of radiation-induced damage in the CNS. Recent developments in neurobiology have made it possible to discriminate different glial cell types and study their relationships and specific functions. These recent developments will be discussed and compared with some "older" literature.

The rat optic nerve has mainly been used for studying glial cell development and diversification because it is a relatively simple part of the CNS. It guides the axons of the retinal ganglion cells from the eye to the brain without actually housing their cell bodies. If the optic nerve is cut just behind the eye and just before the chiasm, single cell suspensions of these optic nerves do not contain viable neurones. Cultures of optic nerve cell suspensions are therefore well suited to study glial cells independently of neuronal influences. Further advantages of studying optic nerve glial cell populations *in vitro* are the possibility to control and manipulate environmental conditions and to identify and characterize cell subpopulations with monoclonal antibodies.

Glial cells and glial cell lines.

The glial cells in the CNS can be divided into two main categories: astrocytes and oligodendrocytes. Astrocytes are cells characterized by specific intracellular intermediate filaments: glial fibrillary acidic protein (GFAP)(Bignami 1972). In the developing rat optic nerve two different astrocytic cell populations could be distinguished on the basis of morphology; growth characteristics; and labeling with ligands directed against polygangliosides (Raff 1983). They were called type-1 and type-2 astrocytes. *In vitro*, type-1 astrocytes have a fibroblast-like morphology, proliferate when stimulated with epidermal growth factor (EGF) and possess the polyligand that is recognized by the monoclonal antibody A_2B_5 (Eisenbarth 1979). Type-2 astrocytes have a process-bearing morphology; are unresponsive to EGF and stain positive with A_2B_5.

Changes in culture media, in an attempt to select for cultures with a high concentration of type-2 astrocytes, led by surprise to cultures enriched for oligodendrocytes (Raff 1983) Mature oligodendrocytes, *in vitro*, have many fine processes, are $A_2B_5^-$ and can be visualized by a monoclonal antibody directed against galactocerebroside (Raff 1978), the major glycolipid in myelin. The observation that changes in the culture medium increased the numbers of either oligodendrocytes or type-2 astrocytes led to the idea that these two different cell types originated from the same precursor cell To provide evidence for this hypothesis single cell suspensions derived from 7-day-old rat optic nerve were subjected to different procedures

First, cell suspensions derived from 7-day-old rat optic nerve were treated with A_2B_5 antibody and complement to lyse $A_2B_5^+$ cells As a result neither type-2 astrocytes nor oligodendrocytes developed in the subsequent culture period It was concluded that both cell types are the descendants of $A_2B_5^+$ cells It was also apparent that no $A_2B_5^+$ cells developed from $A_2B_5^-$ precursor cells after A_2B_5-dependent complement-mediated cytolysis (The existence of an $A_2B_5^-$ pre- O-2A cell has been demonstrated in the cerebral white matter of 6-day-old rats by Grinspan et al (1990) These authors also failed to detect the pre- O-2A progenitor cells in the perinatal rat optic nerve)

Secondly, pre-labeling studies with A_2B_5 were performed to determine the fate of $A_2B_5^+$ cells. Pre-labeled cell suspensions of perinatal rat optic nerves were cultured either in the presence or absence of fetal calf serum (FCS). After 2 days cultures were stained The newly formed type-2 astrocytes and oligodendrocytes were highly positive for A_2B_5 (65%-90%), which demonstrated that they originated from $A_2B_5^+$ precursor cells If, however, oligodendrocytes mature, they lose the A_2B_5 ganglioside

Also studies in which the numbers of cells were counted before and after the administration of FCS pointed in the direction of a common progenitor cell that could be directed either into oligodendrocyte or type-2 astrocyte differentiation dependent on the culture conditions It was even possible *in vitro* to generate cells that co-expressed GFAP and GalC Although this mixed phenotype seems to be the result of *in vitro* manipulation, it emphasizes the close relationship between oligodendrocytes and type-2 astrocytes and gives further evidence that they originate from a common precursor cell Because of the ability of this precursor cell to develop either into an oligodendrocyte or a type-2 astrocyte it was called the O-2A progenitor cell.

Dissociated cultures of embryonic rat brain mimic the *in vivo* situation with respect to the time schedule of appearance of glial cells Pre-labeling studies with RAN-2 of 4-day-old cultures of dissociated 10-day embryonic rat brain revealed

that not O-2A progenitor cells but other precursor cells are the source of type-1 astrocytes. The rat neural antigen-2 (RAN-2) which is defined by a monoclonal antibody (Bartlett 1981) is expressed by type-1 astrocytes, but not by type-2 astrocytes. Type-1 astrocytes (RAN-2^+; $GFAP^+$) appeared to be derived from RAN-2^+; $GFAP^-$ precursor cells (Raff 1981).

In conclusion, there are two distinct glial lineages in the developing rat optic nerve. The O-2A lineage, which gives rise to oligodendrocytes and type-2 astrocytes dependent on the culture conditions and the glial lineage that is responsible for the generation of type-1 astrocytes.

Table 3.1 summarizes some of the staining characteristics and growth factor responses of the different glial cell types in the perinatal rat optic nerve.

Table 3.1

	Type-1 A	O-2A	Oligo	Type-2 A
Antigenic Phenotype				
A2B5	-	+	-	+
Galactocerebroside	-	-	+	-
GFAP	+	-	-	+
RAN-2	+	-	-	-
Response to Growth Factors				
PDGF	-	+	-	-
EGF	+	-	-	-

Glial cell function.

Oligodendrocytes are mainly present in the white matter of the CNS. They enwrap bare axons with insulating myelin sheaths, which allows rapid saltatory conduction of an electrical signal along the myelinated axons. In the peripheral nervous system this task is accomplished by Schwann cells. Disruption of the myelin sheath will result in neurological deficits.

The function of type-2 astrocytes is not fully understood. Type-2 astrocytes send out their processes to nodes of Ranvier and contribute to the structure of the node (ffrench-Constant 1986 a, b; Black 1988). The presence of glutamate receptors at the membrane of type-2 astrocytes suggests glial-neuronal signaling at the node and type-2 astrocytes could well serve as cells that make communication between glial cells and neurons possible (Usowicz 1989).

The function of type-1 astrocytes seems to be diverse (Miller 1989):

1. In the optic nerve type-1 astrocytes are thought to guide the migration of retinal ganglion cell growth cones from the eye to the brain stem and

2. they are associated with a physical barriere, that prevents oligodendrocytes from migrating into the eye.
3. In the CNS type-1 astrocytes seem to have an important role in the formation of the glial limiting membrane which provides a structural framework for the CNS.
4. They induce endothelial cells to form tight junctions that are responsible for the blood brain barriere (BBB). The BBB is a physical barrier which selectively regulates the passage of molecules across the capillaries of the brain and spinal cord.
5. Another function of type-1 astrocytes is the formation of glial scars after injury and
6. they may have a function in immune responses of the CNS (Frohman 1989).
The role of these cells in the proliferation and differentiation of O-2A progenitor cells will be discussed below.

Differentiation of O-2A progenitor cells.

O-2A progenitor cells differentiate *in vitro* in oligodendrocytes or type-2 astrocytes dependent on the culture conditions. The differentiation in oligodendrocytes is not mediated by a specific agent and seems to be the constitutive pathway of O-2A progenitor cells.

The differentiation in type-2 astrocytes, on the other hand, is only possible if specific factors are present in the culture medium. FCS is a source with such an inducing potential (Raff 1983). The differentiation of O-2A progenitor cells in type-2 astrocytes can also be initiated by a protein isolated from the developing rat optic nerve. A rise in the concentration of this protein in the second postnatal week coincides with the appearance of type-2 astrocytes in the rat optic nerve *in vivo* (Hughes 1987). The protein shows great resemblance with ciliary neurotrophic factor (CNTF), a protein that is necessary for the culturing of neurons derived from the chick ciliary ganglion. The source of CNTF is likely to be type-1 astrocytes because purified cultures of these cells release CNTF-like molecules in the culture medium after injury (Lillien 1988). How the production of CNTF by type-1 astrocytes is regulated *in vivo* is still unknown.

Although FCS and CNTF are both able to induce type-2 astrocyte differentiation in O-2A progenitor cells these factors are certainly not identical. CNTF achieves a rapid induction of GFAP formation in only 20-30% of the O-2A progenitor cells and its effect is only temporal. After 3 days *in vitro* O-2A progenitor cells escape from the influence of CNTF and differentiate in oligodendrocytes. Obviously, other factors are needed in addition to CNTF that will result in a permanent differentiation of O-2A progenitor cells in type-2 astrocytes. By contrast, FCS accomplishes type-2 astrocyte differentiation in almost all exposed O-2A

progenitor cells, albeit after several days *in vitro*. In addition, the differentiation of O-2A progenitor cells induced by FCS is irreversible.

Proliferation of O-2A progenitor cells.

In vivo, the appearance of the different glial subtypes in the developing optic nerve is regulated according to a precise time schedule. In the rat optic nerve, type-1 astrocytes are identified at embryonic day 17; oligodendrocytes appear at the day of birth (the gestation period of the rat is 21 days) and type-2 astrocytes are seen at the second post-natal week (Raff 1989). After their first appearance these cells are generated at a relatively high rate over an extended period of time to produce the number of cells necessary for the development of the CNS. The time schedule seen *in vivo* could also be observed in cultures derived from embryonic rat brain (Abney 1981) but is disrupted if cells derived from the perinatal rat optic nerve are cultured in a medium devoid of growth factors. O-2A progenitor cells derived from perinatal rat optic nerve stop dividing within 2-3 days when cultured in a chemically defined medium and differentiate in oligodendrocytes. The normal timing of oligodendrocyte development can be reconstituted by co-culturing optic nerve derived cell suspensions in the presence of purified type-1 astrocytes (Noble 1984; Raff 1985). Also culture medium conditioned by type-1 astrocytes (ACM) appeared to have the ability to prevent the premature differentiation. That platelet derived growth factor (PDGF) is the responsible mitogen for the inhibition of the premature differentiation of O-2A progenitor cells *in vivo* has been demonstrated by several studies (Raff 1988; Pringle 1989). Direct evidence was obtained by inactivation of the proliferative potential of extracts of the rat optic nerve by antibodies directed against PDGF (Noble 1988).

Although the addition of PDGF to the culture medium was able to restore the normal time schedule of differentiation in perinatal rat optic nerve cultures, PDGF was unable to prevent differentiation. After several divisions O-2A progenitor cells withdrew from the mitogenic influence of PDGF and differentiated in oligodendrocytes. Clonal analysis of single O-2A progenitor cells confirmed the restricted proliferative potential of O-2A progenitor cells derived from the perinatal rat optic nerve (Temple 1986). Not only was the proliferative potential of O-2A progenitor cells limited, the progeny of individual O-2A progenitor cells had an identical fate. Even when two sister O-2A progenitor cells were cultured separately their number of cell divisions remained the same. This led to the hypothesis that perinatal O-2A progenitor cells possess an internal clock that counts the number of cell divisions (Temple 1986): When the pre-programmed number of divisions is reached O-2A progenitor cells become irresponsive to PDGF and differentiate in

oligodendrocytes. Further evidence for this theory was the finding that the number of PDGF receptors on O-2A progenitor cells remained unchanged during differentiation. Thus, their diminished responsiveness to PDGF could not be explained by a decreased number of PDGF receptors (Hart 1989 a, b).

The adult O-2A progenitor cell.

The detection of a bipotential glial progenitor cell in the perinatal rat optic nerve had raised the question whether these cells were also present in the adult CNS. Single cell suspensions of adult optic nerve cells cultured *in vitro* also contained cells with the ability to differentiate either in an oligodendrocyte or type-2 astrocyte in the absence or presence of FCS (ffrench-Constant 1986). And analogous to their perinatal counterparts these cells were called O-2A progenitor cells. A more precise characterization of the adult O-2A progenitor cell was performed by Wolswijk and Noble (1989). The perinatal and adult O-2A progenitor cell have in common that their progeny is the same and that they proliferate after stimulation by growth factors produced by purified monolayers of type-1 astrocytes. Adult O-2A progenitor cells, however, differ from perinatal O-2A progenitor cells with respect to morphology, cell cycle time, migration rate, and antigenic phenotype (Table 3.2).

Characteristic	Perinatal O-2A	Adult O-2A
Morphology	bipolar	unipolar
Vimentin	+	-
O_4	-	+
Cell Cycle Time	18± 4 h	65± 18 h
Migration Rate	21 µm/h	4 µm/h

Table 3.2 O-2A progenitor cells characteristics. Table adapted and modified from Wolswijk 1989.

Another essential difference between the perinatal and adult O-2A progenitor cell is their proliferative potential. O-2A progenitor cells derived from the perinatal rat optic nerve could not escape the inevitable process of differentiation after a limited number of cell divisions. The fate of adult O-2A progenitor cells was different. Adult O-2A progenitor cells could give rise simultaneously to oligodendrocytes and new adult O-2A progenitor cells (Noble 1989). This capacity for self-renewal is a distinctive feature of stem cells and the presence of glial stem cells in the adult CNS provides further evidence for a gradual turnover of the glial cell population (see later).

The origin of O-2A progenitor cells.

In the anlage of the optic nerve, O-2A progenitor cells are believed not to originate form the initial matrix cells of the optic nerve. In contrast, O-2A progenitor cells were seen to migrate into the optic nerve from an nearby germinal zone (Small 1987). A likely source of O-2A progenitor cells is the base of the preoptic recess overlying the optic chiasm and it is of interest that just before O-2A progenitor cells are seen in the optic nerve a period of increased mitotic activity is seen in this germinal zone (Small 1987).

The migratory capacity of O-2A progenitor cells has been demonstrated *in vitro* and *in vivo*. Time-lapse cinematography revealed that perinatal O-2A progenitor cells are mobile cells when stimulated with ACM. They become motionless when they differentiate in oligodendrocytes (Small 1987). The mobility of myelinating cells *in vivo* has been shown for example by transplantation of embryonic CNS tissue in the brain of shiverer mutant mice. These mice are genetically unable to form myelin, but after transplantation myelin was found not only at the site of transplantation but also at a considerable distance from it which suggests that oligodendrocytes or their precursor cells were able to move in the CNS (e.g. Gumpel 1989).

After the discovery of adult O-2A progenitor cells the question arose where these cells originated from. That perinatal O-2A progenitor cells were produced in specific germinal zones and migrated from these zones all over the CNS is not that difficult to imagine because the distances in the developing CNS are small and the structure is relatively "loose". It is unlikely that the same structural organization is maintained in the adult CNS. The general opinion is that after the development of the CNS the germinal zones disappear and that glial stem cells are located all over the adult CNS. Some *in vitro* observations have provided evidence that adult O-2A progenitor cells originate from perinatal O-2A progenitor cells. Cultures derived from 3-week-old rat optic nerves were followed with time-lapse cinematography. Cells with the characteristics of perinatal O-2A progenitor cells adopted after several divisions the morphology; slow migration rate and slow cell cycle time of adult O-2A progenitor cells. The appearance of adult O-2A progenitor cells was also seen after serial passaging of perinatally derived optic nerve cell suspensions *in vitro* (Noble 1989).

Additional evidence that adult O-2A progenitor cells are derived from perinatal O-2A progenitor cells is that, in common with oligodendrocytes, they do not have intermediate filaments, stain positive with the O_4 antibody, and are susceptible to the cytolytic action of complement in the absence of antibodies (Wren 1989).

The finding that adult O-2A progenitor cells may originate from perinatal O-2A progenitor cells has changed the initial thought about the bipotentiallity of perinatal O-2A progenitor cells. The old theory was that perinatal O-2A progenitor cells were doomed to differentiate immediately or after a restricted number of divisions and were unable to proliferate indefinitely. It now appears that O-2A progenitor cells derived from the perinatal rat optic nerve are also able to generate adult O-2A progenitor cells as a third option besides differentiation in oligodendrocytes or type-2 astrocytes. Whether all perinatal O-2A progenitor cells are able to give rise to adult O-2A progenitor cells is still uncertain. Another experiment that changed the view about perinatal O-2A progenitor cells is the exposure of these cells to PDGF and fibroblast growth factor (FGF) simultaneously (Bögler 1990). This combination of growth factors caused a continuous self-renewal of perinatal O-2A progenitor cells. Whether this is also happening *in vivo* and whether cells stimulated by PDGF and FGF give rise to adult O-2A progenitor cells is not known. Table 3.3 summarizes the impact of different factors on O-2A progenitor cells derived from the perinatal rat optic nerve (Bögler 1990).

Table 3.3

	Factor(s)	Effect
Perinatal O-2A	-	Oligodendrocyte.
Progenitor Cell	CNTF	Type-2 astrocyte.
	PDGF	Clonal expansion and clonal differentiation in oligodendrocytes.
	FGF	Premature oligodendrocyte differentiation and continued proliferation of oligodendrocytes.
	FGF + PDGF	Continuous self-renewal.
	?	Adult O-2A progenitor cells.

The localization of O-2A progenitor cells.

O-2A progenitor cells were first identified in single cell cultures derived from perinatal rat optic nerve. Subsequently similar cells were found in other regions of the CNS (cerebellum: Levi 1986; cerebrum: Goldman 1986; spinal cord: Raff 1988; spinal cord: this thesis) and in other species, including man. Like optic nerve derived O-2A progenitor cells these cells were able to differentiate in culture either in oligodendrocytes or type-2 astrocytes. O-2A progenitor cells derived from other CNS regions are not so well characterized as O-2A progenitor cells derived from the optic nerve. In this thesis clear differences in radiosensitivity and proliferative capacity between O-2A progenitor cells derived from different regions of the perinatal CNS are described. O-2A progenitor cells seem also to be present

in all regions of the adult CNS (cerebrum: Wolswijk, personal communication; spinal cord: this thesis).

Old data reviewed in the light of the new developments.

Definite proof for the existence of O-2A progenitor cells had to wait until the development of specific antibodies that were able to identify the different glial cell types of the CNS *in vitro*. Before the advent of monoclonal antibodies the CNS was studied with light and electron microscopy. These studies concentrated on the CNS *"in vivo"*, although occasionally *in vitro* experiments were performed (Manuelidis 1971). The recognition of different glial cell types was based on staining characteristics and on morphological grounds (e.g. size and shape of the cell and nucleus; amount of cytoplasma; chromatine density; organelles; cytoplasmic particles).

The combination of light and electron microscopy with autoradiography after tritiated (^{3}H-) thymidine injection made it possible to study proliferation, migration and differentiation of glial cells. And although the techniques were limited and reliable identification of all cells was not always possible, the results of the different studies and the sometimes speculative conclusions clearly showed some foresight. Some of these early findings will be discussed and correlated with the current knowledge about the O-2A lineage.

An important finding was the ^{3}H-thymidine uptake of glial cells in the CNS of young adult mice (Messier 1958). This suggested a low rate of glial turnover in adult animals which has been confirmed by many others (e.g. Altman 1963). This finding did not fit in the prevailing opinion of that time. Glial cells, like neurons, were thought to be mitotically inactive. This opinion was based on the lack of mitotic figures in glial cells in the adult CNS. An observation that dominated the opinion about glial cells, but, as was demonstrated later, was caused by a fixation artefact. The uptake of ^{3}H-thymidine by glial cells was noticed in all parts of the CNS and in all species (Chicken spinal cord: Fujita 1965; Rat and Cat Brain: Altman 1963; Brain of Rhesus Monkey and Mouse: Noetzel 1964). Schultze and Korr (1981) reviewed the cell kinetic data of glial cells in the rodent CNS. They came to similar conclusions for proliferating glial cells in the perinatal and adult CNS: Glioblasts (precursors of oligodendrocytes and astrocytes) had a cell cycle time of 20 h. The growth fraction of glioblasts is low (0.004) and there is a permanent exchange of glioblasts between the growth fraction and the non-growth fraction. It is impossible to determine the growth fraction of O-2A progenitor cells *in vitro* and therefore this can not be compared with *in vivo* values for glioblasts. The cell cycle time, however, has been determined for perinatal and adult O-2A progenitor cells *in vitro* (Wolswijk 1989). For perinatal O-2A progenitor

cells the cell cycle time is approximately 18 h and is similar to values found for perinatal glioblasts *in vivo*. A cell cycle time of 65 h for adult O-2A progenitor cells, however, is considerably longer than the 20 h for the cell cycle time of glioblasts in the adult CNS. The reason for this inconsistency is difficult to explain (e.g. inaccuracy of the determination of the cell cycle time of glioblasts *in vivo*; sub-optimal *in vitro* growth conditions for adult O-2A progenitor cells).

^{3}H-thymidine labeling studies made it possible to determine the fate of proliferating cells. Smart and Leblond (1961) identified in the mouse CNS cells with small dark and medium dark nuclei which function as spongioblasts (glial precursor cells) and differentiated in oligodendrocytes. Whether astrocytes also originated from these spongioblasts or whether astrocytes were derived through oligodendrocytes was not clear from their experiments. Subsequent papers showed evidence for the existence of a common progenitor cell for oligodendrocytes and astrocytes (Vaughn 1968; Privat 1981) as well as the existence of unrelated precursor cells for oligodendrocytes and astrocytes separately (Skoff 1976a,b). With the new evidence of two different types of astrocytes, originating from two different cell lines (Raff 1983), these apparently conflicting data confirm the existence of these two different glial lineages *in vivo*.

More extensive schemes of gliogenesis were presented and they were all based on morphological and autoradiographic studies of the CNS (Paterson 1973). One of these schemes is presented in more detail because it is striking that with relatively simple tools a complex scheme of gliogenesis was proposed that appeared to be very accurate: Vaughn (1969) studied the gliogenesis in the optic nerve of embryonic, perinatal and adult rats. He combined light and electron microscopic sections of adjacent areas of the optic nerve to analyze the morphological transitions between cell varieties. These morphological transitions were studied in a retrograde sequence: cells with a clear identity in the adult optic nerve were linked with less mature cells in the developing CNS, which showed resemblance with the more differentiated cells. This led to the description of different mature and immature glial cell types and their presumed relationships (Figure 3.1). The scheme proposes the existence of a bipotential progenitor cell that is able to generate oligodendrocytes and astrocytes, and could already have been called O-2A progenitor cell. There are two types of astrocytes in the scheme that develop along different pathways. Astrocytes that are already present in the optic nerve before birth and astrocytes that develop after birth and have a common precursor cell together with oligodendrocytes. In the new description of glial lineages (Raff 1983) these astrocytes would have been called type-1 and type-2 astrocytes, respectively. Even the existence of an adult O-2A progenitor cells has

been proposed although the exact nature of these cells could not be determined at that time.

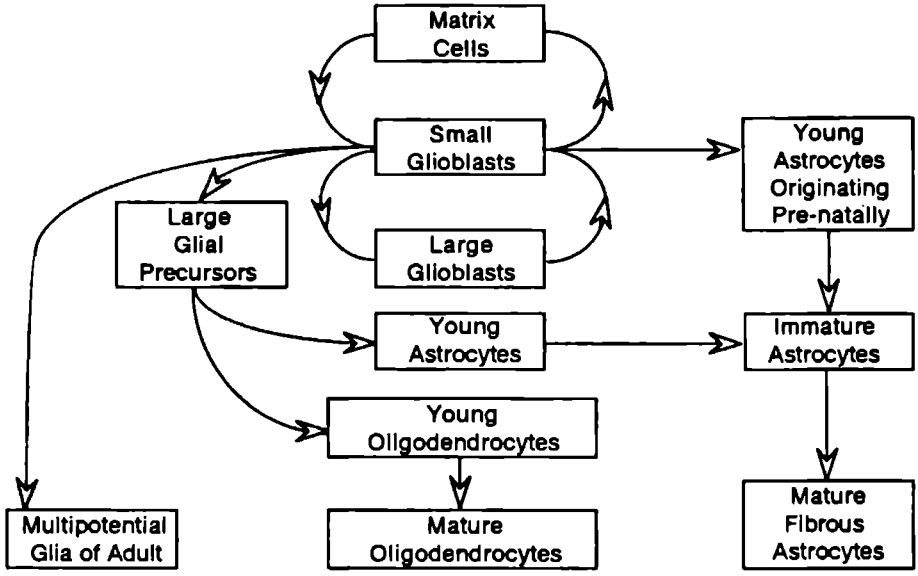

Figure 3.1 Tentative scheme of gliogenesis in developing rat optic nerve. Adapted and modified from Vaughn (1969).

So it appears that the ideas about the different glial cell lineages date from about 25 years ago, and possibly even earlier, and were based on meticulous observations of changing cell characteristics in the developing CNS. *In vitro* studies, with the use of specific antibodies, have verified earlier assumptions on the existence of a perinatal and adult bipotential glial progenitor cells. The greatest merit of the *in vitro* studies of glial cell suspensions derived from the rat optic nerve has been the identification of growth factors involved in glial cell proliferation and differentiation.

CHAPTER 4

CONSEQUENCES OF CELLULAR KINETICS AND TISSUE ORGANIZATION FOR RADIATION-INDUCED DAMAGE

Introduction.

The radiation-induced syndromes and their tentative theories of development are largely based on observed changes in histology and function. In a radiobiological model to explain radiation damage, tissue responses are either associated with the acute death of the target cells (interphase death) or with cell death which will be expressed when the damaged cells try to divide (reproductive cell death). The development of acute and possibly early delayed syndromes may be due to direct cytotoxic damage to the functional cell compartment (eg. oligodendrocytes or type-1 astrocytes; see chapter 3) and recovery will occur after replacement of the defective cells. Reproductive cell death becomes apparent when cells try to divide and appear to be lethally damaged. These lethally damaged cells are therefore unable to produce new cells and when the cell kill has exceeded a certain level the remaining cells will be unable to supply sufficient functional cells to replace the cells that are lost due to natural attrition. This will lead to anatomical or functional defects of the afflicted tissue. If the turnover of functional cells is high, reproductive cell death will be expressed early (= acute-responding tissues). A slow turnover will result in a long latent period between irradiation and effect (= late-responding tissues). The effects of radiation on reproductive cell death can be studied by analyzing the changes in the number of functional cells, but of more interest is the effect of radiation at the stem cell level. In the CNS there is, however, a difficulty in the identification of the different stem cell populations and only recent developments have made it possible to identify glial stem cells *in vivo* and *in vitro* (see chapter 3).

The cellular kinetics of normal rodent CNS has been reviewed by Schultze and Korr (1981). Immature glial cells and endothelial cells incorporate tritiated-thymidine as a sign of continuous proliferation not only during the perinatal period but throughout adult life. These cells are the source of new functional cells and their depletion will result in loss of function and / or integrity of the CNS. The low labeling index for both cell types indicates that the turnover rate of functional cells in the CNS is very low. Therefore, a long latent period between irradiation and expression of damage, inflicted on the reproductive capacity of stem cells, is expected (and is indeed seen after irradiation of the CNS *in vivo*).

Cellular kinetics and irradiation of the CNS.

The cellular kinetics of irradiated rodent CNS has been studied mainly by the groups of Hopewell and Gilmore. Hopewell et al. used different techniques to evaluate the effect of radiation. In their initial experiments, they pretreated adult rodent brains with X-irradiation (2.0 - 40.0 Gy) and scored the cellular effects after subsequent injury with either freezing or infection with Bordetella pertussis (Hopewell 1967; 1970a, b). The normal response of non-irradiated rat brains to freezing injury consisted of necrosis followed by cell division and infiltration of the lesion which started at the periphery. A dose dependent reduction in cellular responses was observed which lasted at least up to 6 months after injury. The nature of the responding cells was believed to be microglia and to a lesser extent astrocytes although no definite methods were used to identify these cells. Similar results were obtained after infection of irradiated adult mouse brain with Bordetella pertussis. Bacterial counts and histological investigations revealed a dose dependent response. Mice infected 3 months after 4.0 Gy of X-rays showed a greater resistance to B. pertussis infection than mice infected immediately after irradiation. This was interpreted as a sign of cellular regeneration. A time dependent recovery was not noticed in the freezing experiments.

Another set of experiments concentrated on the effect of radiation on the subependymal plate. The qualitative (Hopewell 1972) and quantitative (Hubbard 1980) effects of radiation on the cells of the subependymal plate were studied in view of the hypothesis that cellular depletion of the subependymal zone may contribute to the phenomenon of radiation-induced white matter necrosis. The brains of adult rats were irradiated and after doses up to 20.0 Gy a gradual return of the mitotic activity of the subependymal plate cells was observed. The period between irradiation and the return of mitotic activity appeared to be dose dependent: after 2.0 Gy it took approximately 1 month and after 20.0 Gy it took approximately 3 months to reach control values. A dose of 40.0 Gy resulted in a total depopulation of the subependymal plate and the animals died from extensive white matter necrosis. These findings were arguments in favor of the importance of glial stem cells, in this case cells of the subependymal plate, in the development of white matter necrosis. The function of the subependymal plate with respect to the replacement of neuroglia throughout life, however, is unclear in the adult rodent CNS and it seems that also in the human CNS the function of the subependymal plate is restricted (Privat 1972).

Also the rat spinal cord was subjected to quantitative analysis of radiation-induced changes in cell number (Cavanagh 1971; Hubbard 1979). The effects of irradiation were studied after administration of p-Bromophenylacetylurea (pBPAU). pBPAU causes axonal degeneration and the subsequent glial reaction

is maximal at 4 weeks after administration. A dose dependent depression of the cellular reaction, mainly of microglial cells and occasionally of astrocytes, was noticed. Of more interest are the studies that quantified the number of astrocyte and oligodendrocyte nuclei in the rat cervical spinal cord (Hubbard 1979). After a single dose of 40.0 Gy a reduction in both cell types was noticed at 1 month after irradiation but in the month thereafter nuclear density returned to normal or was increased. This effect, however, was only temporary and nuclear density decreased and extensive loss of mainly oligodendrocytes was seen just prior to the development of white matter necrosis. The authors reported no vascular lesions and stated that it was unlikely that white matter necrosis resulted from damage to vessel walls, at least after 40.0 Gy.

The group of Gilmore studied the effect of radiation on the neuroglial cell population of both adult and perinatal CNS. The effect of a single dose of 20.0 Gy to the lumbosacral spinal cord of **3-day-old** rats was investigated after ^{3}H-thymidine injection 2 h before sacrificing the animals (Gilmore 1978). In addition to the normal changes that occur in the labeling index (LI) of these 3-day-old rats, a steeper fall in the LI was observed during the first week after irradiation in the irradiated areas of the spinal cord. In the second week, however, the LI increased above control levels and returned to normal at 23 days post irradiation (p.i.). At 7 and 11 days p.i. the LI in the spinal cord regions adjacent to the irradiated area was increased as compared with non-irradiated controls and it was suggested that glial cells from the non-irradiated parts of the spinal cord were involved in the repopulation of the irradiated zone (Gilmore 1978). The turnover of glial cells (astrocytes, oligodendrocytes and microglia) was also studied after irradiation of the cervical spinal cord of **adult** rats (Hornsey 1981). An early rise in LI of neuroglial cells at 16 to 21 days after irradiation was attributed to an extended cell cycle and a radiation-induced block to cell progression at the G_1/S border. A second increase of the LI was seen at 120 to 125 days after irradiation and seemed to preceed the period at which late radiation damage becomes apparent. The authors considered the increased proliferative activity as an attempt of the irradiated tissue to compensate for radiation-induced cell loss which would lead to functional failure or anatomical dysfunction. These cellular processes were, however, not always able to prevent white matter necrosis 4 to 12 months after a single dose of 20 Gy.

Tissue organization and irradiation of the CNS.

Expression of radiation-induced damage, however, depends not only on the turnover rate of functional cells but also on tissue organization. To understand tissue responses after irradiation and to develop new treatment strategies

identification of the different cell types and the way they are organized is essential. Few tissues are thought to be completely built at the time of birth (or shortly thereafter) to form a static population throughout life without cell renewal. The neurones of the CNS and PNS might be the only exception in mammals. Neurones that die will not be replaced by new ones and will be lost forever. Other cells, however, may take over their function. Cell death of neurones due to irradiation occurs only after very high doses, which are not used in clinical radiotherapy but could be received after a nuclear accident. All other mammalian tissues are thought to consist of functional cells with a more or less restricted life span, which will be replaced by new cells when lost by natural attrition or trauma. On the basis of how cell renewal takes place, tissues can be divided in two fundamentally different types: hierarchical (= type-H) and flexible (= type-F) tissues (Wheldon 1982).

Hierarchical tissues consist of different compartments, which have their own specific function. First, there is a stem cell compartment which is relatively small. Stem cells have the capacity to divide and produce new stem cells as well as daughter cells committed to differentiate. Differentiation is a gradual process and takes place in the second or transit compartment. In this compartment the committed cells undergo several divisions before they become postmitotic functional cells. The functional cells form the third compartment of a hierarchical tissue.

In *flexible* tissues cells are not organized in different compartments but all cells seem to have equal potentials. They all have the capacity to perform their tissue specific functions, but when necessary these cells dedifferentiate and become stem cells that are able to divide and generate new functional cells. Examples of hierarchical tissues (type-H tissues) are skin, the hematopoietic system and intestines. Liver, lung and CNS are proposed to be examples of flexible tissues (type-F tissues) (Wheldon and Michalowski 1986).

The way a tissue is organized determines the sequence of events following a specific cytotoxic injury. In type-F tissues, all cells are susceptible to radiation-induced reproductive cell death and if lethal damage is inflicted the cell will die after one or a few divisions. In type-H tissues, the postmitotic functional cells are only damaged after very high doses and in clinical practice only dividing cells are susceptible to radiation-induced damage. The degree of damage to the stem cell compartment will eventually determine the severity of the permanent injury.

The life span of functional cells will determine the latent period between irradiation and expression of damage. If the turnover rate of functional cells is high, damage will be expressed quickly in a type-H tissue. In a type-H tissue the latent period is more or less dose-independent. In contrast, the expression of

radiation-induced injury in a type-F tissue not only depends on the life span of functional cells but also on the degree of damage.

In studying the cellular events after irradiation of the CNS and in solving the contribution of the oligodendroglia in the development of radiation-induced injury, especially white matter necrosis, it is of interest to know whether the oligodendroglia is organized as a type-F or type-H tissue. The literature supports evidence for both possibilities.

Oligodendroglia: Type-H versus Type-F.

In vitro experiments have provided conclusive evidence that immature glial cells (O-2A progenitor cells) from the perinatal and rat adult CNS are able to differentiate in either oligodendrocytes or type-2 astrocytes (Raff 1983; Wolswijk 1989). These immature precursor cells can be stimulated to divide by specific mitogens and are able to generate new immature cells (self renewal). From these *in vitro* experiments the O-2A lineage seems to be a typical type-H tissue in the perinatal as well as adult CNS. In the literature, however, there is still no common opinion about the origin of myelinating cells in the adult CNS, *in vivo*.

Bunge et al. (1961) were the first to report remyelination after traumatic demyelination of the adult cat spinal cord. Other demyelinating models were developed to study the process of de- and re-myelination. In one of these models demyelination of the superior cerebellar peduncles in 3-week-old mice was induced by a diet containing 0.6% Cuprizone (bis-cycloxaldihydrazone) (Ludwin 1979). The subsequent demyelination is almost complete after 7 to 8 weeks. If animals are placed on a normal diet again, remyelination occurs within 6 weeks. Light and electron microscopy was combined with autoradiography to study the process of remyelination and revealed that demyelination was followed by an increase in macrophages and microglia, both involved in clearing the myelin debris. From about the fifth week, a high mitotic activity of immature glial cells was observed, which preceded the period of remyelination. The author failed to find ^{3}H-thymidine in mature oligodendrocytes. ^{3}H-thymidine, however, was found in remyelinating oligodendrocytes which were labeled during the period of immature glial cell proliferation. A more accurate identification of the cells involved in the process of remyelination was achieved by combining immunocytochemistry with autoradiography *in vivo* after a virus-induced demyelinating event of adult mouse spinal cord (Godfraind 1989).

Cells with the O-2A progenitor phenotype (O_4^+, CNP^-) (CNP = 2',3' cyclic-nucleotide 3' phosphohydrolase is expressed by oligodendrocytes) incorporated ^{3}H-thymidine and increased in number prior to the onset of remyelination. This observation and the appearance of ^{3}H-thymidine, administered during the phase

of demyelination, in newly formed oligodendrocytes suggested that newly formed oligodendrocytes originated from mitotic $O4^+$ CNP^- $GFAP^-$ precursor cells.

Evidence for remyelination through immature precursor cells has also been found in the human CNS. Prineas et al. (1989) investigated lesions of multiple sclerosis in the human brain and spinal cord with immunohistochemical markers and by studying antigens known to be sequentially expressed during development, to determine whether the remyelinating cells were newly generated or were residual mature oligodendrocytes. [During development oligodendrocytes first express GalC and CNP, followed by carbonic anhydrase (CA), myelin basic protein (MBP) and myelin-associated glycoprotein (MAG), before axon ensheathment commences.] Their conclusion was that all oligodendrocytes are destroyed in MS lesions and that the cells responsible for remyelination were not residual postmitotic oligodendrocytes but newly generated cells.

Ghatak et al. (1989) recently presented a histological study indicating that remyelination occurring in the human CNS can lead to full recovery from neurological deficits. A 15-year-old boy presented with headache and visual loss combined with white matter changes on CT- and MRI-scan of the brain. Biopsies of these lesions demonstrated mitoses in small mononuclear cells, presumably oligodendrocytes, in areas of demyelination. Also many oligodendrocytes, often in small clusters, were seen starting to myelinate bare axons. The nature of the demyelinating disease remained unclear, but the neurological abnormalities disappeared completely within several months and serial MRI-scans demonstrated a nearly complete resolution of the white matter abnormalities.

Although the results of these *in vivo* studies confirm the type-H organization of oligodendroglia, other authors have found evidence that also mature oligodendrocytes are able to proliferate and are an important source of new myelinating cells in the adult CNS. Arnella and Herdon (1984) caused by Lysolecithin injection demyelinating lesions in the thoracic spinal cord of 9- to 10-month-old mice. One hour after pulse-labeling with ^3H-thymidine, cells with the characteristics of mature oligodendrocytes were found to have label over their nuclei. Wood and Bunge (1986 a,b) cultured cell suspensions derived from adult rat spinal cord on dorsal root ganglion neurones and observed ^3H-thymidine uptake and mitotic figures in pre-labeled $GalC^+$ cells. The *in vitro* $GalC^+$ dividing cells were only a small fraction (3-5%) of all $GalC^+$ cells and certainly most of them did not express MBP. This indicated that these oligodendrocytes were relatively immature and did not myelinate axons at the time of proliferation. Antibody-complement mediated cell killing with anti-GalC resulted in cultures without oligodendrocytes. In the subsequent culture period only sporadically colonies of oligodendrocytes appeared and the authors concluded that $GalC^+$

oligodendrocytes are the main source of new myelinating cells in the adult CNS and few oligodendrocytes were derived from GalC$^-$ precursor cells. The authors point out that there is a clear difference between the perinatal and adult CNS: GalC$^-$ precursor cells are the source of oligodendrocytes in the perinatal CNS as demonstrated by Raff et al. (1983) and also by Bologna et al. (1982), but GalC$^+$ cells are the main source of new oligodendrocytes in the adult CNS. Wood and Bunge did not consider the possibility that complement alone was cytotoxic to adult and not to perinatal glial precursor cells, as has been demonstrated by Wren and Noble (1989). Again, Wood and Bunge did not mention this finding in their recent publication (1991).

Recently, Bögler et al. (1990) confirmed the proliferative capacity of perinatal GalC$^+$ cells *in vitro* when cultured in the presence of fibroblast growth factor. The question remains whether culture conditions reflect sufficiently the *in vivo* situation. This also applies to cell cultures derived from adult CNS. To obtain single cell suspensions of the CNS its normal architecture becomes totally destroyed and manipulations (such as fluoresence activated cell sorting; Wood 1991) have as a consequence that the many fragile processes of myelinating oligodendrocytes are broken off. If these myelinating cells survive the *in vitro* manipulations they obtain the morphology of round undifferentiated cells which are GalC$^+$ and MBP$^-$ and maybe these cells are capable to divide. It is, however, difficult to imagine that fully mature MBP$^+$ oligodendrocytes which have a highly specialized function *in vivo* and myelinate up to 50 axons are able to withdraw from their function by dedifferentiation and return to the generation of new cells. Furthermore, if mature oligodendrocytes are able to proliferate and if they are the source of new oligodendrocytes, O-2A progenitor cells that are widely present in the adult CNS would have no function.

Conclusion.

Reviewing the literature it may be concluded that oligodendrocytes are normally derived from immature glial precursor cells both in the perinatal and adult CNS. Specific conditions, however, may lead to the division of GalC$^+$ oligodendrocytes, that are relatively immature and not involved in the process of myelination. There is no conclusive evidence that myelinating oligodendrocytes are the main source of new oligodendrocytes in the adult CNS *in vivo*. We suggest, therefore, that the myelinating cells in the perinatal and adult CNS are mainly ordered as an H-type tissue and radiation injury leading to permanent depletion of myelinating cells is likely to be expressed through the stem cell compartment. However, the possibility that the myelinating cells of the CNS belong to an intermediate lineage with characteristics of both Hierarchical and

Flexible tissues can not be excluded. The existence of such lineages has been suggested by Wheldon and Michalowski (1986) on a theoretical basis. By the latter concept all tissues may be considered as intermediate, localized somewhere in the spectrum between truly hierarchical and truly flexible.

CHAPTER 5

AIMS AND OUTLINE OF THE PRESENT STUDY

The central nervous system (CNS) is one of the most critical dose limiting normal tissues in the treatment of diseases with radiotherapy. Different syndromes may develop after irradiation of the CNS, but the syndromes that develop after a relatively long latent period (≥ 4 months) are clinically the most relevant (chapter 1). They may lead to severe disablement and even death. Histological studies of the injured CNS have led to the recognition of different pathological entities and many authors have speculated on their way of development. Elucidation of the mechanisms leading to the chronic effects in the CNS may provide tools to enhance the tolerance of the CNS for radiation. Experimental studies have led to the recognition of treatment variables that drastically influence the radiation tolerance of the CNS (e.g. fraction size; chapter 2). The poor treatment results of for example malignant brain tumors, however, stress the need to explore new ways to enhance the tolerance of the CNS even further. The main problem in this area of research is that the critical steps in the development of radiation-induced damage are unknown. Identification of target cells and factors that influence tissue responses in the development of radiation injury may provide possibilities to intervene.

In recent neurobiological studies new developments have led to the discovery of different glial cell types (chapter 3). *In vitro* analysis elucidated the existence of different glial cell lineages, relationships between different lineages and growth factors involved in cell proliferation and differentiation. Our main interest was raised by the O-2A progenitor cell. This stem cell is present in the perinatal as well as in the adult CNS and is the source of new oligodendrocytes and type-2 astrocytes. The factors that control its proliferation and differentiation are known and provide possibilities for manipulation. Oligodendrocytes are the myelinating cells of the CNS and because one of the delayed syndromes is restricted to the white matter and is characterized by demyelination and necrosis, the precursor cells of oligodendrocytes (O-2A progenitor cells) may be the target cells of this type of radiation injury. This hypothesis is the basis of the present study.

One of the aims was to investigate whether the new developments in neurobiology could be applied in the field of radiobiology. An *in vitro* clonogenic assay was developed (chapter 6) and was used as the main experimental tool to study the effect of radiation on O-2A progenitor cells. This method enabled us to investigate some radiobiological characteristics of O-2A progenitor cells: their

radiosensitivity (chapter nrs. 7; 8), repair capacity (chapter 9) and regeneration profile (chapter 10). Although the main question, whether O-2A progenitor cells are the critical cells in the development of radiation-induced demyelination and white matter necrosis remains unanswered, the present study indicates that radiation responses in the CNS can be studied at the cellular level for well defined cell populations. When this kind of research will be combined with the visualization and quantification of O-2A progenitor cells *in vivo*, the role of these specific glial cells in the development of radiation-induced injury may be determined. It will also provide the opportunity to evaluate the influence of the administration of growth factors or the influence of glial stem cell transplantation on the radiation tolerance of the CNS.

CHAPTER 6

AN *IN VITRO* CLONOGENIC ASSAY TO ASSESS RADIATION DAMAGE IN RAT CNS GLIAL PROGENITOR CELLS

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Abstract.

Normal glial progenitor cells can be isolated from the rat central nervous system (CNS) and cultured in vitro on a monolayer of type-1 astrocytes. These monolayers are able to support and stimulate explanted glial progenitor cells to proliferate. Employing these in vitro interactions of specific glial cell types, an in vivo-in vitro clonogenic assay has been developed. This method offers the possibility to study the intrinsic radiosensitivity, repair, and regeneration of glial progenitor cells after in vitro or in vivo irradiation.

Introduction.

After irradiation the CNS exhibits specific late types of injury that are time- and dose-dependent. An early delayed syndrome is mainly characterized by demyelination and white matter necrosis, mainly restricted to the white matter, while a later syndrome is mainly restricted to pathological vascular changes in both white and grey matter. Although classical histological techniques have greatly contributed to a better understanding of the pathological events involved in the various CNS radiation syndromes, these methods do not allow the identification of the roles of specific potential target cells. The old controversy in radiobiology whether depletion of parenchymal or endothelial cells is responsible for radiation-induced syndromes in late-responding tissues, e.g. the CNS, is therefore unlikely to be resolved when relying solely on histological investigations (Hopewell, 1980; Van der Kogel, 1986).

Recent developments in neurobiology such as the availability of specific antibodies, make it possible to study different glial cell types *in vitro*. In cultures derived from perinatal rat optic nerve two different cell lineages have been characterized (Raff et al, 1983; Raff, 1989). One lineage gives rise to type-1 astrocytes while the O-2A lineage is specialized for myelination (ffrench-Constant and Raff, 1986). So-called O-2A progenitor cells are able to differentiate into oligodendrocytes or type-2 astrocytes, depending on the culture conditions. Oligodendrocytes form myelin and ensheath axons with cytoplasmic membranes. Type-2 astrocytes contact with their processes the nodes of Ranvier, while glutamate receptors on the surface of these cells suggest glial-neuronal signalling at the node. Division of O-2A progenitor cells is stimulated by growth factors produced by type-1 astrocytes (Noble and Murray, 1984) (Figure 6.1). After originally being discovered *in vitro* in cell suspensions derived from perinatal optic nerves, O-2A progenitor cells were subsequently isolated from the CNS of perinatal as well as adult rats (Raff and Lillien,1988; Wolswijk and Noble, 1989). The offspring of these migratory cells are able to replace oligodendrocytes lost due to normal attrition or cytotoxic injury. Radiation-induced depletion of the stem cell compartment in the oligodendrocyte lineage is expected to impair the replacement of myelin-forming cells, resulting in demyelination.

The availability of an *in vitro* clonogenic excision assay allows the quantitative assessment of cytotoxic injury at the stem cell level, giving the possibility to analyse the correlation of changes in this potential target cell population with the occurrence of demyelinating lesions. Using this assay it has been possible to derive cell survival curves and quantitate the presence of viable progenitor cells after *in vivo* irradiation of different regions of the CNS. This *in vitro* analysis of radiation-induced damage of glial progenitor cells seems a powerful

tool to help unravel some of the long-standing questions about the mechanisms of radiation-induced CNS injury.

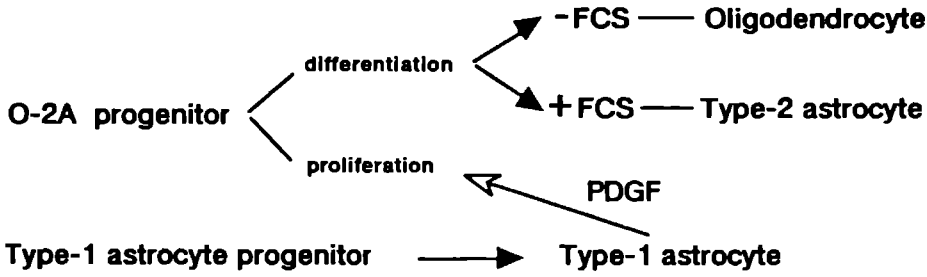


Figure 6.1 The two different glial cell lineages in the perinatal rat optic nerve, as characterized *in vitro*. The first lineage consists of O-2A progenitor cells that can either differentiate into oligodendrocytes in the absence of fetal calf serum (FCS) or differentiate into type-2 astrocytes in the presence of FCS. The second lineage consists of type-1 astrocyte progenitor cells that give rise to type-1 astrocytes. Type-1 astrocytes produce growth factors [e.g. platelet-derived growth factor (Raff et al. 1988)] that stimulate the proliferation of O-2A progenitor cells.

Materials and Methods.

The assay consists of three main procedures.

1. Preparation of monolayers of type-1 astrocytes from the cortex of neonatal rats.
2. Preparation of CNS cell suspensions containing the progenitor cells to be investigated.
3. Immunofluorescent staining with cell type-specific antibodies.

The assay is based on various culture techniques developed for CNS tissue and derived its main input from the work of McCarthy and De Vellis, Raff, Noble and coworkers (McCarthy and De Vellis, 1980; Raff et al, 1983; Noble and Murray, 1984; Wolswijk and Noble, 1989).

Preparation of monolayers of type-1 astrocytes.

The cortices of 1-day-old Wistar rats are dissected out, freed from meninges, and cut into fine pieces. The pieces are treated with collagenase (133 U/ml) in Leibowitz L-15 medium at 37°C. After 1 h, a quarter volume of trypsin (30,000 U/ml) in Ca^{2+}-,Mg^{2+}-free Dulbecco's modified Eagle's medium (DMEM-

CMF) is added for 15 min. After centrifugation the medium is replaced by DMEM-CMF containing EDTA (0.54 mM) and trypsin (7,500 U/ml). After 15 min the reaction is stopped with soybean trypsin inhibitor and DNAse (SBTI-DNAse). Following a 10 min incubation period the the material is centrifuged and the supernatant replaced by DMEM containing 10% fetal calf serum (DMEM plus 10% FCS). The tissue is further dissociated by trituration through a pipette. The resulting cell suspension is seeded onto poly-L-lysine (PLL)-coated tissue culture flasks (75 cm^2) at a density of two cortices per flask. The cells are grown in DMEM plus 10% FCS: 70% of the medium is changed 3 times a week. After 10-14 days a monolayer, consisting predominantly of type-1 astrocytes, has formed, with other cells (e.g. O-2A progenitor cells and oligodendrocytes) growing on top. The cultures are then shaken thoroughly to remove the top cells and treated with cytosine arabinoside (Ara-C, $2x10^{-5}M$) for 2 periods of 2 days, to kill remaining proliferating cells. Finally the type-1 astrocytes are trypsinized and re-plated into smaller PLL-coated flasks (25 cm^2). After 2-4 days a monolayer has formed and the cultures are irradiated with 20 Gy of X-rays to prevent colony formation of any remaining clonogenic cells of the O-2A lineage. Control cultures are always included to evaluate the presence of cells capable of giving rise to colonies of the O-2A lineage. After the above treatment, only sporadically are cells with the characteristics of O-2A progenitor cells seen.

Cultures are maintained in an incubator at 37.5°C in a water-saturated 7.5% CO_2 + 92.5% air atmosphere.

Preparation of CNS cell suspensions and the design of irradiation experiments.

Different parts of the rat (Wistar) CNS (e.g., optic nerve, spinal cord, corpus callosum) can be dissected out and dissociated. To obtain a single cell suspension they are cut into small pieces and treated enzymatically. First they are treated with collagenase in L-15 for 1 h at 37°C. Trypsin is then added for 15-20 min and the cell suspensions centrifuged (500*g*, 5 min). After removal of the supernatant they were resuspended in DMEM-CMF, containing trypsin and EDTA (0.54 mM). Concentrations of collagenase and trypsin differ for the perinatal or adult CNS. For the perinatal CNS the concentrations of collagenase and trypsin are 667 U/ml and 6,000 U/ml, respectively, while for adult CNS 333 U/ml collagenase and 15,000 U/ml trypsin are used. The enzymatic digestion is stopped with SBTI-DNAse and a single cell suspension is obtained by trituration through 25G and 27G needles. Numbers of cells, adjusted to take account of the radiation dose, for perinatal tissues, and measured aliquots for adult tissues (counting cells in dissociated adult tissue is not possible due to the low cell number and the high concentration of myelin) are plated onto monolayers of type-

1 astrocytes. To ensure a high probability of counting colonies derived from single cells, cells are plated at a density of approximately two or three assumed survivors per cm^2. Cells are allowed to adhere for 3-4 h in DMEM plus 10% FCS before changing the medium into a chemically defined medium containing 0.5% FCS [Bottenstein-Sato/0.5% FCS (Bottenstein and Sato 1979)]. Half of the latter medium has previously been conditioned for 3 days by type-1 astrocytes. Employing this system, cultures have been maintained for up to 9 weeks without any signs of deterioration. To obtain cultures in which most of the colonies are ≥ 50 cells, perinatal CNS cultures are maintained for approximately 2 weeks and adult CNS cultures for 4-5 weeks [the cell cycle time of adult progenitor cells is 2-3 times the cell cycle time of perinatal O-2A progenitors (Wolswijk and Noble, 1989)]. Three times a week 30% of the medium is replaced by fresh Bottenstein-Sato/0.5% FCS.

Cells can be irradiated *in vivo* or *in vitro*. If they are irradiated *in vitro* it is done after the adherence period of 3-4 h but before the medium change. Irradiations were performed with 4 MV X-rays at a dose rate of 2.7 Gy min^{-1}.

Immunofluorescence staining and evaluation of cultures.

Cultures are stained directly in the flasks with the monoclonal antibodies A_2B_5 (Eisenbarth et al. 1979) and anti-galactocerebroside (anti-GalC)(Raff et al. 1978) to identify O-2A progenitor cells and oligodendrocytes. A_2B_5 is directed against certain gangliosides present on the membrane of O-2A progenitor cells, type-2 astrocytes and neurones. Cultures are maintained under conditions that allow only O-2A progenitor cells to proliferate, so A_2B_5 is specific for these colonies. GalC is a glycolipid expressed only on the surface of oligodendrocytes in the CNS. A_2B_5 and anti-GalC monoclonal antibodies are added simultaneously to the cultures (hybridoma culture supernatant; dilution 1:2). As second-layer antibodies goat anti-mouse IgM conjugated with fluorescein and goat anti-mouse IgG3 -rhodamine (both diluted 1:200 and obtained from Southern Biotechnology Associates, Alabama) are used to visualize A_2B_5 and anti-GalC antibodies, respectively. After staining, cells are fixed with methanol (-20°C,10 min). Every staining step is followed by three washes with a HEPES/5% bovine serum containing buffer. Finally the bottoms of the culture flasks are cut out, mounted in glycerol containing 22 mM 1,4-diazobicyclo [2,2,2] octane, covered with a large coverslip (18 cm^2) and sealed with nail varnish.

The potential of O-2A progenitor cells to differentiate into type-2 astrocytes was tested by adding FCS (DMEM + 20% FCS) to the cultures. Afterwards, cultures can be stained with A_2B_5 and a (rabbit) serum against glial fibrillary acidic protein (GFAP)(Bignami et al., 1972) to identify type-2 astrocytes (cells positive for

both antibodies). To visualize GFAP a goat anti-rabbit serum conjugated with rhodamine is used (dilution 1:25, Laboratories Inc.,USA). Access to intracellular GFAP was obtained by permeabilizing cell membranes with methanol (-20°C , 10 min) after staining with A_2B_5 and its second-layer antibody.

The cultures are screened with a Zeiss Axiovert microscope equipped for epifluorescence. Figure 6.2 shows a colony derived from adult optic nerve after 3 weeks in culture.

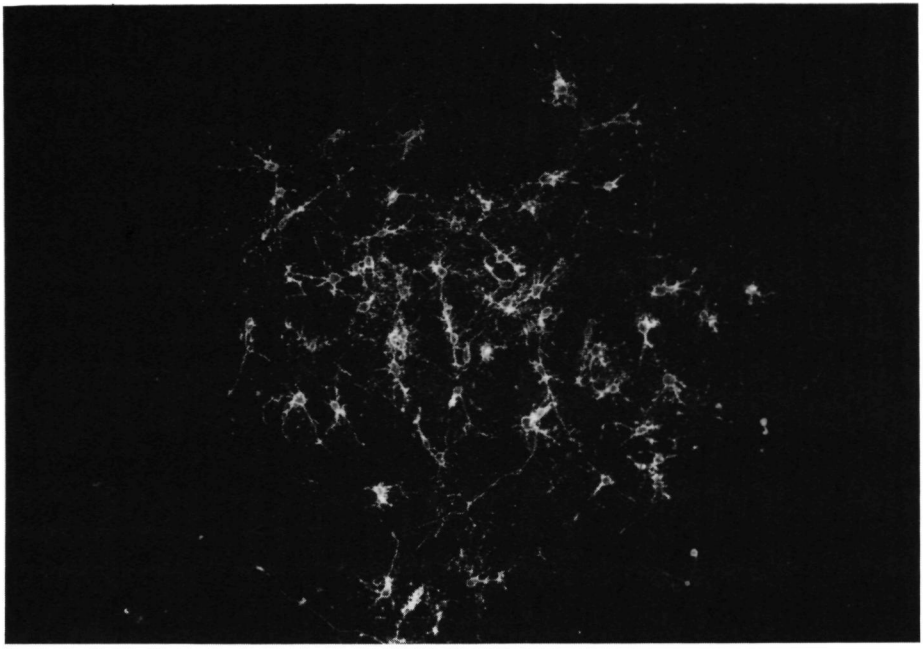


Figure 6.2 A colony of O-2A progenitor cells derived from adult rat (3 months old) optic nerves after 3 weeks in culture. The colony is stained with A_2B_5 monoclonal antibody visualized by anti-IgM conjugated with fluorescein (x100).

Applications.

With the described system it is now possible to perform a quantitative analysis of the clonogenic capacity of glial progenitor cells, after irradiation either *in vitro* or *in vivo*. The acute single dose-survival curve for 1-day-old optic nerve progenitor cells after *in vitro* irradiation is given as an example (Figure 6.3). The number of colonies was counted after 13 days in culture and after correction for colony-forming efficiencies (2.6% to 3.5%), a survival curve was constructed. The combined results of three independent experiments that were performed in duplicate are presented. The obtained survival curve was fitted with the linear-

quadratic (LQ-) model of cell survival, yielding values of the α and β parameters of 0 475 Gy^{-1} and 0.108 Gy^{-2}, respectively

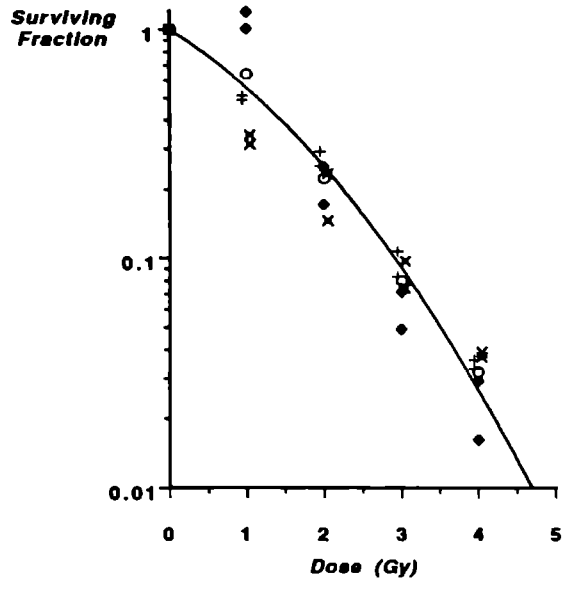


Figure 6.3 Survival curve of perinatal optic nerve O-2A progenitor cells derived from 1-day-old rats Data points of three different experiments performed in duplicate are plotted together with the mean values (open symbols) and fitted according to the LQ-model Cultures were maintained for 13 days

The survival curve obtained after *in vitro* irradiation was compared with survival data obtained from irradiating 1-day-old optic nerves *in vivo* Immediately after whole body irradiation with 2 Gy or 4 Gy of 1-day-old rats, the optic nerves were dissected out and single cell suspensions were prepared Radiation-dose-adjusted cell numbers were plated onto monolayers of type-1 astrocytes and after 13 days *in vitro* the cultures were stained and the number of colonies was counted. A comparison with the survival curve obtained for *in vitro* irradiated perinatal rat optic nerve O-2A progenitor cells did not show a difference in radiosensitivity (Figure 6 4)

Cells surviving irradiation maintained the properties of non-irradiated O-2A progenitor cells ($A_2B_5^+$), such as the ability to differentiate into oligodendrocytes (GalC$^+$) or type-2 astrocytes ($A_2B_5^+$, GFAP$^+$), as can be seen in Figure 6.5. To demonstrate that surviving progenitor cells were still bipotential they were cultured with and without FCS If cultures were maintained in a chemically defined medium without FCS, the development of $A_2B_5^+$, GalC$^-$ O-2A progenitor cells and

$A_2B_5^-$, GalC+ oligodendrocytes was seen within the colonies If after 1 week the medium of these cultures was changed to DMEM plus 20% FCS, many of the cells in almost all the surviving colonies could be induced to differentiate into type-2 astrocytes This demonstrates the preservation of the multipotential character of O-2A progenitor cells in surviving colonies after irradiation

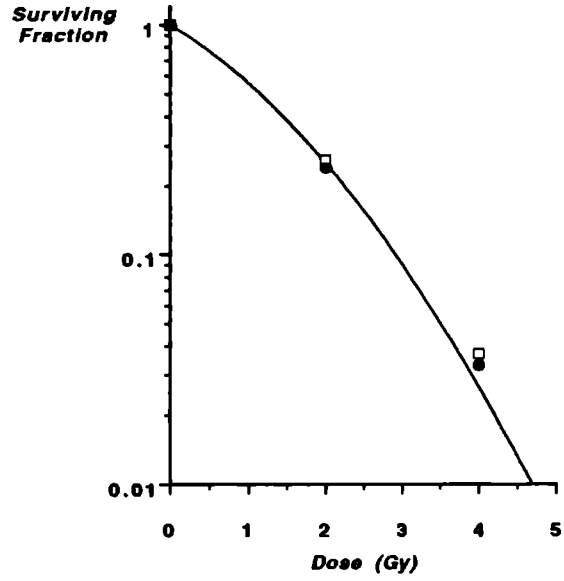
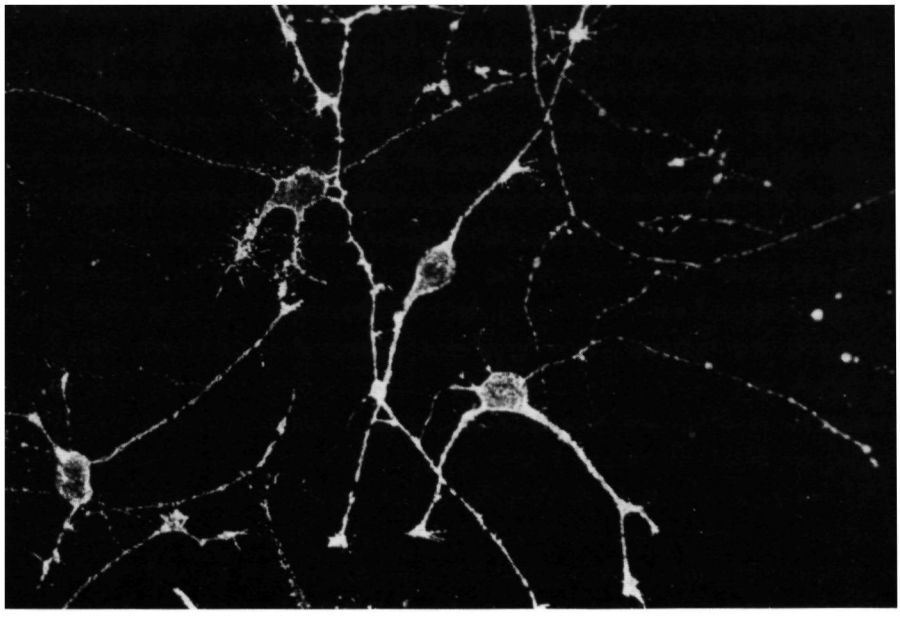
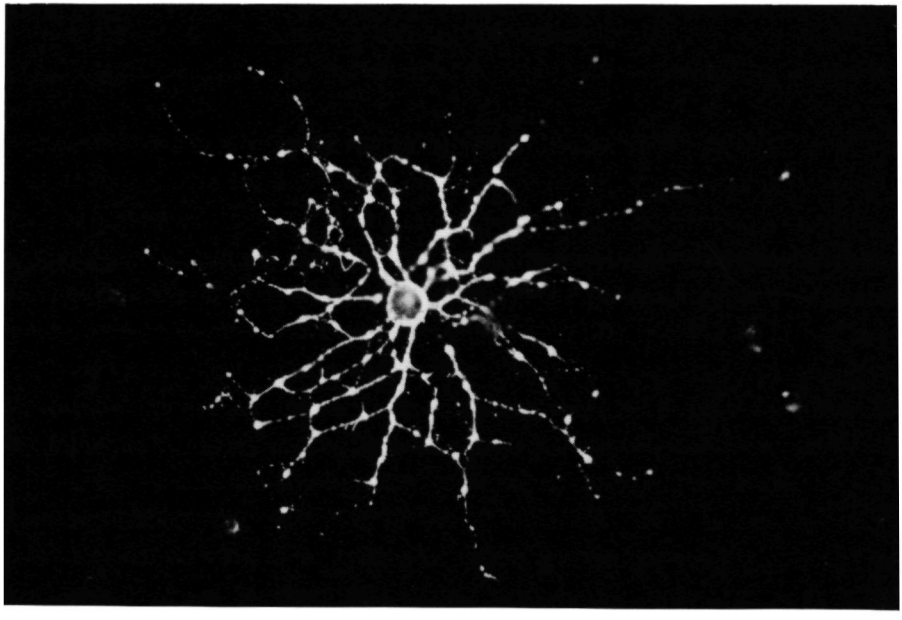


Figure 6.4 Survival data of O-2A progenitor cells derived from 1-day-old rat optic nerves irradiated *in vivo* (0 Gy, 2 Gy and 4 Gy) are compared with the mean survival curve of O-2A progenitor cells derived from 1-day-old rat optic nerves irradiated *in vitro* (Figure 6 3) The experiment was performed twice and the mean values of 4 different culture flasks are plotted

With these methods the hypothesis can be tested that demyelination and white matter necrosis after irradiation of the CNS is due to depletion of O-2A progenitor cells by *in vitro* analysis The intrinsic radiosensitivity, repair and regeneration capacities of O-2A progenitor cells can be studied quantitatively after *in vivo* irradiation and isolation of cells at various time intervals The method has been applied to different regions of the CNS (optic nerve, corpus callosum, spinal cord) in not only perinatal but also adult animals



(a)



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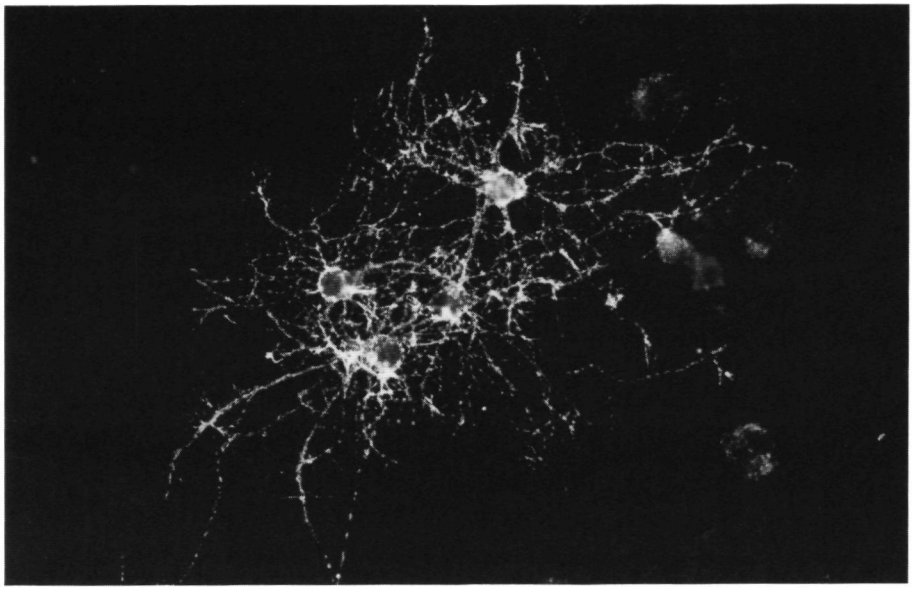
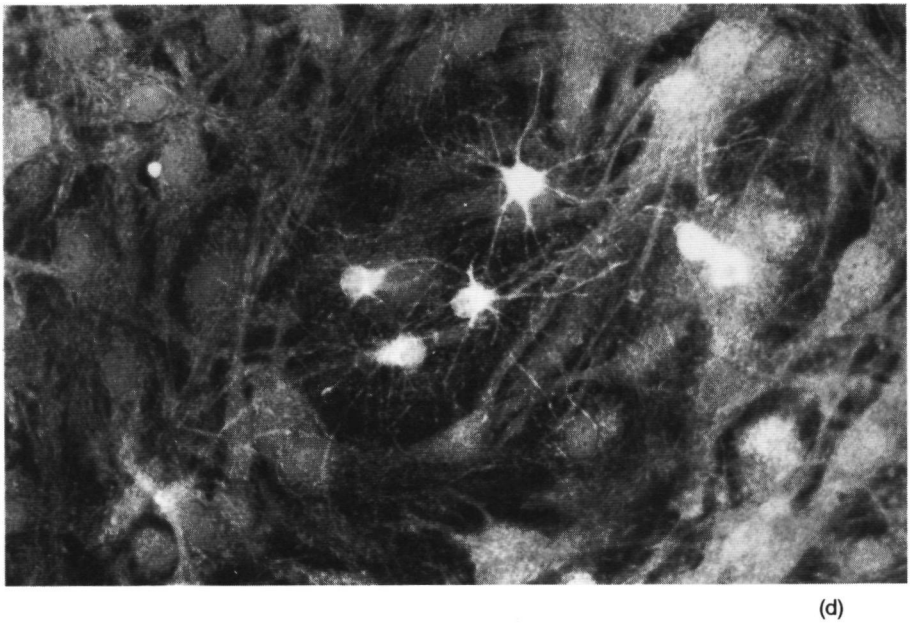


(c)



(d)

Figure 6.5 Cells in colonies of perinatal rat optic nerve that survived 4 Gy of X-rays are shown. (a) shows typical bipolar perinatal O-2A progenitor cells. They are visualized using the A_2B_5 antibody. (b) shows a $GalC^+$ oligodendrocyte with its fine branching processes. (c) and (d) show type-2 astrocytes (4 cells) that are stained with A_2B_5 (c) and an anti-GFAP polyclonal antibody (d). Note that the monolayer of type-1 astrocytes is also positive for GFAP.

CHAPTER 7

RADIOSENSITIVITY OF GLIAL PROGENITOR CELLS OF THE PERINATAL AND ADULT RAT OPTIC NERVE STUDIED BY AN *IN VITRO* CLONOGENIC ASSAY

R.W.M. van der Maazen, I. Verhagen, B.J. Kleiboer and A.J. van der Kogel
Radiotherapy and Oncology, 1991, 20, 258-264

Abstract.

The cellular basis of radiation-induced demyelination and white matter necrosis of the central nervous system (CNS), is poorly understood. Glial cells responsible for myelination in the CNS might be the target cells of this type of damage. Glial cells with stem cell properties derived from the perinatal and adult rat CNS can be cultured in vitro. These cells are able to differentiate into oligodendrocytes or type-2 astrocytes (O-2A) depending on the culture conditions. Growth factors produced by monolayers of type-1 astrocytes inhibit premature differentiation of O-2A progenitor cells and allow colony formation. A method which employs these monolayers of type-1 astrocytes to culture O-2A progenitor cells has been adapted to allow the analysis of colonies of surviving cells after X-irradiation. In vitro survival curves were obtained for glial progenitor cells derived from perinatal and adult optic nerves. The intrinsic radiosensitivity of perinatal and adult O-2A progenitor cells showed a large difference. Perinatal O-2A progenitor cells are quite radiosensitive, in contrast to adult O-2A progenitor cells. For both cell types an inverse relationship was found between the dose and the size of colonies derived from surviving cells. Surviving O-2A progenitor cells maintain their ability to differentiate into oligodendrocytes or type-2 astrocytes. This system to assess radiation-induced damage to glial progenitor cells in vitro seems to have a great potential in unraveling the cellular basis of radiation-induced demyelinating syndromes of the CNS.

Introduction.

The central nervous system (CNS) is often a critical structure in the treatment of malignant diseases with X-rays. If the CNS is irradiated above certain threshold doses functional damage occurs after several months, making the CNS a so-called late reacting tissue. Possible target cells are endothelial cells or glial cells, the parenchymal cells of the CNS. Radiation-induced syndromes, although expressed in similar neurological impairment, differ pathologically (Van der Kogel 1991). Some syndromes are characterized by hemorrhage and vascular abnormalities, and endothelial cells are the most likely target cells. Other syndromes, however, are restricted to the white matter and show an extensive demyelination. This could be due secondary to vascular damage but it is our hypothesis that the depletion of cells responsible for myelination in the CNS (oligodendrocytes and their progenitor cells)(ffrench-Constant and Raff 1986) plays a critical role in this specific pathological condition.

The existence of glial progenitor cells was uncertain until *in vitro* studies unequivocally showed that cells with stem cell properties could be isolated from perinatal rat optic nerves (Raff et al. 1983). These cells were able to differentiate into oligodendrocytes or type-2 astrocytes (O-2A progenitor cells), depending on the culture conditions. If O-2A progenitor cells were grown in serum-free medium they differentiated into oligodendrocytes (if no other growth factors were added). Oligodendrocytes are responsible for the myelination in the CNS. If O-2A progenitor cells were cultured in the presence of 10% fetal calf serum (FCS) they differentiated into type-2 astrocytes, glial cells related to the node of Ranvier. The precise function of type-2 astrocytes, however, is not clear.

The clonal expansion of single O-2A progenitor cells could be studied *in vitro* (Temple and Raff 1985; 1986). In this system, O-2A progenitor cells were grown in serum-free medium on monolayers of purified type-1 astrocytes. Growth factors produced by these monolayers of type-1 astrocytes prevented premature differentiation of proliferating O-2A progenitor cells (Noble and Murray 1984). We have adapted this system for use with larger size tissue culture flasks which allows the seeding of progenitor cells at a sufficiently low density to prevent overlap of colonies (Van der Maazen et al. 1990). Employing this assay, the intrinsic radiosensitivity as well as repair and regeneration characteristics of glial stem cells can be studied after irradiation *in vivo* or *in vitro*.

In this article we present the survival curves for glial progenitor cells isolated from perinatal or adult optic nerves. The optic nerve was chosen as a source of glial cells to investigate radiation-induced damage because it is a pure white matter structure and it is until now, *in vitro*, the best characterized part of the CNS with respect to glial cell relationships and function. In addition to differences

in morphological, antigenetic and growth characteristics [Table 7.1, (Wolswijk and Noble 1989)], a large difference in radiosensitivity was observed for perinatal compared to adult progenitor cells.

Table 7.1 Characteristics of perinatal and adult O-2A progenitor cells [adapted from Wolswijk et al. (1990)].

	Source of O-2A progenitor cells:	
CHARACTERISTIC	PERINATAL OPTIC NERVE	ADULT OPTIC NERVE
-morphology	bipolar	unipolar
-O_4 labeling	negative	positive
-intermediate filaments	positive	negative
-cell cycle time	18 ± 4 hours	65 ± 18 hours
-rate of migration	21.4 ± 1.6 μm/h	4.3 ± 0.7 μm/h

Materials and methods.

In this study, glial progenitor cells were isolated from perinatal or adult rat optic nerves and subsequently grown on monolayers of purified type-1 astrocytes, obtained from cortices of newborn rats. A detailed description of the *in vitro* clonogenic assay is published separately (Van der Maazen 1990).

Preparation of monolayers of type-1 astrocytes.

Cortices of one-day-old Wistar rats were dissected and mechanically dissociated. The pieces were enzymatically digested (incubation with collagenase for 1 h and two 15 min periods with trypsin) and incubated with soybean trypsin inhibitor and DNAse (SBTI-DNAse) for 10 min. The tissue was further dissociated by trituration through a pipet. Cells were seeded in previously poly-L-lysine(PLL)-coated tissue culture flasks and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. Pure type-1 astrocyte monolayers were obtained by shaking the culture flasks thoroughly and treating them with cytosine arabinoside (Ara-C, $2x10^{-5}$M) to get rid of contaminating cells on top of the monolayers of type-1 astrocytes (McCarthy and De Vellis 1980; Noble and Murray 1984). Type-1 astrocytes were subsequently transferred to 25 cm^2 flasks and after monolayer formation the cultures were irradiated (20 Gy) to prevent colony formation of any remaining clonogenic cells of the O-2A lineage.

Cultures were maintained in an incubator at 37.5 °C in a water saturated 7.5% CO_2 + 92.5% air atmosphere.

Preparation of CNS cell suspensions and design of irradiation experiments.

Optic nerves were dissected free of contaminating meninges and dissociated into single cell suspensions with collagenase and trypsin as described previously (Noble and Murray 1984; Wolswijk and Noble 1989). Enzymatic digestion was stopped with SBTI-DNAse and a single cell suspension was obtained by trituration through 25G and 27G needles. Although the general procedure was the same, different concentrations of enzymes were used to dissociate perinatal (age: 1 day) and adult (age: 4 months) optic nerves. For the perinatal optic nerves the concentrations of collagenase and trypsin were 667 U/ml and 6.000 U/ml, respectively, while for adult optic nerves 333 U/ml collagenase and 15.000 U/ml trypsin were used.

To determine the initial percentage of O-2A progenitor cells and oligodendrocytes in suspensions of perinatal optic nerve 10.000 cells were plated on PLL-coated coverslips and allowed to adhere for 1 h. Cells were then stained with appropriate antibodies and fluorochrome conjugated second layer antibodies, as described below. For adult optic nerve the number of cells and degree of differentiation could not be determined because of the high concentration of myelin debris and low cell number.

Radiation-dose adjusted numbers of cells for perinatal optic nerve and measured aliquots for adult optic nerve were plated on monolayers of type-1 astrocytes. To ensure a high probability of single cell origin of colonies, cells were plated at a density of approximately 2-3 survivors per cm^2. Cells were allowed to adhere for 3 to 4 hours in DMEM + 10% FCS. After irradiation with various doses of X-rays the medium was changed into a chemically defined medium containing 0.5% FCS [Bottenstein-Sato/0.5% FCS (Bottenstein and Sato 1979)]. Half of the latter medium had previously been conditioned for 3 days by type-1 astrocytes. Three times a week 30% of the medium was replaced by fresh Bottenstein-Sato/0.5% FCS.

Irradiations were performed with 4 MV X-rays at a dose rate of 2.7 Gy min^{-1}.

Immunofluorescence staining and evaluation of cultures.

The cultures were stained with the monoclonal antibodies A_2B_5 (Eisenbarth et al. 1979) and anti-galactocerebroside (anti-GalC)(Raff et al. 1978). The A_2B_5 antibody is for the culture conditions described specific for O-2A progenitor cells. Anti-GalC specifically stains oligodendrocytes in the CNS. A_2B_5 and anti-GalC monoclonal antibodies were added simultaneously to the flasks (hybridoma

culture supernatant; dilution 1:2). Goat anti-mouse IgM conjugated with fluorescein and goat anti-mouse IgG3 rhodamine (both diluted 1:200 and obtained from Southern Biotechnology Associates, Alabama) were used to visualize A_2B_5 and anti-GalC antibodies, respectively. The staining procedure ended by fixation with methanol (-20°C,10 min). Every staining step was followed by three washing steps with a HEPES/5% bovine serum containing buffer. Finally, the bottoms of the culture flasks were cut out, mounted in glycerol containing 22 mM 1,4-diazobicyclo [2,2,2] octane, covered with a large coverslip (18 cm^2) and sealed with nail varnish.

The cultures were screened with a Zeiss Axiovert microscope equipped for epifluorescence.

Results.

Plating efficiency.

The crude plating efficiency (PE= number of colonies formed divided by the total number of cells plated) for perinatal optic nerve experiments ranged from 2.7% to 4.6%. Because not all the cells of the initial cell suspension are O-2A progenitor cells, cells were plated onto PLL-coated coverslips and stained with A_2B_5 and anti-GalC antibodies after 1 h. The percentage of $A_2B_5^+$ cells ranged from 16 to 24. So in reality the plating efficiency is underestimated. After correction the plating efficiency will rise approximately 5-fold (14%-25%), which is high for an *in vivo-in vitro* assay. The percentage of $GalC^+$ oligodendrocytes was always less than 1.

This procedure could not be used for adult optic nerve material because of the low cell number and the large amount of myelin debris in adult CNS cell suspensions. So the "PE" for adult optic nerve tissue was determined by plating a standard volume of the cell suspension in control culture flasks to obtain the number of viable colony forming cells per volume-unit. The surviving fractions for irradiated cultures were calculated as the ratio of the number of surviving colonies to the unirradiated controls, corrected for the actual volumes used. The numbers of colonies derived in different experiments were highly reproducible.

Colony size distribution and dynamics of colony growth and differentiation.

The effect of radiation on O-2A progenitor cells was studied at the level of cell-survival, number of cells per colony, and differentiation in the colonies. Because perinatal O-2A progenitor cells have a limited proliferative capacity (Temple and Raff 1985; 1986), endpoints have to be defined for the colony sizes at which progenitor cells are thought to be unaffected by radiation with respect to proliferative capacity. The initial criterion was based on data presented in the

literature and was set at the relatively low number of at least 6 $A_2B_5^+$ and/or $GalC^+$ cells per colony. This criterion was used for perinatal optic nerve progenitor cells that were grown for 6 days *in vitro* and for adult optic nerve progenitor cells that were grown for 13 days *in vitro*. It was subsequently found that the colonies kept *in vitro* for these culture periods showed almost no differentiation. Therefore, other experiments were performed in which cultures were kept for longer periods. The influence of culture time and threshold for colony size on survival is demonstrated below.

Radiation has an inverse effect on the colony size of surviving glial progenitor cells. When the cumulative percentages of colonies are plotted as a function of the number of cells per colony, it is evident that surviving colonies become smaller when higher doses of X-rays are given (Figure 7.1 shows representative examples for perinatal and adult optic nerve O-2A progenitor experiments).

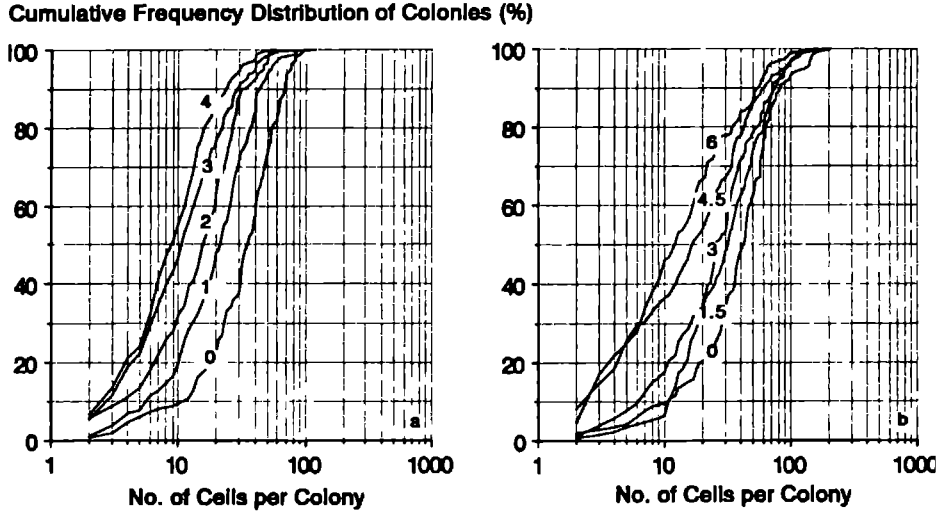


Figure 7.1 Colony size distribution of a representative perinatal (a) and adult (b) optic nerve experiment after 6 days and 13 days *in vitro*. The figures show the cumulative frequency of colonies that have a certain number of cells for the different doses of X-rays (Gy, indicated by numbers) given. The median colony size decreases with higher doses of X-rays.

For perinatal optic nerve cultures, the median cell number at 4 Gy is approximately 1/3-1/4 of the median cell number at 0 Gy, when cultures are stained at day 6 after irradiation. For the experiment shown in Figure 7.1a the median cell number for colonies surviving 4 Gy was 8 cells (3 cell divisions) compared to 32 cells (5 cell

divisions) for unirradiated cultures. Adult optic nerve cultures show the same phenomenon: cells surviving higher doses of X-rays consistently give rise to smaller colonies (Figure 7.1b).

The difference in colony size between irradiated and unirradiated cultures was investigated over a longer period of time for perinatal O-2A progenitor cells. In this experiment the dynamics of colony growth after X-irradiation was studied by a comparison of unirradiated perinatal optic nerve cultures with cultures that were irradiated with 4 Gy, for a period of 2 weeks. With 3-day intervals 5 culture flasks were stained: 2 unirradiated, 2 cultures irradiated with 4 Gy and 1 flask without any optic nerve cells to estimate the background staining of the astrocyte monolayer. At 4 time points the number of colonies, the number of cells per colony and the degree of differentiation were determined. During the first 6 days almost no differentiation into oligodendrocytes is seen within the colonies. Marked differentiation is seen at 9 days and becomes more pronounced at the end of the experiment at 13 days. Another experiment revealed that even after 3 weeks, some colonies (even large ones, of over 500 cells) are seen that show no differentiation at all. The degree of differentiation within colonies is visualized in Figure 7.2, where the differentiation of colonies surviving 4 Gy is compared with the differentiation of unirradiated colonies at 9 and 13 days *in vitro*. The irradiated colonies show a delay in differentiation of approximately 4 days.

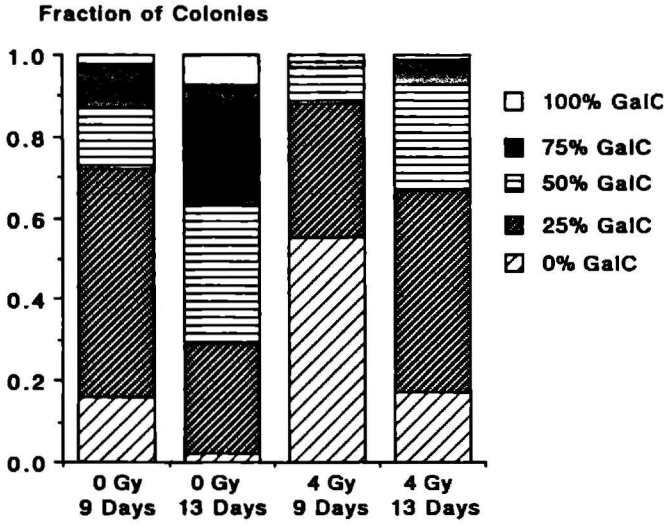


Figure 7.2 Fraction of O-2A colonies derived from perinatal optic nerve that shows a certain percentage of differentiation into oligodendrocytes at 9 and 13 days *in vitro*. O-2A progenitor cells

which survive 4 Gy are still able to differentiate into oligodendrocytes like their non-irradiated controls. The differentiation of the irradiated colonies, however, is delayed for approximately 4 days.

When the size of perinatal optic nerve colonies is examined at the different time points (Figure 7.3) it is clear that the colonies are growing, but the start of differentiation marks the fact that colony growth slows down until it stops when differentiation is complete (Temple and Raff 1986). It can also be noticed that at all time points the colonies from irradiated cells are smaller than the colonies from non-irradiated cells.

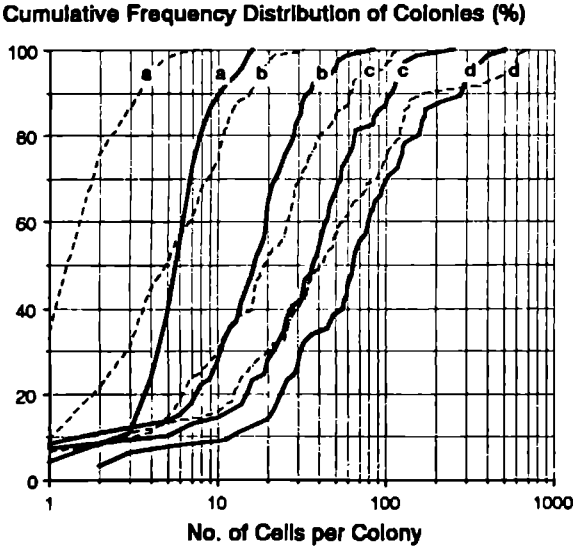


Figure 7.3 Perinatal optic nerve experiment. At different time points irradiated cultures (4 Gy, dotted line) were compared with non-irradiated cultures (filled line). At the time points examined (a=3 days, b=6 days, c=9 days, d=13 days) the colonies are still growing. The median colony size of irradiated cultures is smaller compared to the median colony size of the non-irradiated cultures.

Radiosensitivity of O-2A progenitor cells of perinatal or adult origin.

Cells derived from rat optic nerves were plated on type-1 astrocyte monolayers at the appropriate concentrations for the different doses of X-rays. After 3-4 h the cultures were irradiated and cultures were maintained for various times. Perinatal optic nerve cultures were maintained for at least 6 days. Adult optic nerve cultures were maintained for 13 days. [The mean cell cycle time of adult O-2A progenitor cells is 2-3 times the cell cycle time of perinatal O-2A progenitor cells (Table 7.1; Wolswijk and Noble 1989).] The cultures were stained with A_2B_5 and anti-GalC antibodies and evaluated.

The number of colonies was counted and after correction for plating efficiencies survival curves were constructed. Experiments were performed in duplicate, and repeated 4-5 times. There is a large difference in radiosensitivity of perinatal optic nerve progenitor cells compared with adult optic nerve progenitor cells (Figure 7.4a). This difference is reflected in the values of α and β obtained by fitting the data points according to the linear-quadratic (LQ-) model. The α and β values for the curves in Figure 7.4 are 0.53 Gy^{-1} and 0.068 Gy^{-2} for perinatal optic nerve, and 0.15 Gy^{-1} and 0.028 Gy^{-2} for adult optic nerve progenitor cells.

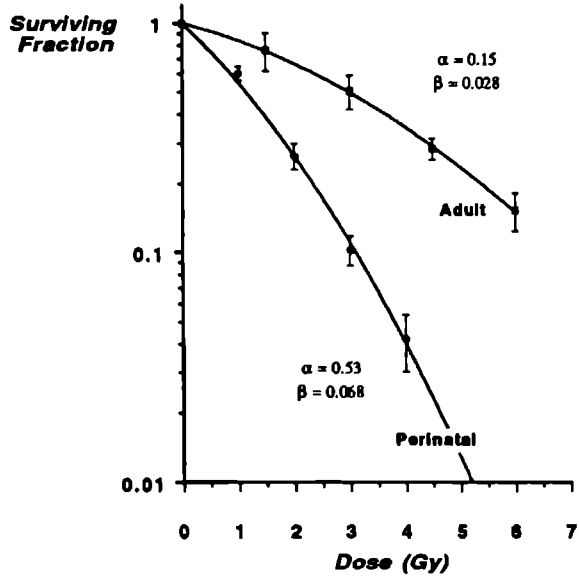


Figure 7.4a Survival curves of O-2A progenitor cells isolated from perinatal or adult optic nerves. Each data point represents mean ± S.D. as derived from at least four separate experiments. Perinatal optic nerve cultures were maintained for 6 days; adult optic nerve cultures for 2 weeks. The ability to form a colony of 6 or more $A2B5^+$ and/or $GalC^+$ cells was used as the threshold for surviving clonogenic cells.

As stated above the selection criterion for surviving O-2A progenitor cells was initially set at a colony forming ability of at least 6 cells. When it was subsequently found that the colonies of perinatal (6-day cultures) and adult (13-day culture) O-2A progenitor cells, showed almost no differentiation and were still growing (Figure 7.3), cultures were kept for longer periods. The mean values of 3 perinatal optic nerve and 2 adult optic nerve experiments, that were kept *in vitro* for a prolonged period of 13 days and 4 weeks, respectively, were compared with

the survival curves obtained after culturing these cells for the shorter period (Figure 7.4b). The selection criterion for surviving colonies was raised to 50 cells or more. The results indicate that survival curves are not greatly affected by raising the criterion to more than 50 cells if cultures are maintained for longer periods.

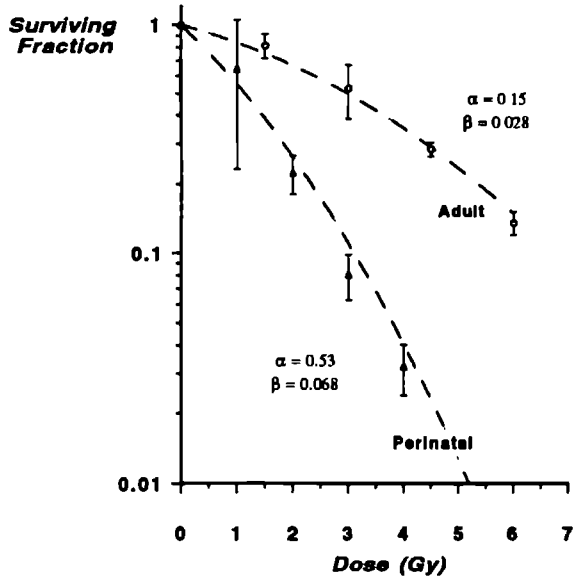


Figure 7.4b The survival curves are redrawn from Figure 7.4a and are compared with mean survival fractions ± S.D. obtained from different experiments, in which the cultures were maintained for longer periods (13 days for perinatal optic nerve experiments and 4 weeks for adult optic nerve experiments) and elevating the threshold to 50 or more $A2B5^+$ and/or $GalC^+$ cells for surviving colonies.

Discussion.

In this study on the radiation response of glial progenitor cells, for the first time a quantitative *in vitro* clonogenic assay has been used. Surviving irradiated cells maintain their normal properties. Like unirradiated control cells, they are able to undergo differentiation into oligodendrocytes or type-2 astrocytes, depending on growth conditions (Van der Maazen et al. 1990). The median colony size is affected by radiation, but this is a phenomenon also seen after irradiation of cell lines (Sinclair 1964).

Normal perinatal O-2A progenitor cells have a limited division potential. Developmental studies of glial cells from optic nerve of perinatal rats have led to the developmental clock hypothesis, i.e. that after a certain number of cell

divisions the whole progeny of a single perinatal O-2A progenitor cell differentiates into oligodendrocytes (Temple and Raff 1986). Therefore, it was necessary to define the endpoints for cell survival for this *in vitro* clonogenic assay. The selection criterion for surviving perinatal clonogenic glial cells was set at 6 cells, if cultures were maintained for 6 days *in vitro*. Survival curves obtained by using this selection criterion were compared with data obtained by using the selection criterion of 50 cells for cultures kept for 13 days *in vitro*. Most of the perinatal O-2A progenitor cells (> 80%) were able to form colonies larger than 50 cells, but after 2 weeks *in vitro*, most of the colonies showed extensive differentiation, indicating that the ultimate colony size had been reached for the majority of the progenitor cells. The same selection criteria were used for adult O-2A progenitor cells that were cultured for 13 days and 4 weeks, respectively.

Comparison of the survival curves revealed a large difference in radiosensitivity between perinatal and adult O-2A progenitor cells derived from optic nerves. The initial slope of the survival curves, represented by the parameter α, were 0.53 Gy^{-1} and 0.15 Gy^{-1} for perinatal and adult O-2A progenitor cells, respectively. The β-values representing the quadratic component were 0.068 Gy^{-2} and 0.028 Gy^{-2} for perinatal and adult optic nerve O-2A progenitor cells. However, β-values derived from acute single dose survival curves are prone to large statistical errors, and are not representative for the repair capacity usually associated with the β-parameter (Peacock et al. 1988).

The large difference in radiosensitivity could be explained by the different position of perinatal and adult progenitor cells in the O-2A lineage. The majority of perinatal optic nerve O-2A progenitor cells is thought to have a restricted proliferative capacity (Temple and Raff 1986). Adult O-2A progenitor cells are considered more close to real stem cells, being capable of self-renewal as well as to give rise to differentiated progeny (Wolwijk and Noble 1989). The phenomenon that cells in different stages of differentiation have different radiosensitivities can also be seen in other tissues such as testis (Van Beek et al. 1986), but is also reported in the rat brain where migrating precursor cells, thought to be more differentiated, were more radiosensitive than non-migratory precursor cells (Altman et al. 1968).

In this article we have shown that it is possible to adapt the *in vitro* culturing of normal O-2A progenitor cells in such a way that it is suitable for a clonal survival assay. This has become possible due to recent developments in neurobiology. Early studies provided some evidence of the existence of a glial progenitor cell, that was able to give rise to differentiated glia (Smart and Leblond 1961), but it lasted until 1983 when Raff et al. (1983) showed unequivocally, *in vitro*, the existence of O-2A progenitor cells in perinatal optic nerve cell suspensions. More

recent studies show that these cells are also present in other regions of the CNS (Raff and Lillien 1988), even in adult animals (Wolswijk and Noble 1989) as confirmed in the present study. When perinatal O-2A progenitor cells are cultured on monolayers of type-1 astrocytes premature differentiation is inhibited (Noble and Murray 1984) and colony formation does occur. Using this method the number of stem cells can be assessed quantitatively. Stem cells, in any tissue, are of great importance in the assessment of radiation-induced damage. The degree of damage to the stem cell compartment determines the possibility of the tissue to recover. Radiation damage is therefore best quantified by the survival of stem cells after radiation. Plain cell numbers do not necessarily reflect the damage inflicted to the stem cell compartment. A recent study reported the radiosensitivity of glial cells in primary mixed cultures derived from whole brains of mice (Barbarese and Barry 1989). After irradiation, numbers of GalC$^+$ and $A_2B_5^+$ cells were counted and compared with unirradiated cultures. It was stated that GalC$^+$ and $A_2B_5^+$ cells do not divide under the described culture conditions. For the former cell type (GalC$^+$ oligodendrocytes) that is probably correct, but $A_2B_5^+$ cells divide and form a layer of O-2A progenitor cells and differentiating oligodendrocytes on top of the forming monolayer of type-1 astrocytes. Other authors even use this technique to obtain large numbers of oligodendrocytes and O-2A progenitor cells. Therefore the counting of cell numbers in the assessment of radiation damage will not give adequate, cell type specific, information.

Effects of ionizing radiation upon glial cells have been studied *in vivo*. Morphological changes (paranodal myelin breakdown, nodal gap widening and Wallerian-type degeneration) were seen within several weeks after irradiation of the cervical spinal cord (Mastaglia et al. 1976). Cell counts after irradiation, with or without ^{3}H-thymidine labeling, showed differences in radiosensitivity of glial cells and allowed comparison between different brain regions (Beal and Hall 1974; Hopewell and Cavanagh 1972; Hubbard and Hopewell 1980; Reyners et al. 1982; Sims et al. 1985). The elucidation of the lineage relationships of glial cells in the rat optic nerve and the discovery of growth factors that control proliferation (platelet derived growth factor)(Raff et al. 1988) and differentiation (ciliairy neurotrophic factor) (Hughes et al. 1988) of glial progenitor cells makes *in vitro* analysis of radiation damage possible. The intrinsic radiosensitivity, repair capacities and regeneration characteristics of O-2A progenitor cells can now be studied at a cellular level. Furthermore, a comparison between different CNS regions and different developmental stages (perinatal - adult) can be made. Hopefully this will lead to a better understanding of demyelinating syndromes seen after irradiation of the CNS.

IRRADIATION *IN VITRO* DISCRIMINATES BETWEEN DIFFERENT O-2A PROGENITOR CELL SUBPOPULATIONS IN THE PERINATAL CENTRAL NERVOUS SYSTEM OF RATS

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Radiation Research, 1991, 128, 64-72

Abstract.

The effects of X-irradiation on oligodendrocyte - type-2 astrocyte (O-2A) progenitor cells derived from different regions of the perinatal central nervous system (CNS) of rats were investigated in vitro. The O-2A progenitor cells can differentiate into either oligodendrocytes or into type-2 astrocytes. The depletion of these cells could lead to demyelination, seen as a delayed reaction after irradiation of the CNS in vivo. To quantify cell survival, O-2A progenitor cells were grown on monolayers of type-1 astrocytes. Monolayers of type-1 astrocytes stimulate O-2A progenitor cells to divide. O-2A progenitor cells were irradiated in vitro and clonogenic cell survival was measured. The O-2A progenitor cells derived from perinatal optic nerve were quite radiosensitive in contrast to O-2A progenitor cells derived from perinatal spinal cord and perinatal corpus callosum. Furthermore, O-2A progenitor cells derived from the optic nerve formed smaller colonies, with most colonies showing early differentiation into oligodendrocytes. In contrast, more than half of the colonies derived from corpus callosum did not show any differentiation after 2 weeks in vitro and kept growing. These differences support the view that perinatal O-2A progenitor cells derived from the optic nerve are committed progenitor cells while the O-2A progenitor cells derived from the perinatal corpus callosum and the perinatal spinal cord have more stem cell properties.

Introduction.

The rat optic nerve is a model system to investigate glial functions and their interrelationship in the white matter of the central nervous system (CNS). The rat optic nerve is used as a source of glial cells because it does not contain neuronal cell bodies. Glial cells are the major constituents of single cell suspensions which are obtained from optic nerves of the perinatal rat, after cutting them free behind the eyes and in front of the chiasm. *In vitro* analysis of glial cells derived from the optic nerves revealed the existence of two different glial lineages with different functions. The lineage that is called after its progeny consists of O-2A progenitor cells that are able to differentiate either into oligodendrocytes or type-2 astrocytes (Raff et al. 1983; Raff 1989). These two differentiated cell types are of importance in myelinated tracts: Oligodendrocytes enwrap axons in membranous sheaths which facilitate fast signal conduction along the axons, and the processes of type-2 astrocytes are in contact with the nodes of Ranvier (ffrench-Constant and Raff 1986). Type-1 astrocytes originating from different glial precursor cells stimulate the proliferation of O-2A progenitor cells by production of growth factors [e.g., PDGF (Noble et al. 1988; Raff et al. 1988)] and are used as a feeder layer for O-2A progenitor cells in *in vitro* studies (Noble and Murray 1984).

A developmental clock for O-2A progenitor cells derived from optic nerves of the perinatal rat has been hypothesized to explain the phenomenon of the limited proliferative potential of these cells *in vitro* which results, after a certain number of divisions, in the differentiation into oligodendrocytes of all the progeny of a single O-2A progenitor cell (Temple and Raff 1985; 1986). In a self-renewing system, this inability to form non-differentiating cells is a property of cells belonging to the proliferative compartment and contrasts with stem cell characteristics. Real stem cells are capable of self-renewal as well as giving rise to cells that are eventually able to differentiate.

The O-2A progenitor cells are important in the normal turnover of oligodendrocytes and type-2 astrocytes and in the regeneration of the O-2A lineage after partial depletion of glial stem cells. Their potential role in radiation-induced demyelination is the subject of our investigations. Depletion of O-2A progenitor cells by ionizing radiation would lead indirectly to demyelination because of the failure to replace oligodendrocytes lost by natural attrition. To study the effect of X-rays on O-2A progenitor cells *in vivo* or *in vitro*, a clonogenic assay was developed based on existing methods to culture O-2A progenitor cells. Using this technique it was found that O-2A progenitor cells derived from the perinatal optic nerve are quite radiosensitive (Van der Maazen et al. 1990; 1991). In the present study, the radiosensitivity of O-2A progenitor cells derived from the perinatal optic nerve was compared with the radiosensitivity of O-2A progenitor

cells derived from perinatal corpus callosum and perinatal spinal cord. Although some authors report the existence of O-2A progenitor cells in regions of the CNS other than the optic nerve (Raff and Lillien 1988), extensive studies dealing with the properties of these cells have not been published. The O-2A progenitor cells are migrating into the developing perinatal optic nerve and do not originate from a germinal center in the optic nerve itself (Small et al. 1987); therefore, there is the possibility that these cells are more differentiated than the O-2A progenitor cells in germinal centers elsewhere in the CNS. In the brain the subependymal plate is such a germinal zone, and although there is not such an area identified in the spinal cord, stem cells are thought to be dispersed throughout the white and grey matter.

The purpose of this study was to investigate the radiosensitivity of perinatal O-2A progenitor cells obtained from different regions of the CNS to find out if a high radiosensitivity was characteristic for all perinatal O-2A progenitor cells or whether this property was restricted to O-2A progenitor cells derived from perinatal optic nerve (Van der Maazen et al. 1991). The results show not only a difference in radiosensitivity, but also a difference in colony size and differentiation pattern. These differences give support to the idea that O-2A progenitor cells derived from different CNS regions belong to different compartments in the O-2A lineage.

Materials and methods.

A detailed description of the quantitative clonogenic assay for O-2A progenitor cells to assess radiation-induced damage *in vitro* has been published separately (Van der Maazen 1990) and was adapted from McCarthy and De Vellis (1980), Noble and Murray (1984), and Wolswijk and Noble (1989).

Briefly, pure monolayers of type-1 astrocytes were derived from the cortex of the brains of 1-day-old Wistar rats. The cortices were dissected and the meninges were carefully removed to avoid contamination by fibroblasts. The cortices were chopped with a scalpel and treated with enzymes (trypsin and collagenase) to obtain a single cell suspension. The single cells were plated on poly-L-lysine (PLL)-coated 75-cm^2 tissue culture flasks and cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS). After 10-14 days, stratification made it possible to separate top cells from bottom cells (type-1 astrocytes) by treating cultures with Ara-C (to kill dividing top cells) and shaking vigorously. Cells were then trypsinized and replated in PLL-coated 25-cm^2 tissue culture flasks. After 3-4 days tight monolayers of type-1 astrocytes were irradiated with 20 Gy (4 MV X-rays; dose rate 2.7 Gy min^{-1}) to prevent colony formation by any remaining O-2A progenitor cells. (A dose of 20 Gy reduces the fraction of

surviving colony-forming O-2A progenitor cells to approximately 10^{-11} to 10^{-17} as extrapolated from the present results.)

For one experiment on the optic nerve, 15 pairs of optic nerves obtained from 1-day-old rats were used. The tissue of 2 spinal cords and the tissue of 4 corpora callosa were needed for a single spinal cord and a single corpus callosum experiment. We used the term "corpus callosum" but the rather crude way of dissecting this part of the CNS yields tissue that contains not only the corpus callosum but also surrounding tissue such as the subependymal plate. The meninges, the predominant source of fibroblasts in the CNS, were carefully removed. Single cell suspensions were made by cutting the tissue in fine pieces and treating the pieces with collagenase and trypsin. After the enzymatic digestion was stopped with soybean trypsin inhibitor and DNAse, a single cell suspension was obtained by trituration through fine needles. Cells were counted with a hemocytometer. 10.000 cells were plated onto PLL-coated glass coverslips and stained with A_2B_5 (Eisenbarth et al. 1979) and anti-galactocerebroside (anti-GalC)(Raff et al. 1978) monoclonal antibodies to determine percentages of O-2A progenitor cells (A_2B_5-positive) and oligodendrocytes (GalC-positive) in the initial cell suspensions.

To determine the intrinsic radiosensitivity *in vitro*, cell numbers that would give rise to approximately 2-3 colonies per cm^2 were plated on monolayers of type-1 astrocytes (feeder cells) and were allowed to adhere for 4 h. Cultures were irradiated with various doses of X-rays (4 MV; dose rate 2.7 Gy min^{-1}) and maintained in a chemically defined medium containing 0.5% FCS [BS/0.5% FCS (Bottenstein and Sato 1979)] to allow colony formation of surviving O-2A progenitor cells. The additional doses used to determine the radiosensitivity of O-2A progenitor cells (1 to 5 Gy) do not affect the monolayer of type-1 astrocytes in its supportive function. A separate experiment showed that there was no difference in the number and size of O-2A progenitor cells cultured on monolayers of type-1 astrocytes preirradiated with either 20 or 40 Gy.

Cultures were stained with A_2B_5 (IgM) and anti-GalC (IgG3) monoclonal antibodies followed by incubation with second-layer antibodies (goat anti-mouse IgM conjugated with fluorescein and goat anti-mouse IgG3 conjugated with rhodamine) directed against A_2B_5 and anti-GalC to visualize O-2A lineage cells. The cultures were screened with a Zeiss Axiovert microscope equipped for epifluorescence, and the number of colonies per flask, the number of cells per colony, and the differentiation in colonies were determined.

To study the capacity of colony-forming cells derived from corpus callosum and spinal cord to differentiate into type-2 astrocytes, cultures were subjected to DMEM plus 20% FCS to induce type-2 astrocyte differentiation. These cultures

were stained with A_2B_5 and anti-glial fibrillary acidic protein (anti-GFAP)(Bignami 1972) antibodies (Table 8.1).

Table 8.1 - STAINING CHARACTERISTICS OF DIFFERENT GLIAL CELL TYPES IN THE CNS

	antibodies		
cell types	A_2B_5	anti-GalC	anti-GFAP
O-2A progenitors	+	-	-
Oligodendrocytes	-	+	-
Type-2 astrocytes	+	-	+
Type-1 astrocytes	-	-	+

Results.

Plating efficiency.

The plating efficiencies (the number of colonies formed divided by the number of cells plated) for the different regions of the CNS ranged from 2.7 to 4.6 % for perinatal optic nerve, 2.4 to 3.6 % for perinatal corpus callosum, and 3.6 to 7.6 % for perinatal spinal cord. Four to 5 independent experiments were performed for each region. Single cell suspensions derived from CNS tissue, however, do not consist purely of O-2A progenitor cells, resulting in an underestimation of the plating efficiency. Therefore the percentage of A_2B_5-positive cells in the initial cell suspensions was determined and plating efficiencies were adjusted to the proportion of A_2B_5-positive cells. The adjusted plating efficiencies ranged from 14 to 25% for optic nerve, 3 to 5.5% for corpus callosum, and 13 to 17% for spinal cord, which are rather high for an *in vivo-in vitro* clonogenic assay. The plating efficiencies for spinal cord and corpus callosum experiments are still underestimated because the cell suspensions derived from these regions contain A_2B_5-positive neurons in addition to A_2B_5-positive O-2A progenitor cells. Neurons, however, do not proliferate or even survive for a long time under the culture conditions described above.

In the cell suspensions of perinatal optic nerve and perinatal corpus callosum the percentage of GalC-positive cells (maturing oligodendrocytes) was always less than one. In perinatal spinal cord cell suspensions the percentage of GalC-positive cells ranged from 1.9 to 5.7. Although GalC-positive cells were present in the initial cell suspension, colonies consisting solely of GalC-positive cells were never seen in cultures stained after 6 days *in vitro*. Thus it seems unlikely that GalC-positive oligodendrocytes in the initial cell suspensions form colonies and they are therefore thought to be of little significance in the

assessment of radiation damage to the O-2A progenitor lineage, using this *in vitro* clonogenic assay.

Radiosensitivity of O-2A progenitor cells derived from different regions of the CNS.

Single cell suspensions derived from the 3 different regions of the CNS were plated on monolayers of type-1 astrocytes at the appropriate concentrations to yield about 2 to 3 surviving colony-forming O-2A progenitor cells per square centimeter. After allowing 4 h for the cells to settle down, the cultures were irradiated and maintained for 6 or 13 days *in vitro*. During these culture periods and up to at least 4-5 weeks (Van der Maazen et al. 1990; 1991) the monolayers of astrocytes do not seem to lose their effectiveness in supporting the growth of progenitor colonies. Cultures were evaluated after staining with A_2B_5 and anti-GalC antibodies.

A traditional cell survival assay is based on the criterion of surviving cells maintaining the capacity of unlimited proliferation (Puck and Marcus 1956). However, single O-2A progenitor cells derived from optic nerves of perinatal rats which were transferred into microwells and studied for their proliferative capacity were found to have a limited ability to divide (Temple and Raff 1986). Therefore the selection criterion for surviving O-2A progenitor cells after irradiation was initially set at a rather low cell number per colony. A colony was defined as a group of at least 6 A_2B_5- and/or GalC-positive cells, if cultures were maintained for 6 days *in vitro*. The number of colonies was counted for irradiated and non-irradiated cultures. The plating efficiency derived from non-irradiated cultures is needed to calculate the surviving fraction of colony-forming cells for the various doses of X-rays. The survival curves for the 3 different CNS regions are shown in Figure 8.1a where the mean surviving fractions of 4 or 5 different experiments for each region are plotted against the dose of X rays. The data were fitted according to the linear-quadratic (LQ-) model of cell survival. This model fits the data according to the equation: $SF= \exp-(\alpha D+\beta D^2)$, in which D is the dose of X-rays and α and β are parameters characterizing the linear and quadratic components of the survival curve. The radiosensitivity of O-2A progenitors derived from perinatal corpus callosum and perinatal spinal cord are identical, but are much lower than the radiosensitivity of O-2A progenitors derived from perinatal optic nerve. The α and β values for the curves presented in Figure 8.1a are 0.53 Gy^{-1} and 0.068 Gy^{-2} for perinatal optic nerve, and 0.21 Gy^{-1} and 0.053 Gy^{-2} for perinatal corpus callosum and perinatal spinal cord, respectively.

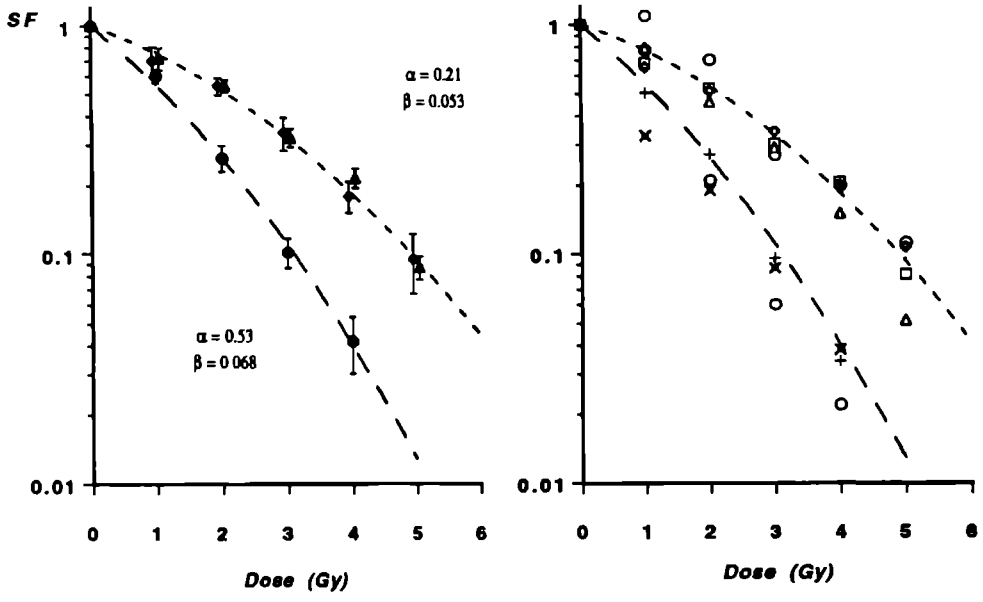


Figure 8.1 a: Survival curves of O-2A progenitor cells derived from perinatal optic nerve, perinatal corpus callosum, and perinatal spinal cord. Single data points represent the mean ± S.D. of four or five independent experiments. The data were fitted according to the LQ-model. The cultures were kept for 6 days *in vitro* and the ability to form a colony of ≥6 $A_2B_5^+$ and/or $GalC^+$ cells was used as a threshold for surviving single cells. Optic nerve (∘); spinal cord (▲); corpus callosum (◆).

b: Survival data for three perinatal optic nerve, two perinatal corpus callosum and two perinatal spinal cord experiments that were kept for 13 days *in vitro* were compared with the survival curves of figure a. The threshold for surviving clonogenic cells was set at ≥50 $A_2B_5^+$ and/or $GalC^+$ cells. Optic nerve (+, x, ⊕); spinal cord (◊, □); corpus callosum (O, Δ).

It was observed that after 6 days *in vitro* the differentiation in the colonies was limited. Cultures maintained for longer periods generated larger colonies. Therefore the survival curves derived from the 6-day-old cultures were compared with survival data obtained from different experiments kept *in vitro* for 13 days. The selection criterion for surviving cells applied to these cultures was set at ≥50 A_2B_5- and/or GalC-positive cells, a number which is usually associated with an unlimited proliferation capacity (Puck and Marcus 1956). Data obtained from 3 different perinatal optic nerve experiments and from 2 different perinatal corpus callosum and 2 different perinatal spinal cord experiments are compared with the survival curves obtained after 6 days *in vitro*. As can be seen in Figure 8.1b, survival curves are not affected significantly by raising the criterion for surviving cells to

more than 50 cells when cultures are maintained for 13 days *in vitro*. The cells that survive after irradiation and are able to form a colony of at least 6 cells after 6 days *in vitro*, develop according to the same pattern as non-irradiated control cells.

Colony size distribution.

The relatively small size of O-2A colonies after 6 days *in vitro* made it possible to count individual cells. The cumulative percentage of colonies for representative experiments of each region of the CNS is plotted against the cell number per colony in Figure 8.2.

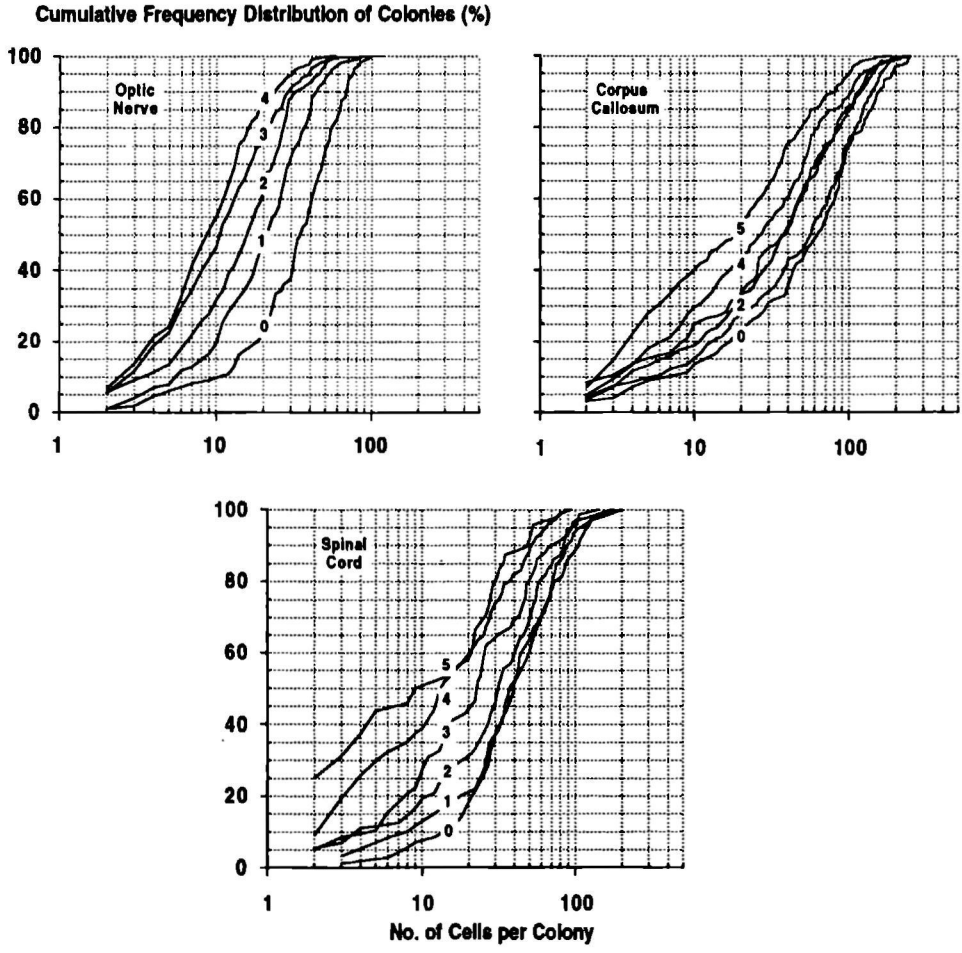


Figure 8.2 Colony size distribution of perinatal optic nerve (a), perinatal corpus callosum (b), and perinatal spinal cord (c). The cumulative frequency of colonies is plotted against the number of cells

per colony for the different doses of X-rays (numbers in plots represent the dose in Gy). Cultures were kept for 6 days *in vitro*.

Cells surviving higher doses of X-rays consistently give rise to smaller colonies. This is true not only for optic nerve progenitor cells but also for corpus callosum and spinal cord progenitor cells. The median colony size for control cultures and cultures surviving 4 Gy is shown in Figure 8.3 for the 3 different regions of the CNS. For perinatal optic nerve the mean value of the median colony sizes of the different experiments is approximately 30 cells for control cultures and 8 cells for cultures surviving 4 Gy. For perinatal spinal cord the comparable values are 40 cells and 12 cells, and for perinatal corpus callosum the 43 cells and 17 cells.

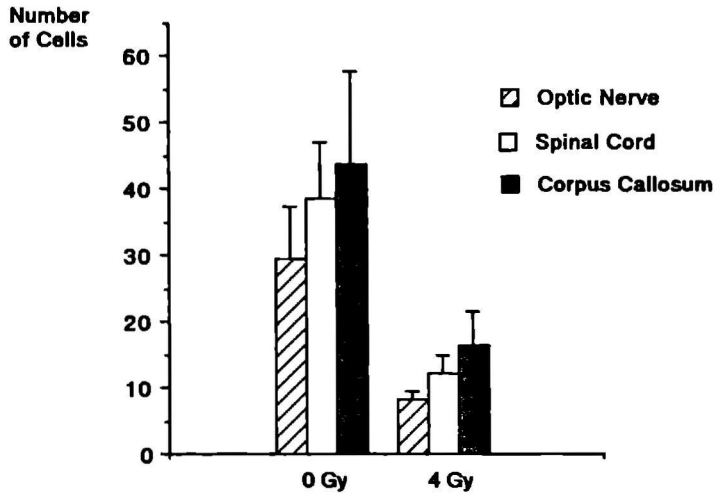


Figure 8.3 The mean values (± S.D.)(four to five different experiments) of the median number of cells in colonies of irradiated (4 Gy) and non-irradiated perinatal optic nerve, perinatal corpus callosum, and perinatal spinal cord cultures obtained from at least four different experiments. There is no statistically significant difference in the mean colony size between non-irradiated optic nerve and corpus callosum colonies (t-Test; p=0.091) and between non-irradiated optic nerve and spinal cord colonies (p=0.147). The differences in mean colony size between the irradiated colonies, however, were statistically different (optic nerve vs corpus callosum: p=0.008) (optic nerve vs spinal cord: p=0.016). Cultures were kept for 6 days *in vitro*.

The differences between the mean values of the median colony sizes of the nonirradiated cultures derived for the different regions of the CNS are not statistically significant (t-Test). However, there is a tendency for O-2A progenitor

cells derived from perinatal corpus callosum and spinal cord to form larger colonies. This is reflected, for instance, in the percentage of progenitor cells that form colonies larger than 100 cells. When the cultures are kept for 6 days *in vitro* almost none of the perinatal optic nerve but approximately 30% of the O-2A progenitor cells derived from the corpus callosum form colonies exceeding 100 cells (non-irradiated cultures)(for the representive examples shown in Figure 8.2).

Differentiation.

The colonies of the cultures maintained for 13 days *in vitro* were too large for counting individual cell numbers. The degree of differentiation in the individual colonies, however, could be estimated. The degree of differentiation was divided into 5 categories: almost no differentiation, and approximately 25, 50, 75, and 100% differentiation into oligodendrocytes. After 13 days *in vitro* >75% of the unirradiated perinatal optic nerve colonies showed a differentiation of 50% or more. Only a few percent of the colonies showed no differentiation at all. This contrasts with the differentiation seen in the unirradiated colonies derived from spinal cord and corpus callosum. Only 30 - 40% of these colonies had a degree of differentiation of >50%, while 30% of the spinal cord colonies and up to 60% of the corpus callosum colonies did not show any differentiation (Figure 8.4). No significant shifts in differentiation were seen in colonies derived from surviving irradiated cells.

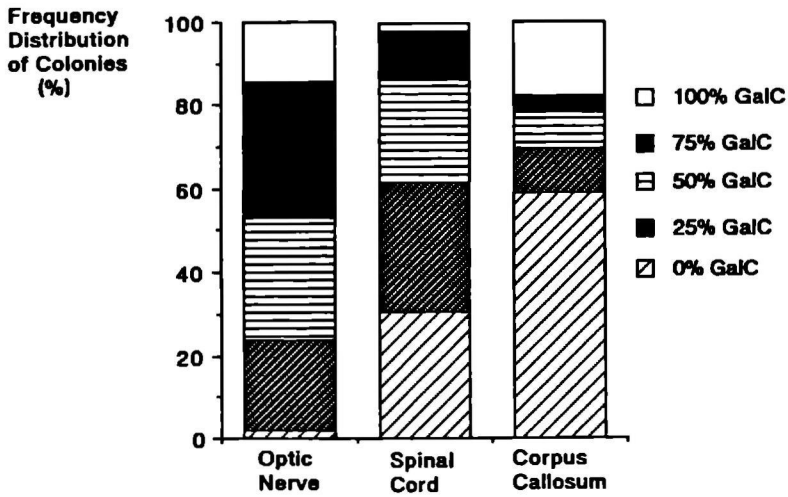


Figure 8.4 Frequency distribution of nonirradiated colonies (%) derived from perinatal optic nerve, perinatal corpus callosum, and perinatal spinal cord related to the percentage of differentiation into

oligodendrocytes within the colonies as indicated by the presence of GalC detected by monoclonal antibodies. Cultures were kept for 13 days *in vitro*.

When the degree of differentiation and the colony size (previous section) for the different regions of the CNS are combined, it is clear that the growth of O-2A progenitor cells derived from perinatal optic nerve is restricted (smaller colonies with a higher degree of differentiation)(Temple and Raff 1986). This contrasts with the proliferative potential of O-2A progenitor cells derived from perinatal spinal cord and perinatal corpus callosum. A considerable percentage of these O-2A progenitor cells form larger colonies than perinatal optic nerve progenitor cells, without any sign of differentiation after 13 days *in vitro* and with continued cell division (data not shown).

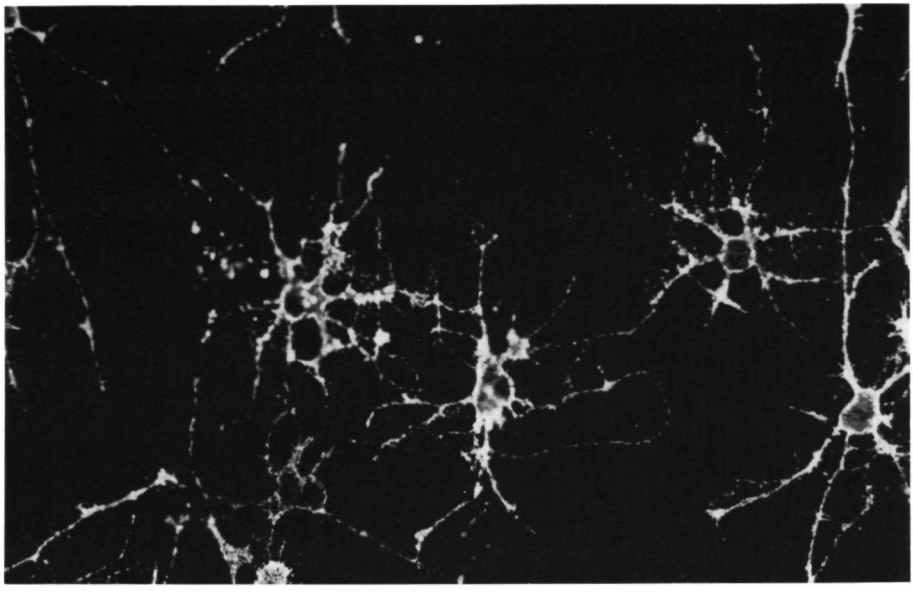
Related to the degree of differentiation is the morphology of O-2A progenitor cells. Most of A_2B_5-positive O-2A progenitor cells in perinatal optic nerve colonies had multiple processes, while many of these cells in corpus callosum and spinal cord colonies retained their bipolar morphology (Figure 8.5).

O-2A properties of A_2B_5-positive colonies.

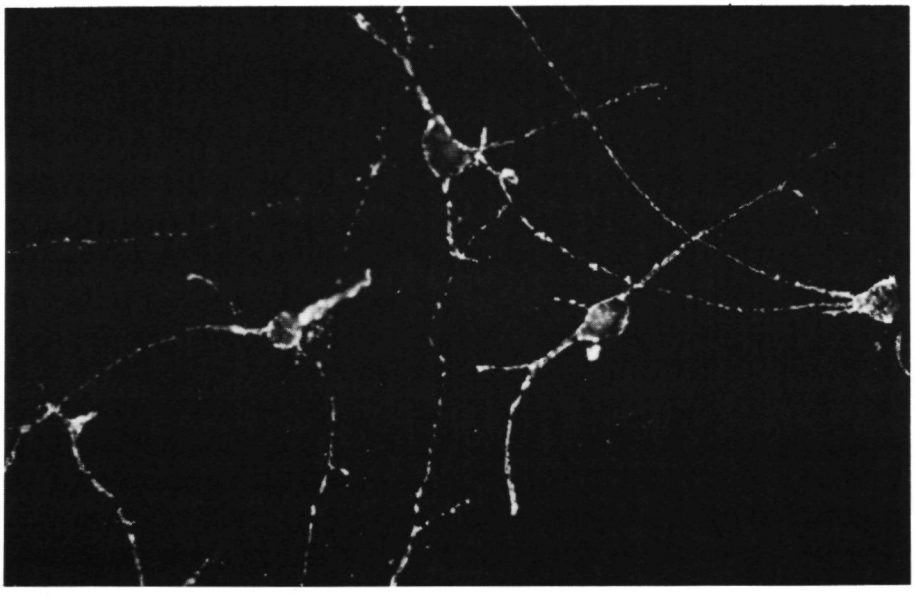
The properties of O-2A progenitor cells derived from perinatal optic nerves have been studied extensively (Raff 1989). Some authors report the existence of O-2A progenitor cells in other regions of the CNS, but detailed studies dealing with the similarities and differences of O-2A progenitor cells derived from different regions have not been published. Therefore, the O-2A properties of A_2B_5-positive colonies derived from perinatal corpus callosum and perinatal spinal cord were determined. Colonies that develop in the first week *in vitro* on monolayers of type-1 astrocytes contain mainly A_2B_5-positive cells. The degree of differentiation into oligodendrocytes increases if the cultures are maintained in BS/0.5% FCS for more than 1 week. If, after 1 week *in vitro*, the chemically defined medium was changed to DMEM + 20% FCS and the cultures were maintained for another week *in vitro*, more than 90% of the colonies contained cells that were GFAP-positive (type-2 astrocytes). So A_2B_5-positive colonies of cells derived from perinatal corpus callosum and spinal cord have the ability to differentiate into either oligodendrocytes or type-2 astrocytes, depending on the culture conditions.

Discussion.

O-2A progenitor cells derived from different regions of the perinatal CNS were cultured *in vitro* and tested for their radiosensitivity. The intrinsic radiosensitivity measured *in vitro* showed a large difference between O-2A progenitor cells derived from optic nerve and O-2A progenitor cells derived from



(a)



(b)

Figure 8.5 O-2A progenitor cells in colonies derived from perinatal optic nerve (a) and perinatal corpus callosum (b). The cultures were kept for 13 days *in vitro* and were stained with A_2B_5 and anti-GalC monoclonal antibodies. The cells on the photographs are only A_2B_5-positive. Optic nerve progenitor cells have multiple processes in contrast to progenitor cells derived from the corpus callosum which retain their bipolar morphology.

spinal cord and corpus callosum. The α values for the survival curves when fitted according to the LQ-model are 0.53 Gy^{-1} for optic nerve and 0.21 Gy^{-1} for corpus callosum and spinal cord. In contrast to the marked difference in α-values, the β-values were more similar (0.05 and 0.07 Gy^{-2}). Differences in intrinsic radiosensitivity between cells belonging to different compartments in a certain lineage have been reported for other tissues such as CNS *in vivo* (Altman et al. 1968), testis (Van Beek et al. 1986), and the hematopoietic system (Nothdurft et al. 1983).

Although a large difference in radiosensitivity was found between O-2A progenitor cells derived from different regions of the CNS, it is very likely that perinatal corpus callosum and spinal cord contain a mixture of O-2A progenitor cells belonging to different maturation stages in the O-2A lineage. These different subpopulations could have quite different intrinsic radiosensitivities. Calculations based on our data indicate that this is not the case for O-2A progenitor cells derived from spinal cord or corpus callosum.

Besides the large difference in radiosensitivity, other differences between O-2A progenitor cells derived from the three regions of the CNS were found (differentiation pattern and colony size). These differences suggest that although these cells have the same differentiation pathways, their position in the developmental lineage is different. O-2A progenitor cells derived from corpus callosum and spinal cord formed larger colonies than O-2A progenitor cells derived from optic nerve. A considerable proportion of these large colonies showed no sign of differentiation at all and were able to divide even more. A difference in colony size between perinatal O-2A progenitor cells derived from different regions of the CNS was also found by Behar et al. (1988), who compared the proliferative capacity of single O-2A progenitor cells derived from the optic nerve and cerebral hemispheres. The mean clone size after 8-10 days *in vitro* for O-2A progenitor cells derived from cerebral hemispheres was almost twice as large as those derived from optic nerves (36 $\pm$ 5 cells vs 20 $\pm$ 4 cells).

The degree of differentiation into oligodendrocytes also differed markedly in the colonies derived from the different regions of the CNS. After 2 weeks *in vitro* almost all colonies in the cultures of perinatal optic nerve tissue showed a more or less advanced degree of differentiation. Studies dealing with single O-2A progenitor cells derived from perinatal optic nerves demonstrated that the onset of differentiation in such a colony will lead to the differentiation of the whole colony (Temple and Raff 1985; 1986). The degree of differentiation in colonies derived from perinatal corpus callosum and spinal cord was less: 30% of spinal cord colonies and 60% of corpus callosum colonies showed no differentiation after 2 weeks *in vitro*. This suggests that, in contrast to the optic nerve, the corpus

callosum and spinal cord contain a considerable proportion of O-2A progenitor cells which retain stem cell properties *in vitro* , i.e., cells with a large proliferative potential without an internal clock that determines the onset of differentiation.

It has been suggested that O-2A progenitor cells derived from the optic nerve are different from O-2A progenitor cells with more stem cell properties such as cells obtained from the optic nerve of adult rats (Noble et al. 1989). Adult O-2A progenitor cells are even more radioresistant than perinatal corpus callosum or spinal cord O-2A progenitor cells (Van der Maazen 1991). The α value derived from the single-dose survival curve for O-2A progenitors cells of adult rats is 0.15 Gy^{-1}, compared to 0.21 Gy^{-1} for O-2A progenitor cells derived from the spinal cord and corpus callosum of perinatal rats. The O-2A progenitor cells derived from adult rats are capable of self-renewal and give rise to differentiated progeny and are considered to be more similar to stem cells than to progenitor cells derived from the optic nerve of perinatal rats (Noble et al. 1989). Thus a decreasing radiosensitivity is seen for O-2A progenitor cells from perinatal optic nerve to perinatal corpus callosum and spinal cord, and finally to progenitor cells from optic nerve and spinal cord of adult rats (O-2A progenitor cells derived from the adult spinal cord have the same intrinsic radiosensitivity as progenitor cells derived from adult optic nerve; unpublished results)(Table 8.2).

Table 8.2 - LQ-SURVIVAL CHARACTERISTICS OF O-2A PROGENITOR CELLS OF DIFFERENT AGE AND ORIGIN.

Source of O-2A progenitor cells	alpha (Gy^{-1})	beta (Gy^{-2})	
perinatal optic nerve	0.53	0.07	
perinatal spinal cord & corpus callosum	0.21	0.05	decreasing sensitivity
adult optic nerve & spinal cord	0.15	0.03	

The present results suggest that O-2A progenitor cells derived from perinatal corpus callosum and spinal cord have more characteristics of stem cells because of their greater proliferative potential and because of the way the timing and extent of differentiation differ from those of O-2A progenitor cells derived from the optic nerve of perinatal rats. The O-2A progenitor cells derived from the optic

nerve do not originate in the optic nerve but migrate into the optic nerve from a nearby germinal zone (Small et al. 1987). The migrating cells are likely to be more differentiated than the nonmigratory cells. The fact that the O-2A progenitor cells derived from the optic nerve have a limited proliferative potential indicates that they have largely lost the ability of self-renewal which is characteristic of stem cells (Temple and Raff 1985; 1986). O-2A progenitor cells derived from perinatal spinal cord and corpus callosum have a greater proliferative potential and differentiate differently *in vitro* (at a later time and clearly not the whole offspring of a single progenitor at the same time). At the moment there are no cellular markers to discriminate between different subsets of perinatal O-2A progenitor cells. A recent study, however, has reported the existence *in vitro* of immature cells derived from the brain of 6-day-old rat that lacked the A_2B_5 antigen but were able to give rise to O-2A offspring (Grinspan et al. 1990). Whether the surviving colonies in corpus callosum and spinal cord cultures are the offspring of such A_2B_5-negative pre- O-2A progenitor cells is not clear, but the study provides further evidence that the colony-forming cells are heterogeneous with respect to antigenicity, dividing potential, and differentiation patterns. The same is true for adult O-2A progenitor cells, which are not only the most radioresistant of the studied glial cells, but can be discriminated from perinatal O-2A progenitor cells by different antibodies (Wolswijk and Noble 1989).

In conclusion, our study supports the presence of different subsets of O-2A progenitor cells in the perinatal CNS, as demonstrated by a marked difference of radiosensitivity and proliferation/differentiation pattern. Whether these different responses have any consequences for the development of radiation injury in brain or spinal cord tissue remains to be investigated.

CHAPTER 9

REPAIR CAPACITY OF ADULT RAT GLIAL PROGENITOR CELLS DETERMINED BY AN *IN VITRO* CLONOGENIC ASSAY AFTER *IN VITRO* OR *IN VIVO* FRACTIONATED IRRADIATION

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Abstract.

Demyelination is one of the pathological conditions identified as a late response of the central nervous system (CNS) to irradiation. We have proposed that radiation-induced depletion of glial stem cells, which are the source of myelinating cells in the CNS, would lead to a lack of replacement of senescent or otherwise damaged oligodendrocytes. This impaired process of cell renewal would result in a decline of oligodendrocytes, i.e. demyelination. In the present study, the repair capacity of glial stem cells was investigated and compared with the repair capacity of the CNS in vivo using functional endpoints. For this purpose, glial stem cells, derived from the adult rat optic nerve, were subjected to fractionated irradiation in vivo and in vitro and their survival was quantified with an in vitro clonogenic assay. The data were analyzed by three different methods, all based on the LQ-model (single dose survival curve; "β_{RR}"; "F_e-plot"). The resulting value of the β-parameter of adult glial stem cells is consistent with values obtained for functional endpoints after irradiation of the CNS in vivo. The α/β-ratio (4.9 Gy - 7.3 Gy) of adult glial stem cells, however, is higher than the α/β-ratio ($\approx$2 Gy) obtained for CNS in vivo and is closer to that of an acute-responding tissue.

Introduction.

At the cellular level radiation-induced damage becomes apparent when in a hierarchical tissue the functional cell compartment is depleted due to natural attrition and is not replenished by newly formed cells (Thames and Hendry 1987). The turnover rate of the functional cells is assumed to be one of the most important determinants of the latent period between irradiation and expression of damage. The central nervous system (CNS) expresses radiation-induced damage after a latent period of several months to years (Van der Kogel 1991) and is a prime example of a so-called late-responding tissue. The cellular mechanisms leading to the different radiation-induced syndromes are still unknown, but with respect to radiation-induced demyelination we have proposed the precursor cells of oligodendrocytes to be potential target cells. These progenitor cells are able to differentiate *in vitro* either into oligodendrocytes or type-2 astrocytes depending on the culture conditions (Raff et al. 1983), hence the name O-2A progenitor cells. Oligodendrocytes are the myelinating cells in the CNS and type-2 astrocytes are involved in the formation of nodes of Ranvier (ffrench-Constant and Raff 1986). O-2A progenitor cells were first isolated from the perinatal rat CNS (Raff et al. 1983) but have also been identified in the adult rat CNS (Wolswijk and Noble 1989).

According to the linear-quadratic dose dependence of cell survival after irradiation (LQ-model), late-responding tissues are characterized *in vivo* by an α/β-ratio of approximately 2 Gy in contrast with α/β-ratio's of close to 10 Gy for acute-responding tissues. The low α/β-ratio observed for the CNS indicates its large fractionation sensitivity and proficiency for repair.

We have developed an *in vitro* clonogenic assay for O-2A progenitor cells derived from the rat CNS (Van der Maazen et al. 1990) and used this assay to determine the repair capacity of adult glial progenitor cells after *in vivo* or *in vitro* fractionated irradiation. The characterization of the radiobiological properties of glial progenitor cells will help to elucidate the role of these cells in the demyelinating syndromes seen after irradiation of the CNS.

Materials and Methods.

The number of surviving O-2A progenitor cells after irradiation can be determined quantitatively by an *in vitro* clonogenic assay as has been described previously (Van der Maazen et al. 1990). Briefly, the assay is based on the interaction between glial cells in a two-cell layer culture system: CNS-derived O-2A progenitor cells are plated on top of purified monolayers of type-1 astrocytes. These monolayers of type-1 astrocytes are obtained from 1-day-old rat brain cortices. Growth factors produced by the monolayers stimulate O-2A progenitor cells to divide (Noble and Murray 1984). Adult O-2A progenitor cells will give rise

to colonies of newly formed O-2A progenitor cells and oligodendrocytes if the culture medium contains < 1% fetal calf serum (FCS). [Higher concentrations of FCS in the culture medium results in differentiation towards type-2 astrocytes.] When plated at a sufficiently low density, O-2A progenitor cells will form colonies originating from a single precursor cell.

In this study, adult O-2A progenitor cells were obtained from optic nerves of 3-month-old rats (Wistar). The optic nerve was chosen as a source of O-2A progenitor cells because these cells are well characterized (Wolswijk and Noble 1989) and the cell yield per pair of optic nerves is quite reproducible. O-2A progenitor cells were irradiated with 4 MV photons either *in vivo* before isolation, or *in vitro* several hours after isolation.

Irradiated or non-irradiated optic nerves were dissected out of the skull and freed from their meninges. The optic nerves were chopped in fine pieces with a scalpel and further digested with trypsin (15,000 U/ml) and collagenase (333 U/ml) and finally triturated through fine needles (25G and 27G) to obtain a single cell suspension. The high content of myelin and cell debris made it impossible to count individual cells. Therefore specific amounts of the single cell suspensions, adjusted to the expected survival levels, were plated on monolayers of type-1 astrocytes. The cultures were maintained for 4 weeks under standard conditions (humidified; 37°C; 5% CO_2) and one-third of the culture medium was changed 3 times a week.

The long cell cycle time of adult O-2A progenitor cells (T_c=2-3 days; Wolswijk and Noble 1989) required the culture time to be increased from 2 to 4 weeks to obtain sufficiently large colonies. The criterion for surviving colonies was set at 50 cells, unless reported otherwise. After the 4-week culture period the cultures were stained with monoclonal antibodies directed against specific surface antigens. The A_2B_5 antibody (IgM) and the anti-galactocerebroside (anti-GalC; IgG3) antibody were used simultaneously and were counterstained with goat anti-mouse IgM conjugated with fluorescein and goat anti-mouse IgG3 conjugated with rhodamine to visualize the positive cells. The A_2B_5 antibody (Eisenbarth et al. 1979) stains O-2A progenitor cells, neurons and type-2 astrocytes, but under the described culture conditions only O-2A progenitor cells will form colonies. The anti-GalC (Raff et al. 1978) antibody reacts with oligodendrocytes. Finally, the cultures were fixed with ice-cold methanol and screened with a Zeiss Axiovert microscope equipped for epifluorescence. The number of colonies and the number of cells per colony were counted.

Actual repair of sublethal damage in O-2A progenitor cells was studied by subjecting these cells to multiple fractions of X-rays and survival was compared with survival after single doses. Irradiation *in vitro* was performed 4 hours after

plating, allowing viable O-2A progenitor cells to attach firmly to the monolayers of type-1 astrocytes. Cultures receiving 2 fractions *in vitro* were irradiated 4 and 10 hours after plating. In this period cells are assumed to be mitotically inactive and the period of 6 hours between irradiations seems to be sufficient for complete sublethal damage repair *in vitro* (Peacock et al. 1988). *In vitro*, split doses of 2x2.0, 2x3.0 and 2x4.0 Gy were compared with single doses of 4.0, 6.0, or 8.0 Gy, respectively. To compensate for individual differences, the optic nerves of 3 animals were pooled for *in vitro* irradiation experiments. The *in vitro* experiment was repeated 4 times.

O-2A progenitor cells were irradiated *in vivo* while the rats received general anesthesia (Ang et al. 1982). The entire heads, including the optic nerves, were irradiated. Dosimetry was performed in a decapitated animal with insertion of thermo-luminescence detectors in the optic tract. Differences in survival of O-2A progenitor cells were compared after a single dose of 8.0 Gy and fractionated doses of 2x4.0 and 4x2.0 Gy. The overall treatment time of the fractionated irradiation regimens was kept at 3 days. Before sacrificing the animals a period of more than 12 hours after the last irradiation was allowed for potentially lethal damage repair (PLDR). The irradiated optic nerves were processed as described above. In 5 different *in vivo* experiments 9 rats were irradiated according to the 4x2.0 Gy regimen. Six animals were irradiated with 2x4.0 Gy and a total of 15 animals were irradiated 1x8.0 Gy. Two to three cultures were obtained from each animal (equals one pair of optic nerves) and the numbers of surviving colonies were determined after the culture period of 4 weeks. Optic nerves of *in vivo* irradiated animals were processed separately.

All studies were performed under the national regulations for animal welfare and with permission of the institutional ethical committee for animal experiments.

Results.

The survival data obtained in this study were analyzed by three different methods, but all according to the LQ-model. First of all the surviving fractions after acute single doses *in vitro* (4-8 Gy) were calculated for the 4 independent experiments and were combined with earlier data (Van der Maazen et al. 1991). The combined data set was fitted according to the LQ-model ($\ln SF = - \alpha d - \beta d^2$), which resulted in a curve (Figure 9.1) characterized by an α-parameter of 0.143 ± 0.016 Gy^{-1} (mean $\pm$ 2 x standard error); a β-parameter of 0.029 ± 0.004 Gy^{-2}; and an α/β-ratio of 4.93 ± 1.23 Gy. The survival data presented in our previous study (Van der Maazen et al. 1991) however, were calculated after a culture period of 2 weeks in contrast to the 4-week culture period in the present study. Comparability

was obtained by raising the criterion for surviving colonies from 6 cells (for a culture period of 2 weeks) to 50 cells (for a culture period of 4 weeks). This adjustment is allowed as has been demonstrated in the previous study (Van der Maazen et al. 1991).

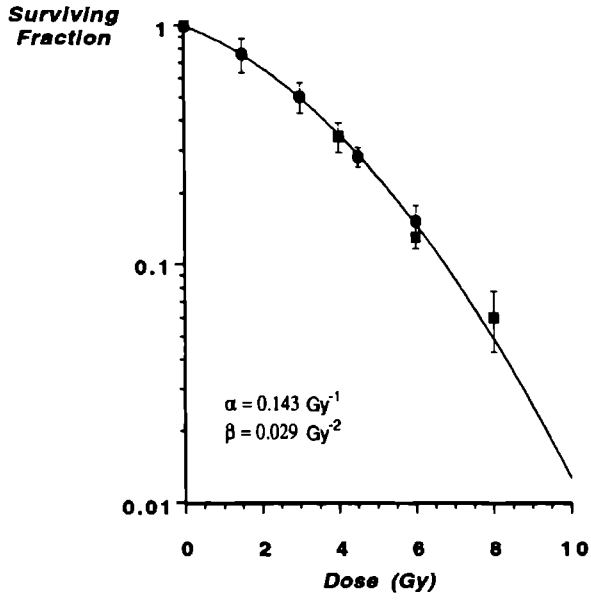

Figure 9.1 Acute single dose survival curve for O-2A progenitor cells irradiated *in vitro*. Earlier data (●; Van der Maazen et al. 1991) were combined with recent data (■) and were fitted according to the LQ-model ($ln\ SF = -\alpha d - \beta d^2$). The curve is characterized by an α-parameter of 0.143 ± 0.016 Gy^{-1} and a β-parameter of 0.029 ± 0.004 Gy^{-2}. Single data points represent mean survival ± standard deviation of 4 independent experiments.

Another method to determine the repair capacity of glial stem cells is to analyze the increased survival after fractionation as compared with survival after single doses according to the method proposed by Peacock et al. (1988). The advantage of this method is that the obtained β-parameter is determined by actual repair of sublethal damage and is independent of the α-parameter. The relationship between the Recovery Ratio (= RR = the ratio of the surviving fraction after fractionated irradiation and the surviving fraction after a single dose) and the β-parameter is expressed by the following equation: $ln(RR) = ((n_2^2 / n_1) - n_2)\ \beta_{RR}\ d_2^2$

(in which: β_{RR} = β-parameter by the method proposed by Peacock et al. (1988); n = number of fractions; d = dose per fraction; and $n_1 \times d_1 = n_2 \times d_2$).

In vitro, the effect of 1x4 0, 1x6 0, and 1x8 0 Gy was compared with 2x2 0; 2x3 0, and 2x4 0 Gy, respectively *In vivo*, 1x8 0 Gy was compared with 2x4 0 and 4x2 0 Gy The RRs for the different dose levels are shown in Table 9 1.

Dose		R R ± stdev	Significance	β_{RR} ± 2 x sterr (Gy^{-2})
In vitro	1x4 0 Gy vs 2x2 0 Gy	1 134 ± 0 127	p = 0 529	*In vitro* :
	1x6 0 Gy vs 2x3 0 Gy	1 751 ± 0 239	p = 0 021	β_{RR} = 0 023 ± 0 0052
	1x8 0 Gy vs 2x4 0 Gy	1 956 ± 0 384	p = 0 001	
In vivo	1x8 0 Gy vs 4x2 0 Gy	2 986 ± 1 563	p = 0 006	*In vivo* :
	1x8 0 Gy vs 2x4 0 Gy	2 059 ± 0 112	p = 0 003	β_{RR} = 0 021 ± 0 0012

Table 9.1 *The mean recovery ratio's ± standard deviation after in vitro and in vivo irradiation The mean colony numbers derived after single dose and fractionated irradiation were compared with Student's t-Test β_{RR} (± 2 x standard error) was calculated according to the formula*
$ln(RR) = ((n_2^2 / n_1) - n_2)\ \beta_{RR}\ d_2^2$

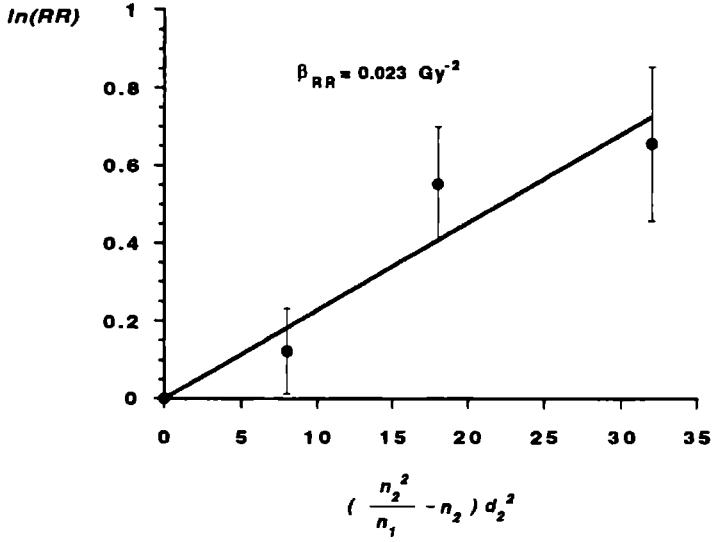

Figure 9.2 Determination of the β-parameter for adult O-2A progenitor cells irradiated *in vitro* according to the method described by Peacock et al (1988) according to the formula $ln(RR) = ((n_2^2 / n_1) - n_2)\ \beta_{RR}\ d_2^2$ Single data points represent the mean value ± standard deviation of 4 independent experiments

When *ln(RR)* is plotted against $((n_2^2 / n_1) - n_2) d_2^2$, β_{RR} is represented by the slope of the line. For O-2A progenitor cells derived from the adult rat optic nerve β_{RR}s of 0.023 ± 0.005 Gy^{-2} (mean ± 2 x standard error) and 0.021 ± 0.0012 Gy^{-2} were obtained after irradiation *in vitro* (Figure 9.2) and *in vivo*, respectively (Table 9.1).

Finally, the survival data were analyzed with a "F_e-plot". The F_e-plot is normally applied when numerical survival data are not available, under the assumption that an equal (level of) effect is caused by an equal level of target cell depletion. This method, however, can also be applied to quantitative survival data according to the equation :

$$-\left(\frac{ln\ SF}{n\ x\ d}\right) = \alpha + \beta d$$

(In which: SF = surviving fraction; n = number of fractions; and d = dose per fraction.)

It allows the combined analysis of both single dose and fractionated survival data to be made. When $-\left(\frac{ln\ S}{n\ x\ d}\right)$ is plotted against the dose per fraction (d), the slope of the line represents β and extrapolation of the line to the ordinate reveals the - α/β ratio. The *in vitro* and *in vivo* survival data were analyzed as one set and an α-value of 0.173 ± 0.0248 Gy^{-1} (mean ± 2 x standard error); a β-value of 0.024 ± 0.0054 Gy^{-2}; and an α/β-ratio of 7.3 ± 2.66 Gy were obtained (Figure 9.3). Separate analyses of the *in vitro* and *in vivo* data did not show a significant difference with the combined fit.

Discussion.

In vitro and *in vivo* survival data of adult O-2A progenitor cells were analyzed according to the LQ-model by 3 different methods: the single dose survival curve; the method proposed by Peacock et al. (1988); and by a "F_e-plot". The absolute value of the β-parameter depended somewhat on the method of analysis, but varied between 0.02 Gy^{-2} and 0.03 Gy^{-2}. These values are consistent with the absolute value of β of 0.025 Gy^{-2} for the CNS *in vivo* (Ruifrok et al. 1992) obtained by the direct data analysis method of Thames et al. (1986) and indicate that these cells are able to repair a substantial amount of SLD.

The α-value, however, is considerably higher for O-2A progenitor cells (0.143 to 0.173 Gy) than for the adult CNS *in vivo* (0.042 Gy; Ruifrok et al. 1992) and consequently the α/β-ratio of O-2A progenitor cells is higher than one would

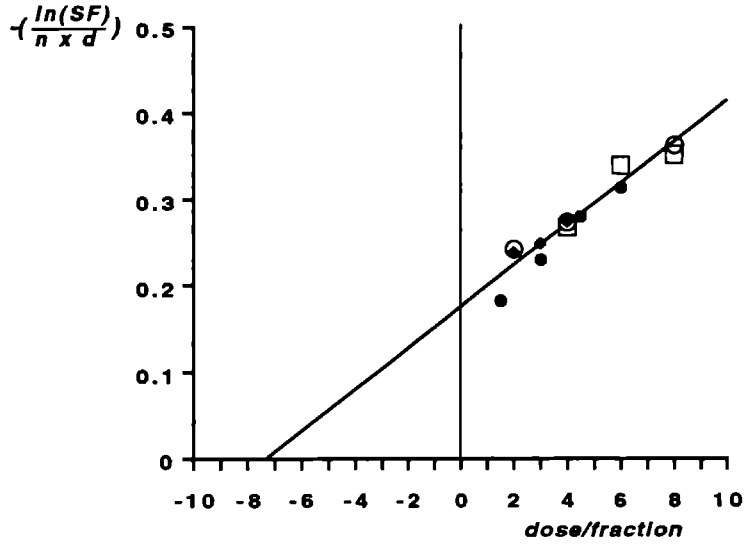

Figure 9.3 "F_e-plot" of survival data after irradiation *in vitro* (● Van der Maazen et al. 1991; □ single dose survival data; ◆ split dose survival data) and *in vivo* (O). Data are fitted according to the equation: $-(\frac{ln\ SF}{n\ x\ d}) = \alpha + \beta d$. For O-2A progenitor cells absolute values of 0.173 ± 0.0248 Gy^{-1} and 0.024 ± 0.0054 Gy^{-2} were found for α and β, respectively.

expect for a late-responding tissue. *In vivo* the α/β-ratio for rat cervical spinal cord is approximately 2 Gy (Van der Kogel 1991) whilst for adult O-2A progenitor cells the α/β-ratio ranged from 4.9 Gy to 7.3 Gy. The question therefore arises whether O-2A progenitor cells are the target cells of radiation-induced CNS injury because a high α/β-ratio is compatible with repair characteristics of an acute-responding tissue. A possible explanation for this apparent inconsistency is the difference in proliferative state of the target cells. Longer times available for the repair of radiation-induced damage between irradiation and entering into the cell cycle could explain the lower absolute value for α for the adult CNS when compared with the α-value for isolated O-2A progenitor cells *in vitro*. In contrast with the *in vivo* situation, all O-2A progenitor cells *in vitro* are stimulated constantly to divide and less time will be available for repair. The proliferative state of the O-2A progenitor cells *in vitro* could be compared with the proliferative state of the target cells of the developing CNS *in vivo* . Recently, Ruifrok et al. (1992) demonstrated that indeed the α/β-ratio for the developing CNS was significantly higher than the α/β-ratio for the adult CNS (α/β-ratio is 4.5 Gy for 1-week-old rats) and makes it plausible that the proliferative state of the target cells determines their fractionation sensitivity. For the kidney, which is another example of a late-responding tissue, it

has also been noticed that the proliferative state of the target cells, after irradiation, can influence the fractionation sensitivity. When the α/β-ratio was measured shortly after the precipitation of radiation injury, kidney epithelium revealed an α/β-ratio of 12.5 Gy consistent with values obtained for acute-responding tissues (Ewen and Hendry 1989). However, this was not observed using an *in vitro* assay performed at different times after *in vivo* irradiation (Jen and Hendry 1993).

The assumption that in a late-responding tissue the majority of the stem cell population is in a resting phase of the cell cycle is, however, not completely in agreement with the unexpected early and fast repopulation of O-2A progenitor cells after irradiation of the adult rat optic nerve (Van der Maazen 1992). This quick repopulation of O-2A progenitor cells suggests that radiation damage inflicted upon these stem cells is detected almost immediately and is restored independent from the functional cell compartment (= oligodendrocytes). The precise time interval that elapses between irradiation and entrance into the cell cycle has not been studied but could give answers to the questions raised.

In vivo and *in vitro* experiments have made it plausible that glial cells in the CNS are organized according to different hierarchical systems. With respect to demyelination seen after irradiation of the CNS the O-2A lineage seems most likely to be involved. Godfraind et al. (1989) investigated the cellular responses in the CNS after a hepatitis mouse virus infection which is directly cytotoxic to oligodendrocytes. O-2A progenitor cells were capable to replenish the glial stem cell pool and the functional cell compartment (= oligodendrocytes) with improvement of the neurological deficits within weeks. The latent period between irradiation and demyelination in the CNS makes it clear that radiation is not directly cytotoxic to oligodendrocytes. The proposed target cells are their precursors and radiation damage will be expressed when the normal loss of functional cells is not compensated for. The present study on the LQ-parameters of O-2A progenitor cells has revealed values for the β-parameter that are consistent with values for CNS irradiated *in vivo*, but an α/β-ratio which tends towards values usually found for rapidly proliferating tissues.

CHAPTER 10

REPOPULATION OF O-2A PROGENITOR CELLS AFTER IRRADIATION OF THE ADULT RAT OPTIC NERVE ANALYZED BY AN *IN VITRO* CLONOGENIC ASSAY

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Abstract.

In the central nervous system (CNS) O-2A (Oligodendrocyte - type 2 Astrocyte) progenitor cells have been proposed as potential target cells, and their depletion by irradiation will cause demyelination. The extent and time course of repopulation of these glial stem cells were studied in the adult rat optic nerve after irradiation in vivo. The number of O-2A progenitor cells was measured quantitatively by an in vitro clonogenic assay. Although the CNS is typically a late-responding tissue, repopulation was initiated almost immediately after irradiation and after several weeks a plateau was reached that lasted up to 6 months. Single doses of 4 -12 Gy of X-rays caused a permanent reduction in the number of O-2A progenitor cells.

An analysis of the colony size of O-2A progenitor cells showed a sustained reduction in the number of offspring of cells surviving a dose of 12 Gy. In addition, the colony size of unirradiated progenitors diminished with increasing age of the animals.

Introduction.

The rate and extent of repair of normal tissues will determine the possibility of retreatment after an initial course of radiotherapy. The normal central nervous system (CNS) in the treatment field is the critical structure that limits the radiation dose to primary CNS tumors and tumors in its neighborhood. When the radiation dose exceeds a critical level, the CNS becomes irreparably damaged. Understanding the cellular mechanisms involved in radiation damage in the CNS will give the opportunity to manipulate the conditions to increase tolerance and will offer a scientific foundation on which decisions about retreatment could be based.

Examination of injured CNS reveals that at least two different radiation-induced syndromes can be determined. The pathological effects are dominated either by demyelination or by vascular abnormalities and seem to be related to total dose and latent period (Van der Kogel 1980). We hypothesized that the demyelinating syndromes are not a secondary effect of vascular damage but are due to the depletion of myelinating cells. Recently, precursors of the myelinating cells in the perinatal rat optic nerve have been described (Raff et al. 1983). These cells were called O-2A progenitor cells because of their ability to differentiate into oligodendrocytes or type-2 astrocytes. Similar O-2A progenitor cells exist in the adult CNS and are capable of self-renewal, albeit at a low rate (Wolswijk and Noble 1989). This observation is in agreement with thymidine labeling studies which demonstrated a slow turnover of glial cells in the adult CNS(Smart and Leblond 1961). As self-renewing precursor cells of oligodendrocytes, adult O-2A progenitor cells were hypothesized to be an important population of target cells for radiation-induced demyelination (Van der Maazen et al. 1990). Studying the long-term effect of a single dose of radiation on this putative target cell population will contribute to the understanding of the cellular effects of radiation on the CNS.

To measure quantitatively the effect of radiation on the O-2A progenitor pool, we have developed an *in vitro* clonogenic assay to determine the number of surviving glial precursor cells after *in vivo* irradiation (Van der Maazen et al. 1990). In the present study the optic nerves of adult rats were irradiated with different doses of X-rays and the number of clonogenic O-2A progenitor cells was determined at up to 6 months after irradiation. A clear dose-response relationship was observed, and it appeared that at 6 months there was no complete recovery of the O-2A progenitor pool after relatively moderate doses of 8 and 12 Gy. Analysis of the colonies suggests that there is a delayed expression of radiation-induced cytotoxicity. In addition, the progeny of adult progenitors seems to be reduced with age.

Materials and Methods.

Effects of a single dose of radiation on the O-2A progenitor cell population of the adult rat optic nerve have been studied. Rats (Wistar) were irradiated at the age of 3 months with X-rays from a linear accelerator (4 MV), including most of the head and both optic nerves. The rats received general anaesthesia during irradiation (Ang et al. 1982). Dosimetry '*in vivo*' was performed with TLDs (thermoluminescent dosimeters) inserted in the optic tract of a decapitated animal.

The number of surviving O-2A progenitor cells was assessed by an *in vitro* clonogenic assay. This assay has been described before (Van der Maazen 1990) and will be discussed briefly. Growth factors produced by monolayers of type-1 astrocytes stimulate single adult O-2A progenitor cells to form colonies, consisting of new O-2A progenitor cells and oligodendrocytes. Monolayers of type-1 astrocytes were prepared from newborn rat cerebral cortices according to the guidelines of McCarthy and De Vellis (1980), with small modifications. The optic nerve of the adult rat was chosen as a source of O-2A progenitor cells because these cells are well characterized and the number of colonies derived from one nerve is highly reproducible. After the rats were sacrificed, the optic nerves were collected and freed from their meninges. With a scalpel they were cut in small pieces and subjected to treatment with trypsin (15000 U/ml) and collagenase (333 U/ml). After enzymatic treatment the little pieces of optic nerve were triturated through fine needles (25G and 27G) to obtain single cell suspensions. The counting of individual cells in these cell suspensions was very unreliable due to the high content of myelin and cell debris. Therefore, the cell suspension of one pair of optic nerves was diluted to a volume of 2 ml. The yield of O-2A progenitor cells derived from one optic nerve was quite reproducible, allowing the plating of "standardized" amounts of the optic nerve cell suspension [yield: an average of 750 ± 250 (± standard deviation) viable O-2A progenitor cells per pair of optic nerves]. The "standardized" amounts of optic nerve cell suspensions were plated on monolayers of type-1 astrocytes and were grown for 4 weeks in a chemically defined medium (Bottenstein and Sato 1979) supplemented with 0.5% fetal calf serum under standard conditions (37°C; 5%CO_2; humidified). For the counting of colonies, the cultures were incubated with monoclonal antibodies directed against surface antigens of O-2A progenitor cells [A_2B_5 = IgM; Eisenbarth et al. 1979) and oligodendrocytes (anti-galactocerebroside = IgG3 (Raff et al. 1978)]. The cultures were counterstained with goat anti-mouse IgM conjugated with fluorescein and goat anti-mouse IgG3 conjugated with rhodamine to visualize O-2A progenitor cells and oligodendrocytes. The determination of the number of colonies and the number of cells per colony was performed with a Zeiss Axiovert microscope equipped for epifluorescence.

The present study deals with the effect of 4, 8, or 12 Gy of X-rays on the O-2A progenitor cell population in the adult rat optic nerve. The number of O-2A progenitor cells was determined by means of the method described above at several time intervals after irradiation (immediately, 1 day, 1 week, 2 weeks, 3 weeks, 2 months, 3 months, 4.5 months and 6 months after irradiation). All animals (in total 120) were irradiated at the age of 3 months. For each dose, three animals were used at each specific time interval. Each animal yielded two or three cultures, depending on the number of recovered O-2A progenitor cells. The mean colony number of three identically treated animals was determined and the fraction of recovered O-2A progenitor cells was calculated as a fraction of the number of colonies obtained from three non-irradiated control animals (whose optic nerve cell suspensions were cultured on the monolayers of type-1 astrocytes of the same batch). The criterion for surviving O-2A progenitor cells after irradiation was set at the ability to form a colony of at least six cells. This low number of cells per colony was chosen because the cell cycle time of adult O-2A progenitor cells is rather long (Wolswijk and Noble 1989) and, as will be discussed below, to compensate for the effect of aging on colony size.

Results.

The fractions of recovered O-2A progenitor cells are shown in Figure 10.1 for a period of 6 months after irradiation with doses of 4, 8, and 12 Gy. There is a clear dose-response relationship and the initial drop in cell number is close to the survival level predicted by previous experiments (Table 10.1), which yielded an α coefficient of 0.15 Gy^{-1} and a β coefficient of 0.03 Gy^{-2} for *in vitro* survival data according to the linear-quadratic (LQ-) model (Van der Maazen et al. 1991).

Table 10.1 - SURVIVING FRACTIONS (S.F.) OF O-2A PROGENITOR CELLS

Dose	Predicted S.F	Observed S.F.
4 Gy	0.340	0.319
8 Gy	0.044	0.054
12 Gy	0.002	0.013

Comparison of surviving fractions of adult O-2A progenitor cells observed after irradiation in vivo and immediate analysis in vitro with surviving fractions predicted by the LQ-model with an α coefficient of 0.15 Gy^{-1} and a β coefficient of 0.03 Gy^{-2} as derived from in vitro single-dose survival data (Van der Maazen et al. 1991). The observed surviving fractions were analyzed with Student's t-Test (0 Gy vs. 4, 8, 12 Gy; 4 Gy vs. 8, 12 Gy; and 8 Gy vs. 12 Gy) and were found to be significantly different from each other ($p < 0.001$).

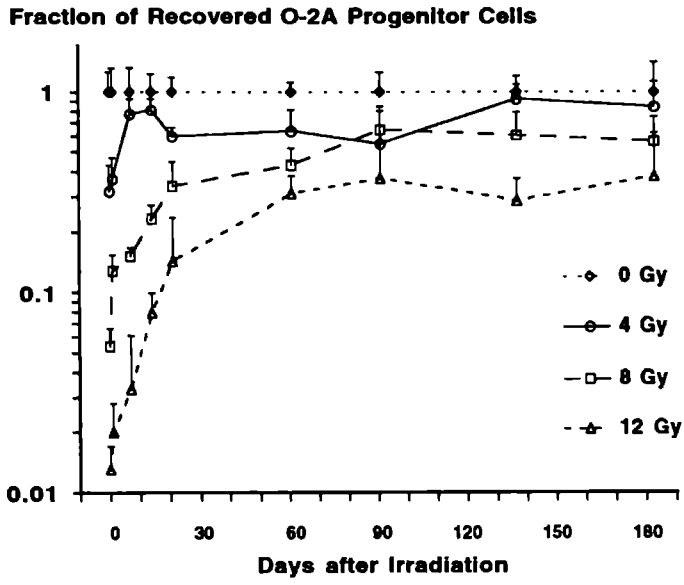


Figure 10.1 Repopulation of O-2A progenitor cells plotted as the fraction of recovered cells after irradiation *in vivo* of adult rat optic nerve with 4 (◊), 8 (O), and 12 (Δ) Gy of X rays. The surviving glial stem cells were determined *in vitro* with a clonogenic assay. The mean surviving fraction ± standard deviation is plotted against the time after irradiation.

In the first 24 h after irradiation there is a rise in survival due to potentially lethal damage (PLD) repair. After 24 h the increase in the number of O-2A progenitor cells is likely to be due to repopulation. Three weeks after irradiation the first wave of regeneration of O-2A progenitor cells seems to be almost complete, while at 2 months a plateau is reached lasting for at least 6 months after irradiation. At 6 months after a single dose of X-rays the number of O-2A progenitor cells in the irradiated optic nerves had not reached the levels in untreated optic nerves. At 6 months after irradiation the numbers of recovered O-2A progenior cells were compared with Student's t-Test (0 Gy vs. 4, 8, 12 Gy; 4 Gy vs. 8, 12 Gy and 8 Gy vs. 12 Gy) and were found statistically different ($p < 0.05$). Only the number of O-2A progenitor cells at 6 months after 4 Gy was not significantly different from 0 Gy ($p = 0.179$). The extent of repopulation seems to be determined by the size of the initial dose and consequent cell loss.

At day 0; 3 weeks; 2 months; and 6 months after irradiation the sizes of colonies derived from control animals and from animals irradiated with 12 Gy were compared. At all time points the nonirradiated colonies are larger than the colonies obtained after 12 Gy (Figure 10.2), indicating that even at 6 months

radiation-induced damage is still expressed by surviving O-2A progenitor cells. When the colony size of nonirradiated colonies is examined over the period of 6 months after irradiation it is obvious that at the age of 3 months > 80% of the O-2A progenitor cells is able to form a colony of more than 50 cells in a 4-week culture period. This contrasts with only approximately 50% of the O-2A progenitor cells that is able to reach this size when the rats are 9 months old. Thus, the reproductive capacity of O-2A progenitor cells seems to diminish with age, an observation not reported before.

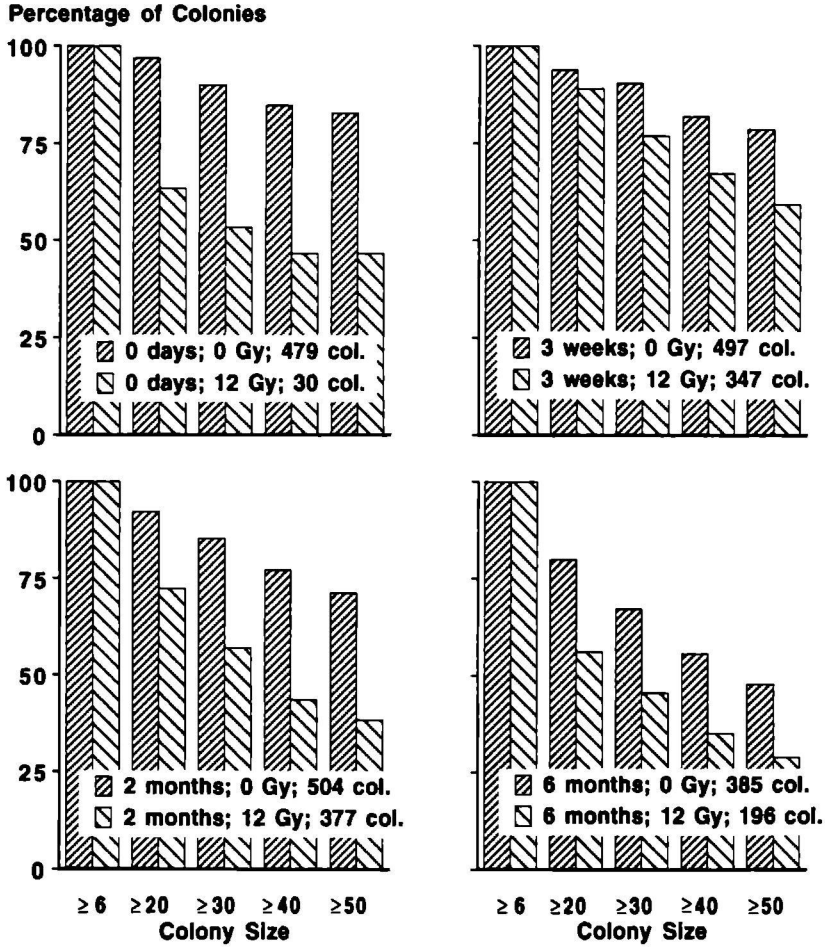


Figure 10.2 Proliferative capacity of adult O-2A progenitor cells derived from nonirradiated and irradiated (12 Gy) rat optic nerves determined immediately (a); 3 weeks (b); 2 months (c); and 6 months after irradiation (d). The height of the bars represents the percentages of surviving clonogenic O-2A progenitor cells able to generate an offspring of at least 6, 20, 30, 40, and 50 cells after a culture period of 4 weeks. Irradiation and age have a negative influence on colony size.

Discussion.

Irradiation of the adult rat CNS *in vivo* resulted in a decrease, followed by a rapid increase in the number of glial stem cells during the first 3 weeks after irradiation to reach a plateau at approximately 2 months, which lasted up to 6 months. The plateau depends on the initial dose and it is remarkable that after relatively moderate doses of X-rays there is a permanent reduction in the number of glial stem cells (at least up to 6 months). If the O-2A progenitor cell population is the target cell population of radiation-induced demyelination, one would expect that a permanent decrease in cell number and progeny would be reflected in a reduced tolerance for retreatment. Studies on rat spinal cord tolerance seem to confirm this assumption. Retreatment at 6 months after an initial dose close to the tolerance level reveals almost no recovery, while lower initial doses permit retreatment, which indicates that a substantial amount of restoration of the stem cell compartment has been achieved (Van der Kogel 1991). It is still uncertain if longer periods after irradiation will lead to full recovery of radiation damage in the CNS as is seen, for example, for acute reactions of mouse skin (Terry et al. 1989).

Different mechanisms contribute to the rise in the number of surviving stem cells after irradiaton. Immediately after irradiation repair of intracellular damage takes place and dependent on the method of investigation it is called PLD repair or sublethal damage (SLD) repair. It is still unclear if these two forms of repair are a reflection of the same process or are indeed two independent cellular mechanisms. In split-dose or fractionated irradiations with 24-h intervals, the rat spinal cord showed a remarkable capacity to repair sublethal damage (Van der Kogel 1991; White and Hornsey 1978). After this period of 24 h, which until recently was accepted to be sufficient for complete repair of intracellular radiation damage, no further increase in radiotolerance could be detected for weeks to months.

Recently, several authors have subjected the issue of 'regeneration versus repair' to further analysis. Jen and Hendry (1991) reported the existence of a slow repair component in kidney clonogens. An increase in surviving clonogens, assayed *in vitro*, was seen in the period from 12 h to 6 weeks after X-irradiation, but not after neutron irradiation. It was concluded that the increased survival was due to slow repair of intracellular damage rather than to regeneration. Fisher et al. (1988) studied the long-term repair of mouse hepatocytes after irradiation *in vivo*. At 11 months after irradiation an increase in clonogenicity of hepatocytes was found which could be expressed by a dose-modifying factor (MDF = D_o after 11 months / D_o immediate after irradiation) of 3.5. An increase in the labeling index (LI) as a sign of repopulation was not observed for the whole liver population in the 11-month period, so the increase in clonogenicity was attributed to a slow

repair component and not to repopulation. The LI for the whole liver, however, could be an underestimation of the LI for the subpopulation of clonogenic hepatocytes that are assayed with the so-called "fat pad assay". Repopulation could therefore have played a larger role in the recovery process of hepatocytes than concluded by the authors.

Animal studies using functional endpoints after irradiation also provide evidence for the existence of a slow intracellular repair component (Hopewell and Van den Aardweg 1988; Van Rongen et al. 1991). Of special interest is the study by Ang et al. (1992), who investigated the repair kinetics of sublethal lesions in the rat spinal cord after irradiation. The experimental data were best fitted with a biexponential repair model. The proportion of injury repaired by the slow component, with a half-time of repair of 4 h, was estimated to be 65%. This would suggest that at 24 h after irradiation repair is almost complete. We did not investigate the existence of a slow repair component in O-2A progenitor cells, but if *in vivo* experiments indicate that 24 h between irradiations is sufficient in the CNS for both rapid and slow repair processes, a further rise in surviving clonogenic O-2A progenitor cells between 24 h and 2 months after irradiation is likely to be due to repopulation.

It is striking that the initial recovery of O-2A progenitor cells occurs so quickly after irradiation. Probably, after radiation-induced loss of O-2A progenitor cells, the number of these cells is regulated by homeostatic mechanisms independent of the slowly turning-over functional cell compartment (= oligodendrocytes). On the other hand, O-2A progenitor cells are also stimulated to proliferate by a loss of oligodendrocytes as has been shown *in vivo* using a virus-mediated acute demyelination model (Godfraind et al. 1989). The rapid increase of the number of O-2A progenitor cells coincides with an increased labeling index of glial cells in the rat spinal cord after a single dose of 20 Gy (Hornsey et al. 1981): The mean value of the LI in the cervical spinal cord for unirradiated rats was 3.0% ± 0.5. After 20 Gy an immediate fall in LI to almost zero was observed at 1 to 6 days after irradiation, followed by a rise to almost 9% during the period 16 to 21 days after irradiation. The early rise in LI and the concomitant rise in O-2A progenitor cells could be expected to be accompanied by an increased tolerance of the CNS as early as a few weeks after an initial radiation dose. At this point there is a difference between the time at which recovery is predicted to start by this *in vitro* analysis and the time at which functional restoration of radiation damage can be demonstrated *in vivo*. Retreatment data on rat spinal cord *in vivo* show that functional restoration of radiation damage occurs mainly between 10 and 20 weeks after an initial dose of X-rays (Van der Kogel 1991). Further experiments

will be aimed at investigating to what extent early glial stem cell proliferation contributes to the replacement of functional oligodendrocytes.

Analysis of the colony sizes derived from unirradiated animals revealed an age-dependent decrease in the size of the colonies produced by the progenitor cells. At the age of 3 months more than 80% of the unirradiated colonies contained more than 50 cells after a culture period of 4 weeks. This percentage gradually decreased to approximately 50% for O-2A progenitor cells obtained from 9-month-old rats. At this moment the biological implications of this finding are unknown, but it could well be that older animals are more susceptible to radiation damage or show a decreased regeneration capacity compared to younger animals. To avoid this influence of age in establishing a regeneration profile, a rather low colony-size limit for surviving O-2A progenitor cells was chosen. The effect of age on the proliferative capacity of adult O-2A progenitor cells has not been reported before but it has been previously described for haematopoietic stem cells (Mauch et al. 1982).

Another phenomenon which became clear from the analysis of colony sizes is the delayed expression of radiation damage by surviving cells. At all times after irradiation (even at 6 months) the colony size of O-2A progenitor cells irradiated with 12 Gy is smaller compared with the colony size of unirradiated O-2A progenitor cells. This finding supports the view of Seymour et al. (1986) that among cells in a colony derived from a single surviving cell after irradiation, non-colony-forming cells are found. This is an important finding in the evaluation of the effect of X-rays on O-2A progenitor cells. Not only the number of surviving cells but also the proliferative potential of these cells will determine the impact of radiation on tissue integrity.

The rapid initial recovery observed in this study suggests the presence of a homeostatic mechanism which regulates the number of cells in the O-2A progenitor pool in the adult rat optic nerve, independent of the number of mature oligodendrocytes. After radiation-induced cell depletion, mechanisms are activated to restore the original cell number. It would be important to understand these mechanisms to manipulate them in order to reduce the toxicity of radiation or chemotherapeutic agents. The mediators in the restoration process are likely to be growth factors, some of which have been identified in the CNS. Platelet-derived growth factor appears to play an important role in the onset of proliferation (Noble et al. 1988) and, it may be possible to relate the increase of O-2A progenitor cells to an earlier or concomitant rise in cytokines. Ultimately, the administration of growth factors before or after irradiation could reduce the adverse effects of radio- and/or chemotherapy, possibly allowing more intensive treatments or retreatments of recurring tumors.

APPENDIX 1

SURVIVAL CURVE MATHEMATICAL MODELS

From the many models that have been proposed to fit clonogenic cell survival data after irradiation only two will be discussed: the single hit-multitarget model and the linear-quadratic model. Both models are frequently used in the literature to fit survival curves to experimental data and do this acurately. In the recent literature, however, preference is given to the linear-quadratic model because of its simplicity, biophysical basis and its applicability to predict tissue responses (see appendix 2). Examples of other or more expanded models are: incomplete repair model (Thames 1985); accumulation model (Roesch 1978); repair saturation model (Green 1972); repair interaction model (Harder 1984); lethal-potentially lethal damage model (Curtis 1986); repair misrepair model (Tobias 1985).

The Single Hit Multitarget Model.

This model combines the single hit and the multitarget model and is based on Poisson statistics. The single hit model assumes that, as the name already indicates, a cell is inactivated when hit at a single sensitive spot. One such lesion is sufficient for cell death. An exponential curve describes the relationship between cell survival and absorbed dose. The curve becomes a straight line when plotted on a semilog scale.

$$SF = \frac{N}{N_0} = exp\ -(\frac{D}{D_0})$$

$$ln\ SF = -(\frac{1}{D_0})\ D$$

(In which: SF = surviving fraction; N = number of surviving clonogenic cells; N_0 = number of irradiated clonogenic cells; D = single dose; D_0 = the dose that would have killed all the cells if they were all hit at their lethal spot. However, due to Poisson statistics of dose distribution some cells will not be hit and others once or more. If $D = D_0$ the SF = exp(-1) = 0.37.)

The multitarget model was developed to deal with the "shoulder" in experimental survival data. For many cells it appeared that at low doses radiation was not as effective in cell killing as it was at higher doses. This phenomenon was explained by the theory that there are several targets in a cell that need to be inactivated before the cell is killed.

According to Poisson statistics, the probability that a single target is not hit by radiation is (= surviving fraction in the single hit model: see above):

$$p(0) = exp\ -(\frac{D}{D_0})$$

The probability that one target is hit is:

$$p(1) = 1 - exp\ -(\frac{D}{D_0})$$

The probability that n targets are hit is:

$$p(n) = (1 - exp\ -(\frac{D}{D_0}))^n$$

The surviving fraction is:

$$SF = 1 - (1 - exp\ -(\frac{D}{D_0}))^n$$

(In which: n = the number of targets or extrapolation number.)

The width of the shoulder is defined by the extrapolation number and the quasi threshold dose (= D_q). The D_q relates to the extrapolation number as:

$$D_q = D_0 \bullet ln\ n$$

The single hit-multitarget model combines the two models and gives a better fit of experimental data:

$$SF = (exp\ -(\frac{D}{D'_0})) \bullet (1 - (1 - exp\ -(\frac{D}{D'_0}))^n)$$

The Linear-Quadratic Model.

The linear-quadratic model (LQ-model) describes cell survival as a resultant of two processes. It resembles the single hit-multitarget model because the first part of the curve is determined by the linear component and as the dose increases the survival is increasingly influenced by the second (=quadratic) component. In contrast with the single hit-multitarget model the LQ-model does not produce a straight line at high doses. When plotted on a semilog scale the curve keeps on bending. The two components of the LQ-model have been linked with lethal lesions occuring at the subcellular level. Chadwick and Leenhouts (1981) and Kellerer and Rossi (1978) suggested that a lethal lesion is the accumulation of two sublethal events. A single hit may induce both sublesions necessary to kill the cell or two hits are needed to inactivate both sublesions. These two processes represent the linear and the quadratic part of the survival curve. Chadwick and Leenhouts associated radiation-induced double strand DNA breaks with cell death and considered a single hit lethal event to be a single double strand break and a two hit lethal event a double single strand break.

The linear component of the LQ-model has been associated with the intrinsic radiosensitivity of the cells. The curve becomes steeper if the cells are more sensitive. The quadratic component has been linked with the ability of the cells to repair radiation-induced damage.

The LQ-equation:

$$SF = \frac{N}{N_0} = exp\ (-\alpha D - \beta D^2)$$

$$ln\ SF = -\alpha D - \beta D^2$$

(In which: N = the number of surviving clonogenic cells; N_0 = the number of irradiated clonogenic cells; α and β are the target cell survival parameters; D = single dose; αD = the alpha component; βD^2 is the beta component.)

The formula can be rewritten for fractionated irradiation as:

$$ln\ SF = n \bullet (-\alpha d - \beta d^2)$$
$$ln\ SF = D \bullet (-\alpha - \beta d)$$

(In which: D = the total dose = n • d; n = the number of fractions; d = the dose per fraction.)

The α/β-ratio is the dose that leads to an equal contribution of the linear and quadratic component to the cell kill.

When single dose survival data are fitted according to the LQ-model absolute values for the α- and β-parameter can be derived. Although the determination of these parameters is in principal very accurate, small changes in the surviving fractions have large consequences with respect to the absolute values of these parameters. Especially when the cells are characterized by a large intrinsic radiosensitivity (high α-value) the determination of the β-parameter becomes difficult (Peacock et al. 1988). Another disadvantage of this method is that the β-parameter, which is a measure for the repair capacity of the target cells, is not determined by actual increased survival after fractionated irradiation. Peacock et al. (1988) proposed a method to determine the β-parameter after fractionated irradiation based on actually repaired radiation damage. The increase of survival after fractionated irradiation can be expressed as the recovery ratio (= RR). The recovery ratio is by definition not dependent on the α-component but is determined by the β-component. The relation between the recovery ratio and the β-parameter can be deduced as follows:

$$Recovery\ Ratio = RR = \frac{SF_2}{SF_1}$$

$$ln\ RR = ln\ SF_2 - ln\ SF_1$$

$$\ln RR = n_2 \bullet (-\alpha d_2 - \beta d_2^2) - n_1 \bullet (-\alpha d_1 - \beta d_1^2)$$

$$d_1 \bullet n_1 = d_2 \bullet n_2 \quad <\text{------}> \quad d_1 = \frac{n_2}{n_1} \bullet d_2$$

$$\ln RR = \{n_2 \bullet (-\alpha d_2 - \beta d_2^2)\} - \{n_1 \bullet [-\alpha \bullet \frac{n_2}{n_1} \bullet d_2 - \beta (\frac{n_2}{n_1} \bullet d_2)^2]\}$$

$$\ln RR = -n_2 \bullet \beta \bullet d_2^2 + n_1 \bullet \beta \bullet (\frac{n_2}{n_1} \bullet d_2)^2$$

$$\ln RR = -n_2 \bullet \beta \bullet d_2^2 + n_1 \bullet \beta \bullet (\frac{n_2^2}{n_1^2}) \bullet d_2^2$$

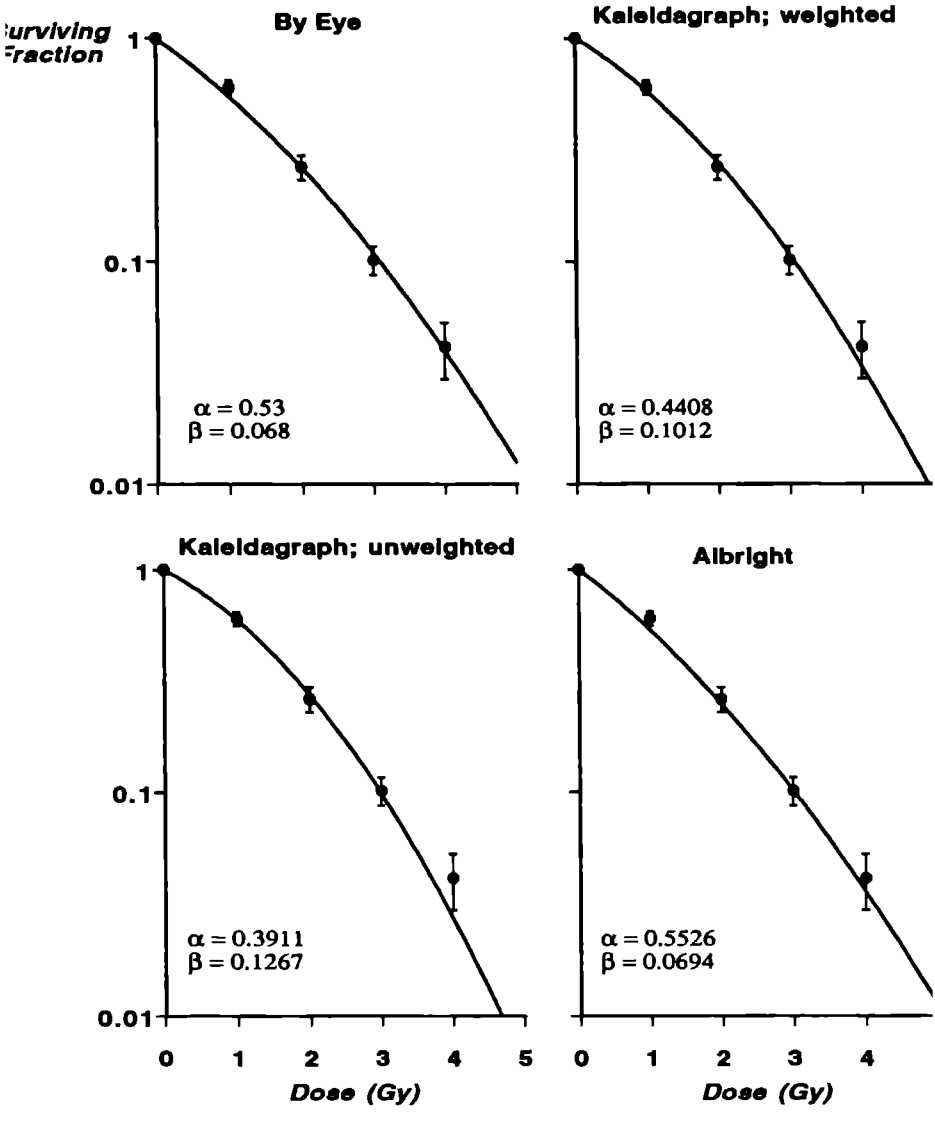
$$\ln RR = \{ -n_2 + n_1 \bullet (\frac{n_2^2}{n_1^2})\} \bullet \beta \bullet d_2^2$$

$$\ln RR = (\frac{n_2^2}{n_1} - n_2) \bullet \beta \bullet d_2^2$$

This general formula allows the determination of the β-parameter for cells that have been exposed to different fractionation regimens. The β-parameter determined by this method has been called the β_{RR}. The lnRR can be plotted against $(\frac{n_2^2}{n_1} - n_2) \bullet d_2^2$ and the fitted straight line allows the calculation of the β_{RR} (= the slope of this line).

Another method to determine the β-parameter independently from the α-parameter is to fit the acute single dose survival curve according to the LQ-model with a fixed α-parameter derived from low dose rate experiments (Steel 1991). Irradiation with a continuous low dose rate allows cells to repair almost all sublethal damage. Survival is therefore only determined by the linear component of the LQ-equation (α-parameter). With this 'low dose rate derived' α-parameter an acute single dose survival curve can be fitted according to the LQ-model and will

give an independently derived β-parameter. The facilities, however, to perform low dose rate experiments are not available in Nijmegen.



Graphical presentation of the different Survival Curve Models: a. Single Hit Model; b. Multitarge model; c. Single Hit Multitarget Model; and d. Linear Quadratic model.

APPENDIX 2
TOLERANCE FORMALISMS

Isoeffect models are mathematical models used in radiobiology and radiotherapy to describe the relation between the total dose and the treatment variables, which are thought to influence treatment outcome. They are based on experimental and clinical observations and are attempts to predict the outcome of changes in the treatment schedule. Isoeffect models allow the calculation of different radiation schemes with an equal probability of a specific tissue response (=isoeffective). If the model predicts differences in the radiation response of tumors and normal tissues, it can also be used to design a treatment protocol with the highest therapeutic ratio.

Isoeffect models can be divided into two main categories (Thames and Hendry 1987):
1. the power-law formalisms and
2. isoeffect models based on cell survival.

Power-law formalisms.

Power-law formalisms are characterized by an equation in which the total accumulated dose to achieve a specific tissue response is correlated with either the overall treatment time or the number of fractions or both:

$$D = c \cdot N^p \cdot T^q$$

(In which: D = the total dose; c = a constant; N = the number of fractions; T is the overall treatment time and p and q are the exponents of the N and T variables.)

These formalisms were derived from the Strandqvist formula (1944). Strandqvist plotted on a double log scale the total dose to achieve cure of skin and lip carcinoma against the overall treatment time and extrapolated the following relationship:

$$D = c \cdot T^{0.22}$$

The equation related treatment outcome only to overall treatment time. Cohen (1949) analyzed the radiation response of normal skin and came to the conclusion that normal skin reacted differently to radiation than skin carcinomas and he proposed different values for the T exponents (0.33 and 0.22, respectively). Experimental data on pig skin (Fowler 1963), however, could not be explained by this relatively simple model and the fraction number (which is related to the fraction size) was proposed as an additional parameter that determines treatment outcome. Ellis (1967) was one of the first to recognize this problem and postulated the following formalism:

$$D = NSD \bullet N^{0.24} \bullet T^{0.11}$$

(In which: D = the total dose; NSD = the nominal single (or standard) dose = the "calculated" single dose for a certain biological effect = a constant; N = the number of fractions; and T = the overall treatment time.)

This formalism correlated treatment outcome with the overall treatment time and the number of fractions. Although the equation was derived from observations of skin responses to irradiation and based on very limited data (Thames and Hendry 1987), it was used by others to predict the radiation response for most other tissues. This led to unpredicted side effects especially for the so-called late-responding tissues. Therefore, these late-responding tissues were subjected to radiation experiments to determine the influence of the N- and T-parameters and their exponents on radiation tolerance. For the rat cervical spinal cord the overall treatment time seemed to have no effect on the radiation response, if the overall treatment time did not exceed a period of 6 to 8 weeks and if fractions were spaced sufficiently to allow complete repair of sublethal damage (review: Van der Kogel 1991). Another difference with the skin data was the effect of fraction size on treatment outcome. The rat CNS compared to skin was much more sensitive than skin to changes in the fraction size: if the fraction size increased the isoeffective dose (D) decreased much faster for spinal cord than for skin. This increased sensitivity for changes in fraction size is reflected in the absolute value of the N-exponent: 0.24 for skin and 0.42 for CNS (Van der Kogel 1979; Sheline 1980). For the rat cervical spinal cord the power-law equation became:

$$D = c \bullet N^{0.42}$$

(In which: D = the total dose; c = a constant; N = the number of fractions.)

For the lumbosacral spinal cord a slight deviation from the above formula was observed and is probably a reflection of the different underlying pathophysiological mechanisms (radiculopathy in stead of myelopathy)(Van der Kogel 1979):

$$D = c \bullet N^{0.38} \bullet T^{0.02}$$

Although the new power-law formalisms for the CNS predicted radiation tolerance much more accurate than the original Ellis formalism, inconsistencies

remained between the predicted values and the actual experimental data (Van der Kogel 1991). These inconsistencies were most pronounced at both ends of the curve and could have been anticipated if the implications of the model were realized: the power-law formalisms predict an infinitely increasing tolerance if the dose per fraction decreases and this is unlikely to happen in reality (although this is difficult to confirm in an experimental set up).

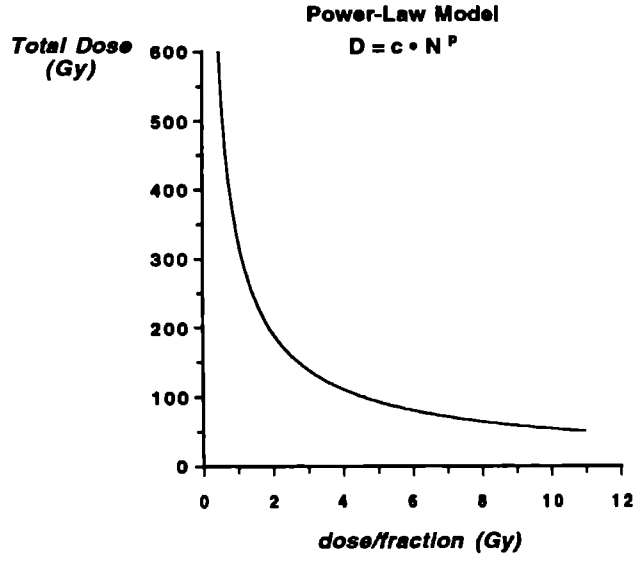


Figure A2.1 power-law model

Isoeffect models based on cellular survival.

Models based on cellular survival to describe tissue responses were introduced as the concept emerged that biological responses after irradiation were related to the survival of target cells responsible for the function or integrity of the tissue. Different radiation schedules with equal responses are assumed to inactivate the same number of target cells and are therefore isoeffective at the tissue response level. As it was possible to describe *in vitro* clonogenic survival data according to several models, the use of these models was extrapolated to predict tissue responses *in vivo*.

There are many models that can be used to fit *in vitro* clonogenic survival data (see appendix 1). With respect to the prediction of tissue responses the linear-quadratic model has gained a lot of attention because of its simplicity and biophysical basis. The linear-quadratic model for clonogenic cell survival can be written as:

$$ln\ SF = -(\alpha \cdot D + \beta \cdot D^2)$$

(In which: SF = the surviving fraction; D = the total dose given as a single fraction; α and β are the target cell survival parameters.)

This equation can be rewritten for multiple equal fractions as:

$$ln\ SF = -n(\alpha \cdot d + \beta \cdot d^2)$$

(In which: n = the number of fractions; d = the dose per fraction.)

This formula can be adapted to describe *in vivo* tissue responses by replacing the -ln(SF) by E (= full effect) (Douglas and Fowler 1976). The survival of clonogenic target cells and tissue response are used as synonyms:

$$E = n(\alpha \cdot d + \beta \cdot d^2)$$

This formula was rewritten to obtain a visual representation: the F_e-plot:

$$E = \alpha \cdot nd + \beta \cdot nd^2$$

$$\frac{E}{nd} = \alpha + \beta \cdot d$$

$$\frac{1}{nd} = \frac{\alpha}{E} + \left(\frac{\beta}{E}\right) \cdot d$$

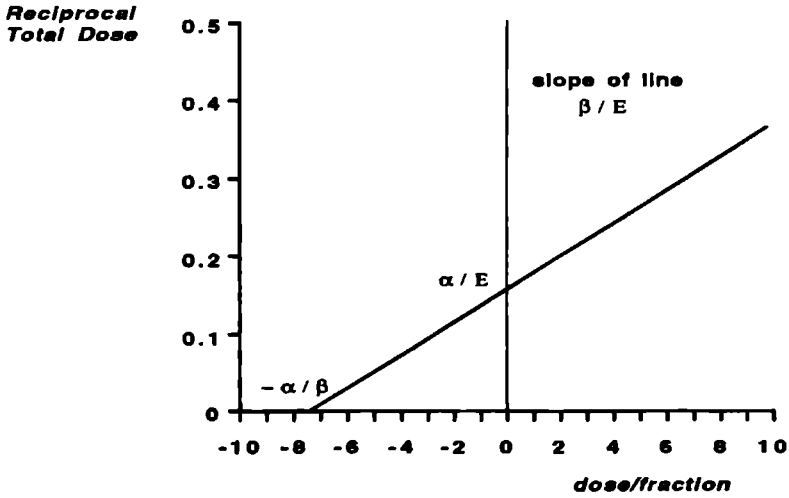


Figure A2.2 Douglas - Fowler plot.

The F_e-plot describes the relationship between the reciprocal of the total dose and the dose per fraction as a straight line. It allows the determination of the α/β-ratio without knowing the actual survival of the target cells. The α/β-ratio can be determined either by calculating the ratio of α/E and β/E or by extrapolating the straight line back to the ordinate where it will intersect the ordinate at $-\alpha/\beta$. The α/β-ratio appeared to be an important value to characterize different tissues. On the basis of the α/β-value, tissues were divided in two main categories: tissues that are sensitive or relatively insensitive for fraction size changes. If the α/β-ratio of a tissue is known, simple calculations can provide isoeffective radiation treatment regimens. Different ways to perform these calculations have been proposed, but they are based on the same principle:

ETD - formula (Extrapolated Total Dose) (Barendsen 1982):

$$ETD = \frac{E}{\alpha} = n \cdot d \cdot (1 + \frac{d}{\alpha/\beta})$$

TE - formula (Total Effect) (Thames and Hendry 1987):

$$TE = \frac{E}{\beta} = n \cdot d \cdot (\alpha/\beta + d)$$

In these formulas ETD and TE represent calculated values that characterize a specific reference schedule with a known probability for an effect to occur. Variation of e.g. the number of fractions will allow the calculation of the fraction size and the new treatment protocol will be isoeffective with the reference schedule. It also allows to determine the total effectiveness of two radiation schedules with different variables because ETD and TE values may be added:

$$TE = PE_1 + PE_2$$

$$D_r (\alpha/\beta + d_r) = D_1 (\alpha/\beta + d_1) + D_2 (\alpha/\beta + d_2)$$

(In which: TE = total effect of the reference schedule; PE = partial effect of the first part of the treatment; PE2 = the partial effect of the second part of the treatment; r = reference schedule.)

These LQ-based tolerance formalisms can be adjusted to deal with corrections for incomplete repair or continuous low dose rate irradiation (Thames and Hendry 1987).

APPENDIX 3

FITTING CELL-SURVIVAL CURVES

In view of the many problems associated with the analysis of radiation response parameters, as a first approach survival curves can be simply fitted by eye. At the beginning of our experimental work survival data were analyzed by this method and curves were estimated using semi-log plots of surviving fraction versus dose. Although some authors consider this as a reliable method, the need was felt to analyze the data with a more mathematically based model. Many models are used to analyze survival data but the "linear-quadratic" model (LQ-model) has some advantages (its simplicity; its convenience; and its biophysical basis) and is at the moment the most accepted method to analyze cellular survival data. We used this model to analyze the survival data of O-2A progenitor cells after *in vivo* or *in vitro* irradiation.

First, we fitted the survival curves by eye with the LQ-formula as the underlying model:

$$\text{Surviving Fraction} = \exp(-\alpha d - \beta d^2).$$

By varying the values of α and β, the best fit was determined. Subsequently, we used a Macintosh computer programme (KaleidaGraph) for the fitting procedure. This programme fits a curve by an iterative least square analysis routine. A later version of the programme allowed to fit the variables by using an internal weight [1/(standard error)^2] for each variable. Values which show little deviation from the mean will have smaller standard error values and consequently a larger internal weight.

Later on, the computer programmes developed by N. Albright (1987) were used for the analysis of cellular dose-survival data obtained for O-2A progenitor cells. N. Albright developed several computer programmes which allow the user to build a data base, calculate cell survival with a correction for cell multiplicity at the time of irradiation, and fit various survival models to the data by iteratively weighted least squares. Fitting survival models, e.g. the LQ-model, to the data by iteratively weighted least squares assumes that the data points represent statistically independent measurements. Furthermore, the data are weighted to improve the accuracy of fits of cell survival curves to dose-survival data. The estimated variance in the log of cell survival is the basis for the weights used in weighted least squares and standard errors. The weight model includes 5 factors:

a. Poisson distribution of the number of viable cells plated;

b. Dependence of colony counting errors on dose;

c. Variation in the number of surviving colonies between replicate platings for the same data point;
d. An additive constant to account for dilution volume errors, etc. and
e. A factor to make the average of the variances of the log of colony survival calculated from the model equal the actual variance of the data points about a fitted curve.

As mentioned above different methods were used to determine the absolute values for α and β. In our first manuscript on adult O-2A progenitor cells (chapter 7) the initial survival data were fitted by eye (but according to the LQ-model). In a later manuscript (chapter 9) we combined these data with newly obtained survival data and the combined data set was analyzed with iterative least squares (KaleidaGraph) without using an internal weight for each variable. Although it has not been presented before the combined data set was also analyzed by the programmes developed by N. Albright. The absolute values for α and β obtained by these different ways of analysis are shown in Table A3.1 and it can be seen that small changes in the absolute values of α and β have large consequences for the α/β-ratio.

Table A3.1.

METHOD OF ANALYSIS	α-value (Gy^{-1})	β-value (Gy^{-2})	α/β-ratio (Gy)
By eye	0.15	0.028	5.4
KaleidaGraph	0.143	0.029	4.9
According to Albright	0.18	0.025	7.2

As stated above, the values presented in Table A3.1 were not obtained for the same data set ("the by eye analysis was based on a smaller amount of data"). Therefore another data set is used to illustrate the effect of different fitting programmes on the absolute values of α and β. The survival data of perinatal O-2A progenitor cells were chosen because this data set was fitted by eye (chapter 6) at a time we did not have access to the other two computer programmes. Subsequently, the data set was also fitted by iterative least squares analysis as performed by Kaleidagraph and the computer programmes developed by N. Albright. Kaleidagraph offers the possibility to fit the variables unweighted or to fit the data with a weight relative to the standard error. The fitting procedure developed by N. ALbright makes also use of iteratively weighted least squares but the weight is determined by a more sophisticated model including a statistically robust method of error estimates (S.E. or 95% confidence limits). The differently fitted curves are seen in Figure A3.1. It seems that the curve which is generated by iterative least squares analysis without weighting (Kaleidagraph) provides the

poorest fit. The other three methods provide curves that are almost identical (by eye), however, with values of α and β that differ considerably (and consequently a large difference in α/β-ratio). The differences are partly explained by the relative small number of data but also indicate that α/β-ratios derived from single dose survival curves may be different due to the method of analysis.

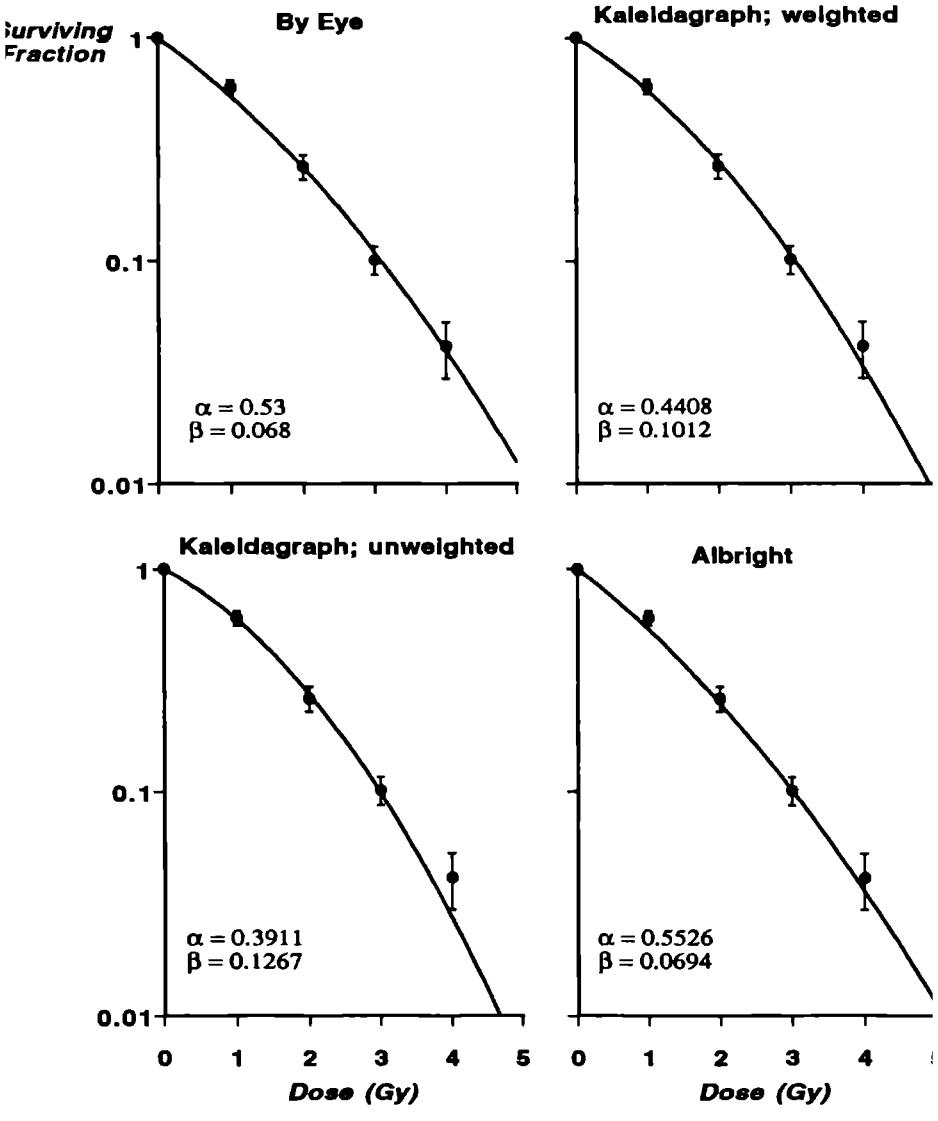


Figure A3.1 Survival data of perinatal O-2A progenitor cells. Each data point represents mean ± S.D. as derived from four separate experiments. The data were fitted according to the LQ-model by four different methods.

APPENDIX 4

REAGENTS AND TISSUE CULTURE MEDIA

Reagents.

-*ANTI-FADE*. Anti-fade is a solution of 2.5 g 1,4-diazobicyclo-octane (Sigma D 2522) dissolved in 100 ml glycerol on a stirrer overnight at 37^{0}C. It is stored at 4^{0}C and protected against light.

-*COLLAGENASE*. Collagenase (Sigma C 0130) is prepared as a stock solution of 2000 U/ml in Leibwitz L-15 medium. The stock solution is filter sterilized (0.22 µm filter, Millipore SLGV 025 BS) and stored in 0.5 ml aliquots at -20^{0}C.

-*EDTA*. Sodium EthyleneDiamine Tetracetic Acid (Sigma E 8008) is prepared as a stock solution of 1 % (1 g per 100 ml) in DMEM-CMF. The stock solution is filter sterilized (0.22 µm filter, Millipore SLGV 025 BS) and stored in 0.2 ml aliquots at -20^{0}C. The final concentration of 0.54 mM (0.02 % w/v) is obtained by adding 9.8 ml DMEM-CMF to 0.2 ml stock solution.

-*L-GLUTAMINE*. Stock solution of 200 mM L-glutamine (Gibco 043-5030H). Filter sterilized (0.22 µm filter, Millipore SLGV 025 BS) and stored at -20^{0}C.

-*INSULIN*. Bovine pancreas insuline (Sigma I 6634) is dissolved in 0.01 M HCl to obtain a final concentration of 11.7 IU/ml. It is stored in 10 ml aliquots at -20^{0}C.

-*POLY-L-LYSINE*. Stock solution is prepared by adding 50 ml sterile water to jar (Sigma P 6282). It is stored in 2 and 9 ml aliquots at -20^{0}C. The stock is diluted 1:5 with sterile water and 1.5 ml is needed for the coating of 25 cm^2 flasks and 3 ml for 75 cm^2 flasks.

-*SATO-MIX*. A stock solution is prepared containing 6 different reagents:

1) 194.28 ml PBS + 5.72 ml *ALBUMIN* (Path-o-cyte 4, Miles Laboratories)
2) 200 ml sterilized water + 322 mg *PUTRESCINE* (Sigma P 5780)
3) 20 ml sterilized water + 6.74 mg *TRI-IODO-THYRONINE* (Sigma T 1775) + small drop of NaOH.
4) 20 ml 100 % ethanol + 8 mg *L-THYROXINE* (Sigma T 1775) + small drop of HCl
5) 20 ml 100 % ethanol + 12.46 mg *PROGESTERONE* (Sigma P 8783)
6) 20 ml PBS + 7.74 mg *SODIUM SELENITE* (Sigma S 1382)

Solutions 3) and 4) are prepared on a magnetic stirrer.
Solutions 1) to 4) are added together with 2 ml of both solution 5) and 6)

Aliquots of 11 ml are stored at -20^{0}C and are to be added to 500 ml medium.

-*SBTI / DNASE-MIX.* A stock solution is prepared containing equal volumes of 3 different solutions.

1) *SOYBEAN TRYPSIN INHIBITOR* (SBTI)(Sigma T 6522) at a final concentration of 5200 U / ml DMEM

2) *BOVINE PANCREAS DNASE I* (Sigma D 4527) at a final concentration of 74 Kunitz Units / ml DMEM

3) *BOVINE SERUM ALBUMINE* (Fraction V, Sigma 4919) at a final concentration of 3 mg / ml DMEM

Equal volumes of these 3 solutions are added together, filter sterilized (0.22 µm filter, Millipore SLGV 025 BS), and stored at -20^{0}C.

-*TRANSFERRIN.* Bovine Transferrin (Sigma T 8027) is prepared as a stock solution of 10 mg / ml sterilized water. It is stored in 5 ml aliquots at -20^{0}C.

-*TRYPSIN.* Bovine Pancreas Trypsin type III (Sigma T 8253) is prepared as a stock solution of 120,000 U / ml DMEM-CMF and stored in 1.5 ml aliquots at -20^{0}C. The final concentration is 30,000 U / ml DMEM-CMF (diluted 1 : 4).

Tissue Culture Media.

-*PBS.* Phosphate Buffered Saline contains 8 g NaCl (Merck 6404), 2.3 g Na_2HPO_4•$2H_2O$ (Merck 6580), and 0.4 g KH_2PO_4 (Merck 4871) in 1 l sterilized water.

-*STAINING BUFFER.* Hanks Balanced Salts (without sodium bicarbonate, Gibco 076-01200A) are dissolved in a small amount of distilled water. A Hepes buffer is added (5.2 g sodium salt, Sigma H 7006; 4.76 g free acid, Sigma H 3375) together with 50 ml heat inactivated (56^{0}C, 30 min.) donor calf serum and 2 ml 10% sodium azide (Sigma S 2002). Destilled water is added to a volume of 1 l.

-*DULBECCO'S MODIFIED EAGLE'S MEDIUM* (DMEM; Gibco 074-2100) is purchased as a powder and prepared according to the prescriptions of the manifacturer. It conains 110mg/l sodium pyruvate; 3.7 g/l sodium bicarbonate, and 4.5 g/l glucose. Gentamicine (0.5 ml from a stock solution of 50 mg/ml, Gibco 043-5750) and L-Glutamine (5 ml from a 200 mM stock solution, Gibco 043-5030H) are added.

-*DMEM + 10% HEAT INACTIVATED FETAL CALF SERUM* (DMEM + 10% FCS).

-*DMEM - CALCIUM AND MAGNESIUM FREE* (DMEM-CMF; Gibco 074-02100) is purchased as a powder and prepared according to the prescriptions of the manifacturer. Gentamicine (0.5 ml from a stock solution of 50 mg/ml, Gibco 043-5750) is added.

-*SATO* culture medium contains 460 ml DMEM, 2.5 ml heat inactivated FCS, 10 ml bovine Insulin (sigma I 6634), 5 ml bovine Transferrin (Sigma T 8027) and 10 ml SATO-mix.

-*LEIBOWITZ L-15 MEDIUM* (Gibco 074-01300A) is purchased as a powder and prepared according to the prescriptions of the manifacturer. Gentamicine (0.5 ml from a stock solution of 50 mg/ml, Gibco 043-5750) is added.

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ABSTRACT

Curative radiotherapy of malignant and benign tumors of or in the neighborhood of the central nervous system (CNS) is limited by the tolerance of the surrounding normal CNS. The severity of the negative side effects limits the (biological effective) dose that may be administered to the target volume. Various syndromes can be distinguished but of clinical importance are the irreversible late delayed syndromes (chapter 1). Two different delayed syndromes may be distinguished, based on pathological criteria. Although there is no consensus, there is evidence that the two syndromes are caused by different pathogenetic cellular mechanisms (chapter 1). The relatively early lesions are characterized by demyelination and white matter necrosis and are attributed to the loss of myelinating cells (oligodendrocytes). The other syndrome usually develops later and is characterized by vascular abnormalities in both the white and gray matter. It is thought to be the result of a defective vasculature. The long latent periods between irradiation and the development of injury indicates that the CNS does not express radiation-induced damage immediately but that injury becomes apparent when the gradual loss of critical structures is not compensated for. Although it is realized that the development of radiation-induced damage of the CNS might be more complex and that the distinction between the delayed syndromes is not always as clear-cut as stated above, such simplifications are necessary to make the problem accessible for more basic investigations. Consequently, in this study the following **hypothesis** about **the development of demyelination and white matter necrosis** has been proposed:

> *Irradiation of the CNS kills glial stem cells which are responsible for the production of new oligodendrocytes. The level of cell kill will determine whether the surviving fraction of stem cells is capable to generate sufficient oligodendrocytes to compensate for their normal or radiation-induced loss. Insufficient replacement will lead to functional deficits (paresis; paralysis) characterized by demyelination.*

Although this hypothesis has been proposed earlier, new developments in immunology and neurobiology have made it possible to investigate radiation injury of the CNS at the cellular level (chapter 3).

In vitro analysis of single cell suspensions derived from the rat optic nerve led to the discovery of a glial cell lineage which is responsible for the myelination of axons in the CNS. The stem cells in this lineage were called O-2A progenitor cells because they were able to generate oligodendrocytes as well as type-2 astrocytes. Both cell types are mainly found in the white matter and are involved in

fast signal conduction along axons. Although O-2A progenitor cells were first discovered in the developing CNS and seemed to have a limited proliferative potential, they were also isolated from other parts of the perinatal CNS as well as from the adult CNS and these cells appeared to have stem cell properties (chapter 3). In view of the proposed hypothesis O-2A progenitor cells could well serve as the target cells which depletion by X-rays could result in an insufficient replacement of oligodendrocytes and consequently lead to impaired (re)myelination (≈ demyelination).

In this study, an *in vitro* clonogenic assay has been developed to determine quantitatively the effect of radiation on O-2A progenitor cells (chapter 6). The assay is based on a two cell layer culture system. The basic layer is formed by a monolayer of type-1 astrocytes which serves as a feeder layer for O-2A progenitor cells plated on top of them. Growth factors produced by the type-1 astrocytes stimulate O-2A progenitor cells to divide and when plated at a low density colonies are formed with a high probability of originating from a single stem cell. This assay offers the possibility to investigate radiobiological characteristics of O-2A progenitor cells irradiated either *in vitro* or *in vivo*. If O-2A progenitor cells are indeed the target cells for radiation-induced injury their radiosensitivity (chapter 7 and 8); repair capacity (chapter 9) and repopulation profile (chapter 10) should be in agreement with clinical and experimental data obtained for the "CNS *in vivo*".

In clinical and experimental studies, the development of paralytic signs has been used as an endpoint to investigate the influence of treatment variables on the radiation tolerance of the CNS (chapter 2). The CNS appeared to be rather insensitive for radiation damage (with large differences between species) and a prime example of a late-responding tissue. These findings can be quantified by two parameters described by the "linear-quadratic" (LQ-) equation: the α-and β-component [Surviving Fraction = $\exp(-\alpha d - \beta d^2)$](appendix 1 and 2). These two parameters determine the sensitivity of the target cells for radiation injury and their capacity to repair sublethal damage. Late-responding tissues are characterized by a low α/β-ratio (≈ 1.5-3 Gy) in contrast to acute-responding tissues which have a high α/β-ratio (≈ 5-15 Gy).

The radiosensitivity of O-2A progenitor cells determined *in vitro* depended on the degree of differentiation. Committed O-2A progenitor cells, as derived from the perinatal rat optic nerve, were far more sensitive than O-2A progenitor cells derived from the adult rat optic nerve (chapter 7). The latter are considered to be stem cells with self-renewal properties. O-2A progenitor cells derived from stem cell areas of the perinatal CNS exhibited an intermediate radiosensitivity: far more resistant than committed progenitor cells, but more sensitive than adult stem cells (chapter 8).

The repair capacity of adult O-2A progenitor cells has been analyzed by three different methods but all according to the LQ-model (the single dose survival curve; a method proposed by Peacock et al. 1988; and by a modified "F_e-plot"; chapter 9). The absolute value of the β-parameter obtained by this analysis is consistent with values of β obtained for the "CNS *in vivo*" and indicates that O-2A progenitor cells are able to repair a substantial amount of sublethal damage.

Although the analysis according to the LQ-model assumes that tissue responses are directly related to cell survival, one should keep in mind that LQ-parameters obtained for the "CNS *in vivo*" are based on dose response curves (with paralysis as functional endpoint) and LQ-parameters found for O-2A progenitor cells are based on surviving cell numbers. Although the *in vivo* response may be based on changes in the number of O-2A progenitor cells, the two experimental models are completely different and investigate radiation effects at different levels. When nevertheless a comparison was made this revealed that the α-value of O-2A progenitor cells was low (≈0.155 Gy^{-1}) and that the α-value of the "CNS *in vivo*" was even lower (≈0.042 Gy^{-1}). The β-values, on the other hand, were more or less identical (0.025 Gy^{-2}). As a consequence, the α/β-ratio for adult O-2A progenitor cells was higher than for the "CNS *in vivo*"and is compatible with values found for acute-responding tissues. One could argue that therefore O-2A progenitor cells can not be the target cells of radiation-induced demyelination, but the difference in the α/β-ratio may be due to experimental design. For, in contrast to the *in vivo* situation, all O-2A progenitor cells *in vitro* are stimulated to divide. The α/β-ratio determined for O-2A progenitor cells *in vitro* may therefore be compatible with values found for acute-responding tissues. A situation *in vivo* which may resemble the *in vitro* assay with respect to the stimulation and proliferation of all available glial stem cells is the developing CNS. The fractionation sensitivity of the developing CNS may therefore mimic the fractionation sensitivity of an acute-responding tissue. This assumption has been recently confirmed for the spinal cord of 1-week-old rats which appeared to have a significantly higher α/β-ratio (4.5 Gy) than the mature CNS (2 Gy). This suggests that the proliferative state of the target cells is one of the main factors which determines the fractionation sensitivity of a tissue.

Retreatment of the CNS may be possible if radiation-induced injury is restored at the stem cell level. Experimental data show that recovery of the CNS takes place but that the level of recovery seems to be determined by the level of injury caused by the initial irradiation (chapter 2). Similar observations were made on the repopulation of O-2A progenitor cells after irradiation of the adult optic nerve: the level of recovery is less if the dose of X-rays is higher (chapter 10). The time course of repopulation of O-2A progenitor cells, however, seemed not to be in

agreement with the "*in vivo*" functional endpoints. Repopulation of glial stem cells occurred rather fast. Within 2-3 weeks after irradiation a steady state was reached which remained for at least 6 months. In adult rat CNS *in vivo*, however, repair of radiation-induced damage could only be demonstrated at approximately 2 months after irradiation.

For the retreatment of the CNS, not only the fraction of surviving stem cells is of importance but also the proliferative potential of these cells. *In vitro* analysis of the colonies formed by surviving O-2A progenitor cells after irradiation, either *in vitro* or *in vivo*, revealed an adverse effect of the dose on the colony size. Cells surviving higher doses of X-rays formed smaller colonies (chapter 7; 8; 10). Whether this effect is of biological importance is still unknown.

The radiosensitivity, repair and repopulation profile of glial stem cells have been investigated and were compared with clinical and experimental data of the "CNS *in vivo*". Although similarities were found, not all results were consistent and the underlying mechanisms of some observations can only be speculative. More data are necessary not only for O-2A progenitor cells but also for other lineages and cell types in the CNS (e.g., type-1 astrocytes; endothelial cells). This information is needed for a thorough understanding of the cascade of events that occurs after irradiation of the CNS. Complete knowledge of the cellular and humoral responses will elucidate critical steps in the development of radiation-induced injury and might offer opportunities to intervene before irreparable tissue damage (necrosis) occurs.

SAMENVATTING

De tolerantie van het normale centrale zenuwstelsel (CZS) is de beperkende factor bij de curatieve bestraling van kwaadaardige en goedaardige tumoren van en in de buurt van het CZS. De ernst en het irreversibele karakter van de bijwerkingen die kunnen optreden bepaalt de bestralingsdosis die kan worden toegediend. Na bestraling van het CZS zijn verschillende ziektebeelden vastgesteld maar van klinisch belang zijn met name de bijwerkingen die vanaf 4 tot 6 maanden kunnen optreden. Op basis van pathologische criteria kunnen twee verschillende entiteiten onderscheiden worden. Ofschoon de meningen nog verdeeld zijn bestaat er het idee dat aan deze twee entiteiten verschillende cellulaire mechanismen ten grondslag liggen. De relatief vroegtijdig (vanaf 4 maanden) optredende afwijkingen worden pathologisch gekenmerkt door demyelinisatie en versterf van witte stof in het CZS. Deze afwijkingen worden toegeschreven aan het verlies van myeliniserende cellen (oligodendrocyten). Het tweede ziektebeeld dat meestal later optreedt (vanaf ongeveer 1 jaar) wordt gekenmerkt door vaat-afwijkingen die zowel in de witte als grijze stof van het CZS gelocaliseerd zijn. Deze afwijkingen worden toegeschreven aan directe effecten van bestraling op de cellen waaruit de bloedvaten zijn opgebouwd. De lange latentie-tijd tussen het aanrichten van bestralingsschade en de uiting ervan geeft aan dat de cellen die actief de myelinisatie van neuronen verzorgen en snelle prikkelgeleiding langs de zenuwen mogelijk maken niet in eerste instantie door bestraling worden aangedaan. Deze cellen zijn echter onderhevig aan een natuurlijk verouderingsproces en dienen op den duur vervangen te worden door nieuwe cellen. Wanneer deze vervanging echter niet plaatsvindt omdat dit proces wèl door bestraling is beschadigd, zal er functieverlies van het weefsel optreden hetgeen zich kan uiten in verlammingsverschijnselen. Het tijdstip waarop dit functieverlies optreedt hangt dus af van de snelheid waarmee de functionele, myeliniserende cellen vervangen worden. Ofschoon een twee-deling als bovenomschreven misschien artificieel is en de werkelijkheid zeker complexer, maakt een dergelijke vereenvoudiging het probleem toegankelijk voor verder onderzoek. Als uitganspunt voor dit proefschrift werd daarom de volgende **hypothese** geformuleerd:

Demyelinisatie, als late reactie op bestraling van het CZS, wordt veroorzaakt door celdood van gliale stamcellen die verantwoordelijk zijn voor de vervanging van verouderde of beschadigde oligodendrocyten. Het absolute aantal van deze stamcellen dat wordt uitgeschakeld bepaalt of er voldoende cellen overblijven om te voorzien in de aanmaak van nieuwe oligodendrocyten. Als het aanbod achterblijft bij de vraag zal dit leiden tot

stoornissen in de functie van de oligodendrocyten (= snelle prikkelgeleiding langs zenuwcellen). Klinisch zal dit leiden tot neurologische uitvalsverschijnselen.

Ofschoon deze hypothese niet nieuw is hebben recente ontwikkelingen in de immunologie en de neurobiologie het mogelijk gemaakt deze hypothese op cellulair niveau te toetsen.

Het nauwkeurig *in vitro* bestuderen van cellen afkomstig uit de oogzenuw van pasgeboren ratjes leidde tot een identificatie van celtypen die zich op een hiërarchische wijze tot elkaar verhouden. Uit stamcellen onstaan door een proces van multiplicatie en differentiatie uiteindelijk cellen (oligodendrocyten) die de myelinisatie van neuronen in het CZS verzorgen. De naam O-2A progenitor cellen danken deze stamcellen aan hun vermogen te differentiëren in of oligodendrocyten of type-2 astrocyten. Deze beide celtypen zijn essentieel voor de snelle prikkeloverdracht in met name de witte stof van het CZS. Latere studies toonden aan dat O-2A progenitor cellen niet alleen voorkomen in de oogzenuw van pasgeboren ratjes, maar universeel aantoonbaar zijn in het gehele CZS van pasgeboren en volwassen dieren. Wel lijkt er een onderscheid te bestaan tussen cellen met een min of meer beperkt vermogen en cellen met een onbeperkt vermogen tot multiplicatie. Deze laatste cellen worden met recht stamcellen genoemd en zijn in het kader van de te toetsen hypothese de potentiële kandidaten die uitgeschakeld zouden kunnen worden door bestraling. Als gevolg hiervan zal er geen of onvoldoende vervanging plaatsvinden van verouderde of gedode oligodendrocyten hetgeen zal leiden tot gestoorde (re-)myelinisatie (= demyelinisatie).

Ter bestudering van het effect van bestraling op O-2A progenitor cellen werd een *in vitro* clonogene assay ontwikkeld. Deze assay is gebaseerd op de wisselwerking van twee verschillende celtypen. Als ondersteunende laag wordt gebruik gemaakt van type-1 astrocyten. Op deze laag van cellen worden de te onderzoeken O-2A progenitor cellen gekweekt. Groeifactoren die door de type-1 astrocyten worden geproduceerd zetten de O-2A progenitor cellen aan tot deling. Als de afzonderlijke O-2A progenitor cellen voldoende ver uit elkaar liggen zullen zich kolonies van O-2A progenitor cellen en oligodendrocyten ontwikkelen die onstaan zijn uit één voorloper cel. Op deze manier kan het effect van bestraling op de gliale stamcel populatie quantitatief onderzocht worden. Dit kan gebeuren zowel na bestraling *in vitro* als *in vivo*. Als de hypothese, dat late bestralingsgeïnduceerde demyelinisatie veroorzaakt wordt door depletie van O-2A progenitor cellen, klopt dan zullen de bestralingskarakteristieken van dit

celtype in overeenstemming moeten zijn met klinische en experimentele bestralingsgegevens van het "CZS *in vivo*".

In *"in vivo"* experimentele studies wordt de frequentie van optreden van verlammingsverschijnselen gescoord om het effect van modificaties van bestralingsbehandelingen te testen op de tolerantie van het CZS. Hieruit is naar voren gekomen dat het CZS relatief ongevoelig is voor stralenschade (met grote verschillen tussen diverse species) en een modelvoorbeeld voor een weefsel dat stralenschade pas na een lange tijd tot expressie brengt. Het effect van bestraling op stamcel niveau kan beshreven worden met een wiskundig model waarbij de overleving van cellen na bestraling gecorreleerd wordt aan een lineaire (α) en een quadratische (β) component. De ratio van deze twee waarden zegt iets over de mogelijkheid van de bestraalde cellen om een gedeelte van de bestralingsschade, toegebracht tijdens een gefractioneerde behandeling, te herstellen (= fractioneringsgevoeligheid). Laat-reagerende weefsels worden vaak gekenmerkt door een lage α/β-ratio ($\approx$1.5-3 Gy) in tegenstelling tot acuut-reagerende weefsels met een relatief hoge α/β-ratio ($\approx$5-15 Gy).

De gevoeligheid van O-2A progenitor cellen voor de cytotoxische werking van straling is wisselend en lijkt te berusten op de mate van gedifferentieerdheid van deze voorloper cellen. Verder uitgerijpte cellen, zoals O-2A progenitor cellen verkregen uit de oogzenuwen van pasgeboren ratjes bleken gevoeliger te zijn voor bestraling dan O-2A progenitor cellen uit de oogzenuwen van volwassen ratten. O-2A progenitor cellen uit het perinatale CZS met nog wèl duidelijk het vermogen tot ongelimiteerde proliferatie vertoonden een intermediaire stralengevoeligheid: duidelijk resistenter dan O-2A progenitor cellen afkomstig uit de perinatale oogzenuw en gevoeliger dan O-2A progenitor cellen uit het volwassen CZS.

Het vermogen van volwassen O-2A progenitor cellen om stralenschade te herstellen werd geanalyseerd op drie verschillende manieren (uit de overlevingscurve; volgens de methode zoals deze beshreven is door Peacock et al. 1988; en volgens een gemodificeerde "F_e-plot"). Deze drie methoden berusten echter wel alle drie op het LQ-model. De absolute waarde die voor de β-parameter op deze wijze werd verkregen komt overeen met de waarden voor β die voor "CZS *in vivo*" bekend zijn. Dit geeft aan dat O-2A progenitor cellen in staat zijn een behoorlijke hoeveelheid sublethale stralenschade te herstellen.

Analyse volgens het LQ-model neemt echter aan dat door bestraling geïnduceerde weefselreacties direct gecorreleerd zijn aan absolute aantallen in cel-overleving. Toch mag men niet uit het oog verliezen dat analyse van weefselreacties en de analyse van absolute cel-overleving op een ander niveau plaatsvindt en dat wellicht een vergelijking van resultaten die op deze twee

uiteenlopende manieren verkregen zijn niet geoorloofd is. Voert men een dergelijke vergelijking toch uit dan blijkt dat de β-parameter nagenoeg gelijk is voor O-2A progenitor cellen en "CZS *in vivo*" maar dat daarentegen de α-parameter duidelijk verschillend is. Het gevolg is dat de α/β-ratio voor volwassen O-2A progenitor cellen hoger is dan men voor een laat-reagerend weefsel zou verwachten en dat men op grond van deze bevinding de te toetsen hypothese zou kunnen verwerpen. De verschillen in onderzoeksmethoden echter kunnen verantwoordelijk zijn voor de verschillen in de α/β-ratio. De proliferatie kinetiek van O-2A progenitor cellen *in vitro* verschilt namelijk duidelijk met de proliferatie kinetiek van gliale stamcellen *in vivo*. *In vitro* vindt een constante stimulatie tot deling van alle O-2A progenitor cellen plaats terwijl *in vivo* slechts een klein gedeelte van de gliale stamcellen actief prolifereert. De situatie *in vitro* is wellicht vergelijkbaar met het zich ontwikkelende CZS *in vivo* waar een beroep gedaan wordt op vele stamcellen om te voorzien in de vraag naar gliale cellen die het CZS gaan vormen. De α/β-ratio van het CZS van 1-week-oude ratjes *in vivo* is in vergelijking met het volwassen CZS *in vivo* significant hoger en bereikt een waarde die vergelijkbaar is met die van O-2A progenitor cellen *in vitro*. Dit geeft aan dat de proliferatieve status van een weefsel een belangrijke factor is die de fractioneringsgevoeligheid van het weefsel mede bepaalt.

Herbestraling van het CZS is mogelijk indien het weefsel zich herstelt na een eerdere bestraling. Experimentele gegevens wijzen in de richting dat er inderdaad herstel optreedt maar dat de hoogte ervan afhangt van de mate van schade die door de eerste bestralingsbehandeling teweeg is gebracht. Er vindt namelijk minder herstel plaats naarmate de dosis van de voorafgaande bestraling hoger is geweest. Hetzelfde werd waargenomen na bestraling van O-2A progenitor cellen in de oogzenuw van volwassen ratten *in vivo*. De periode waarin dit herstel optreedt lijkt echter te verschillen. Het herstel van het aantal O-2A progenitor cellen speelt zich af in de eerste drie weken na bestraling terwijl het herstel in functionele reserve van het CZS in volwassen ratten pas na ongeveer 2 maanden *in vivo* kan worden aangetoond.

Voor een herbestraling is mogelijk niet alleen het aantal overlevende cellen van belang maar ook de proliferatieve mogelijkheden van deze cellen. *In vitro* analyse van de kolonie grootte van (*in vitro* en *in vivo*) bestraalde O-2A progenitor cellen laat zien dat er een omgekeerde relatie bestaat tussen kolonie grootte en bestralingsdosis. Of dit ook daadwerkelijk klinische consequenties heeft is niet bekend.

Concluderend kan vermeld worden dat de stralengevoeligheid, herstelcapaciteit en repopulatie van gliale stamcellen werd bestudeerd en de uitkomsten werden vergeleken met resultaten die bekend zijn voor het "CZS *in*

vivo". Ofschoon er overeenkomsten zijn, zijn er ook een aantal resultaten die niet direct met elkaar in overeenstemming te brengen zijn. Op dit moment kan men hieromtrent alleen maar speculeren. Meer onderzoek zal nodig zijn naar de effecten van bestraling op O-2A progenitor cellen maar ook naar de effecten van bestraling op de andere cellen van het CZS (b.v. type-1 astrocyten, endotheel cellen). Deze gegevens zijn nodig om een goed inzicht te krijgen in de cellulaire mechanismen die leiden tot late schade in het CZS. Kennis van de cellulaire en humorale respons na bestraling zullen kritische stappen in de ontwikkeling van bestralingsschade aan het licht brengen en wellicht mogelijkheden bieden om dit proces te beinvloeden voordat onomkeerbare beschadiging (necrose) is opgetreden.

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Richard Wilhelmus Maria van der Maazen werd op 5 april 1959 te Goirle geboren. Hij behaalde in 1977 het gymnasium-β diploma aan het St. Odulphus Lyceum te Tilburg. In september van hetzelfde jaar begon hij met de studie Geneeskunde aan de Katholieke Universiteit van Nijmegen en werd op 29 juni 1984 tot arts bevorderd. Van april 1985 tot april 1986 was hij werkzaam op het laboratorium van de afdeling hematologie van het St. Radboud Ziekenhuis onder leiding van Dr. T.J.M. de Witte en deed onderzoek naar het ontstaan van lymfocyten uit hematopoëtische voorloper cellen. In april 1986 begon hij zijn opleiding tot radiotherapeut die hij in 1988 voor een periode van $2^1/_2$ jaar onderbrak om zich te wijden aan experimenteel onderzoek. De start van dit onderzoek werd gemaakt in Londen onder leiding van Dr. G. Wolswijk en Dr. M. Noble (neurobiologen werkzaam in een van de Ludwig Institutes for Cancer Research). Na 1 jaar werd het onderzoek voortgezet in het Radiobiologisch Laboratorium van het Instituut voor Radiotherapie te Nijmegen (hoofd: Prof. dr. A.J. van der Kogel). In september 1990 werd de opleiding tot radiotherapeut hervat en in april 1992 vond registratie als specialist plaats (opleider: Prof. dr. W.A.J. van Daal). Vanaf april 1992 is hij als staflid verbonden aan het Instituut voor Radiotherapie van het Academisch Ziekenhuis St. Radboud te Nijmegen.

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