THE ROLE OF p53 IN RADIATION-INDUCED APOPTOSIS IN THE ADULT MOUSE CNS

by

Brenda Marilyn Chow

A thesis submitted in conformity with the requirements for the degree of Master of Science, Graduate Department of Medical Biophysics, University of Toronto

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The Role of p53 in Radiation-Induced Apoptosis of the Adult Mouse CNS

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Abstract

Central nervous system (CNS) tumours are treated with radiation. However, radiation-induced injuries can be severe. Loss of oligodendrocytes through apoptosis may contribute to these injuries. The p53 transcription factor may mediate radiation-induced apoptosis in these cells. To determine the involvement of p53 in the adult CNS, p53 wildtype, heterozygous and knockout mice were irradiated. In p53 +/+- mice, glial cells upregulated p53 after irradiation and oligodendrocytes and subependymal cells underwent apoptosis. CNS cells in p53 -/- mice were resistant to XRT-induced apoptosis, demonstrating a requirement for p53. Preliminary studies with primary cultures demonstrated that astrocytes were resistant to radiation-induced apoptosis and showed neither p53 nuclear accumulation nor nuclear translocation following radiation.

Future studies will determine the contribution of p53-dependent radiation-induced apoptosis in the adult CNS to the development of late radiation injury and the regulation of p53 localization following radiation.
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Introduction
1.0 Introduction

Benign or malignant tumours of the central nervous system (CNS) can lead to distortion of neural tissue and can greatly impair neurological function. Radiation therapy is a common strategy for treatment of CNS tumours. Unfortunately, CNS tissue is radiosensitive, and radiation treatment may lead to serious side effects, including the loss of neurological function, which may occur months to years after radiotherapy. One of the target cells of radiation-induced injury is believed to be the oligodendrocyte, the sole myelinating cell type of the CNS. We have previously demonstrated that oligodendrocytes undergo apoptosis following irradiation, leading to a significant loss in cell number within 24 h (Li et al., 1996b). I hypothesize that the loss of these cells may contribute to the severity of late radiation-induced damage. Elucidation of the molecular mechanism by which oligodendrocytes undergo apoptosis may present new strategies for modulation of the apoptotic response, and may relieve acute and/or delayed radiation-induced injury. The involvement of the tumour suppressor gene, p53, has been implicated in the apoptotic pathway of intestinal crypt cells and thymocytes following radiation. Nuclear accumulation of p53 has also been reported in oligodendrocytes following cytokine treatment and oxidative stress. In the work described this thesis, I investigated whether irradiated oligodendrocytes undergo apoptosis in a p53-dependent manner. This chapter provides the background for these experiments, and subsequent chapters report the experimental findings and suggest future studies to extend the present work.
1.1 Cancer of the Central Nervous System

Cancer arising in the central nervous system (CNS) leads to a severe compromise in neurological function, whether the tumour is benign or malignant. Unlike tumours arising in other parts of the body, tumour growth within the CNS is constrained by the skull and the vertebrae of the spinal column. Brain tumour mass can lead to specific neurological deficits due to compression of cranial nerves or brain substance. Tumour growth can also lead to an increase of intracranial pressure, and local electrochemical instability, possibly leading to the development of seizures. In the spinal cord, these "mass effects" can lead to disruption of sensory, motor and pain pathways as well as autonomic changes (Thapar and Laws, 1995).

Surgery is often the initial therapeutic strategy, both to obtain a histologic diagnosis, and to relieve pressure on brain and spinal cord structures. Radiation therapy is frequently given after surgery to relieve symptoms and to prevent tumour recurrence. In some cases, radiotherapy is also given when the tumour is too deep-seated to remove by surgical resection, or alternatively, if surgical resection is not possible due to the close apposition of the tumour to neural structures. Unfortunately, the tolerance of normal CNS tissues to radiation is generally lower than the dose required to completely eradicate most CNS tumours, and early and late effects of radiation can have devastating consequences (Sheline et al., 1980, see section 1.6). The pathogenesis of these injuries is still unclear (van der Kogel, 1991; Schultheiss et al., 1995).
1.2 The Central Nervous System

The central nervous system (CNS) is a complex system that controls processing of sensory information and is responsible for involuntary and voluntary behaviours as well as consciousness and higher thought processes. In mammals, the CNS is anatomically composed of the spinal cord and brain. The spinal cord is a column of nervous tissue that is arranged in transverse segments and protected by bony vertebrae. Motor and sensory information is relayed to specific regions within a segment of the spinal cord from the periphery. The information is first relayed to clusters of cell bodies, called nuclei, then relayed to the brain via longitudinal tracts (Nolte, 1993).

The brain is divided into the brainstem, cerebellum and cerebrum. The function of many regions of the brain is still unknown, but some specific functions have been assigned to particular brain regions. The brainstem controls many involuntary functions, such as breathing. The cerebellum is largely responsible for balance and co-ordination. Much is unknown about the function of the cerebrum, but the division of the cerebrum into four lobes, the frontal, parietal, temporal and occipital lobes, has made understanding of its function easier. The frontal lobe is responsible for higher thought processes, while certain areas of the parietal lobe are responsible for speech and spatial ability. The temporal lobes are important for memory, and visual processing occurs in the occipital lobe. The ascending spinal cord tracts convey information to various brain structures, which integrate and interpret the information and relay a response to various effector organs via the descending spinal cord tracts (Nolte, 1993).

Tissues of the CNS can be divided into grey or white matter. Grey matter consists primarily of cell bodies of neurons that integrate and process information. White matter,
in contrast, has a preponderance of myelinated processes. Myelin is composed of approximately 75% lipid and 25% protein (Compston et al., 1997) and gives white matter its characteristic fatty white appearance. White matter generally contains tracts of myelinated cellular processes that relay information to pertinent nervous system centres. Thus, grey matter processes information, and white matter transmits this information to other nervous system centres (Nolte, 1993).

1.3 Cells of the CNS

CNS cells may be generally divided into neuronal or glial cell types. Many types of neurons exist in the CNS, but all have a similar function. Glial cells, however, vary greatly in morphology and function. Glial cells of the CNS include microglia, astrocytes, subependymal cells and oligodendrocytes.

1.3.1 Neurons

Neurons are crucial to the integrity and smooth function of the CNS. Several different types of neurons exist in the CNS, but all neurons have a characteristic cell body with protruding spikes, called dendrites, and one or more long processes, called axons. One special property of a neuron is its ability to maintain a difference in electric potential across its membrane. During its resting state, the neuron maintains a more negative environment inside the cell than outside. The difference in electric potential is sustained by a selective permeability of the membrane for sodium, potassium and chloride ions. In addition, ion channels transport ions against their concentration gradients. When a stimulus from the external or internal environment impinges upon the cell, the polarity of
the membrane changes; the inside of the cell becomes more positive than the outside of
the cell. If the change in polarity, called depolarization, is large enough in magnitude to
exceed a specific threshold, a succession of depolarizations, known as action potentials,
are propagated down the length of the axon. A chain of action potentials can travel down
one axon and stimulate another neuron until the stimulus reaches the CNS, where sensory
processing occurs (Hall, 1992).

1.3.2 Microglia

Microglia constitute an estimated 5 - 15% of all CNS cells (Rezaie and Male, 1999). Microglia are functionally similar to macrophages and undergo different
morphological changes, corresponding to distinct functional states, throughout their
lifetime (Rezaie and Male, 1999). In the earliest stage of microglial development,
microglia are characteristically ameboid and closely resemble macrophages (Perry et al.,
1985). These ameboid microglia were shown to phagocytose debris, resulting from cell
death, in the late embryonic and postnatal stages of development (Ferrer et al., 1990;
Hume et al., 1983). Ramified microglia are present in the adult CNS as a relatively
quiescent cell population (Perry and Gordon, 1988), but in response to stress, these cells
are capable of proliferation and can respond to a site of injury several hundred
micrometres distant (Moore and Thanos, 1996). Microglia, like macrophages, can
process and present internalized antigens (Hickey and Kimura, 1988) and can also induce
T-cell proliferation in vitro (Frei et al., 1987).
1.3.3 Astrocytes

Astrocytes are numerous in the CNS. It has been estimated that they comprise up to 20% of the total cell volume of the cerebral cortex (Tacconi, 1998). Astrocytes play a number of different roles in the CNS, and while some astrocytic functions are well characterized, much remains unknown about these cells. Among their many roles, astrocytes have been reported to assist in the maintenance of the blood-brain barrier (Hayashi et al., 1997; Janzer and Raff, 1987; Tao-Cheng and Brightman, 1988), regulate brain energy metabolism (Pellerin and Magistretti, 1994), and participate in the antioxidant brain processes (Makar et al., 1994).

1.3.4 Subependymal Cells

Subependymal cells are undifferentiated cells surrounding the ventricles of the brain. Their role is largely unknown but they have been postulated to act as a pool of precursor cells that may repopulate different cell populations following CNS injury. These cells have been shown to differentiate into astrocytes following trauma (Holmin et al., 1997), and a subpopulation of subependymal cells demonstrates properties of neural stem cells (Chiasson et al., 1999; Morshead et al., 1994). Subependymal cells have been shown to respond to various growth factors by proliferating, migrating and differentiating into neuronal, astrocyte and oligodendrocyte cell lineages (Craig et al., 1996).

1.3.5 Oligodendrocytes

An important glial cell type is the oligodendrocyte. Conduction of action potentials from the source of the stimulus to the CNS must occur quickly, but the distance
over which the signal must travel can be considerable. In order to speed conduction velocities, oligodendrocytes aid in action potential propagation.

Oligodendrocytes are found only in the CNS, and possess very elaborate cytoplasmic sheets, consisting of the several different types of lipids and proteins that make up myelin. These cells may ensheath up to 50 neuronal axons at a time (Butt and Ransom, 1989), tightly wrapping and compacting their cytoplasmic processes in concentric circles around discrete lengths of the axon, and leaving gaps of bare axon between myelinated lengths (Figure 1.1). The patches of exposed axon are called the Nodes of Ranvier, whereas the myelinated portions are called the internodes. As action potentials propagate along a myelinated axon, they are unable to penetrate the myelinated internodes. Instead, in a process known as saltatory conduction, the action potential jumps from one node of Ranvier to the next, effectively decreasing the total distance the signal must traverse (Compston et al., 1997; Hall, 1992).

Under normal circumstances, action potential propagation is fast and efficient. However, in the case of CNS injury, such as that induced by radiation, demyelination (Sheline et al., 1980) and vascular damage (Rubin et al., 1994; Stewart et al., 1995) prevent the efficient functioning of the CNS, leading to neurological deficits. The cytotoxic effects of radiation have been attributed to its ability to induce DNA damage.

1.4 Ionizing Radiation

Electromagnetic radiation consists of a stream of photons that have wave and particle-like properties and that, in a vacuum, move at the speed of light. Photons have a characteristic energy, wavelength and frequency and are arranged in a continuum, called
Figure 1.1 Oligodendrocyte myelination. Oligodendrocytes can myelinate many axons (A) by wrapping and compacting cytoplasmic processes around an axon (B). Reproduced from Hall, 1992.
the electromagnetic spectrum, according to their energy levels. Ranges of photon energies are referred to by conventional names. For example, UV photons have energies of a few electron volts (eV) to 100 eV. X-ray and γ-ray photons have energies generally greater than 100 eV. X-ray and γ-ray photons are called ionizing radiations because the energy per photon is large enough that they are capable of displacing an electron from an atom or molecule to which it is bound. The atom or molecule then becomes an ion, because of its charged state (Hill, 1992).

Interaction of electromagnetic ionizing radiation (XRT) with matter results in the deposition of energy through three principal processes: the photoelectric effect, the Compton process and pair production. Which process will dominate depends upon the energy of the photons. In the photoelectric effect, which dominates at an energy range of 10 - 100 keV, the energy from a photon is completely absorbed by the atom, resulting in the ejection of an inner shell electron from its orbit. At an energy range of 100 keV - 10 MeV, the Compton process dominates. In this process, the partial transfer of energy from an incident photon to an outer shell electron results in the displacement of the electron from its orbital and scattering of a less-energetic emergent photon. Both the excited, free electron and the emergent photon can continue to ionize other atoms or molecules. When photons have more than 1.02 MeV of energy, energy can be given up by pair production. In this process the high-energy photon is absorbed by the atom and an electron and a positron pair is produced (Bristow and Hill, 1998).

In clinical radiotherapy, which utilizes an energy range of 100 keV to 20 MeV, the Compton process is the dominant mechanism for energy deposition in tissues. The fast-moving, free electrons produced by the Compton process can induce direct or
indirect damage to biologically important molecules in cells. The free electrons can themselves interact with biological molecules, such as DNA, and cause direct damage. Indirect damage can be caused by the production of harmful free radicals within the cell. Free radicals are highly reactive species with unpaired electrons in their outer orbit. In cells, the most common free radical is produced by the ionization of water, which forms the ion radical $\text{H}_2\text{O}^+$. This highly reactive species can interact with a second water molecule, which then produces the $\text{H}_3\text{O}^+$ ion and the highly reactive $\text{OH}^-$ radical. The $\text{OH}^-$ radical may subsequently interact with biological molecules (Hall, 1994).

The random nature of energy deposition by excited free electrons suggests that any molecule in the cell may be subject to damage. This molecular damage can lead to inhibition of biochemical processes, such as DNA, RNA or protein synthesis. XRT-induced damage to cellular membranes can trigger signal transduction pathways leading to cell death (Fuks et al., 1995). However, evidence strongly suggests that XRT kills cells primarily by induction of irreparable DNA damage (Nunez et al., 1996; Schwartz et al., 1996). The free electrons or the free radicals produced by XRT can interact with DNA, causing single- and double-strand breaks in the sugar phosphate backbone. DNA bases may also be altered or lost and the formation of cross-links between DNA strands or between DNA and chromosomal proteins also leads to impairment of DNA function (Bristow and Hill, 1998).

The cell's enzymatic repair processes, which can repair single- and double-strand breaks, are the primary line of defence against DNA damage. Cells also possess thiol compounds, such as glutathione and cysteine, which contain sulphydryl groups that can react with free radicals and decrease their reactivity. What determines if the cell survives
the DNA damage induced by XRT and other stresses is the ability of the cell to repair DNA damage (Schwartz et al., 1996; Schwartz et al., 1988). This ability is often cell type specific. It has been shown that neurons, which undergo apoptosis following XRT, repair DNA damage more slowly than astrocytes, which are resistant to XRT-induced apoptosis (Gobbel et al., 1998).

1.5 Radiation Therapy

The ability of XRT to kill cells is exploited in XRT therapy for malignant tumours. Tumour control can be understood in terms of the response of individual cells making up the tumour. It is now generally accepted that tumours develop by clonal expansion of stem cells (Chambers and Hill, 1998; Mackillop et al., 1983). These tumour stem cells have unlimited proliferative capacity and make up a fraction of the total tumour cell population. Tumour control is achieved when all tumour stem cells have been killed, or their proliferative capacity is eradicated. One measure of a tumour's capacity for self-renewal can be gauged through a clonogenic assay, in which irradiated cells are plated at low density and their ability to repopulate, or form colonies, is assessed. Another assay for tumour control is the growth delay assay, in which treated and untreated tumour cells are injected into an animal and the latency to tumour regrowth is assessed (Wong and Hill, 1998).

Radiotherapy can cause cell death in two principal ways. In interphase cell death, irradiated cells die without first dividing. Interphase death can occur by apoptosis (see section 1.9) or necrosis. By contrast, in mitotic-linked cell death, cells may undergo one or two abortive cell cycles before dying. It is believed that the major mode of cell kill
following XRT is due to mitotic-linked death. The significance of apoptosis in clinical radiotherapy is controversial. Some studies have demonstrated that delaying the apoptotic event (Yin and Schimke, 1995) or increasing the rate of induction of apoptosis (Aldridge et al., 1995) in tumour cells does not affect clonogenic survival. However, other reports have demonstrated that tumour cells which showed significant levels of apoptosis tended to be more radiosensitive (Meyn et al., 1993; Stephens et al., 1991). XRT-induced apoptosis was found to contribute to the cytotoxic effects of XRT in glioblastoma cells (Haas-Kogan et al., 1996) and prostate tumour cells (Algan et al., 1996).

The total dose of XRT that can be delivered to the tumour is limited by the tolerance of normal tissues to XRT. The effect of XRT on normal tissue can be divided into early and late responses. Early responses generally occur within weeks of XRT, while late responses are observed months following XRT. Early responses to XRT are observed in tissues with a high rate of cell renewal, such as the skin and gastrointestinal tract. Most cells express XRT-induced damage during mitosis, so cycling cells would be expected to show greater effects of XRT early after treatment (Wong and Hill, 1998).

Late responses to XRT are generally observed in organs whose cells normally divide infrequently or not at all. Late responses may also involve XRT-induced damage to connective tissues and vasculature, possibly leading to impaired circulation, which may subsequently lead to secondary death of parenchymal cells due to nutrient deprivation. Loss of parenchymal cells may trigger a compensatory proliferation of other parenchymal cells, which, when they express their XRT damage, could lead to massive cell death and functional failure of the organ (Wong and Hill, 1998).
Radiotherapy is usually given in daily fractions of 2 Gy, 5 days a week, over a 5 to 7 week period. Fractionation allows repair of sublethal damage, repopulation of cells, redistribution of cells from a more radioreistant phase of the cell cycle to a more radiosensitive phase, and reoxygenation of hypoxic tissue (Wong and Hill, 1998). It is believed that these processes improve the efficacy of radiation treatments, primarily by preferentially sensitizing tumour cells to XRT.

1.6 Radiation-induced Injury in the CNS

The CNS is a major dose-limiting organ in clinical radiotherapy. Early and late clinical responses to the CNS have been well described following XRT (Sheline et al., 1980). In the acute response, which occurs during the course of XRT, patients report symptoms of fatigue, nausea, vomiting and headache (Loeffler et al., 1990; Sheline et al., 1980). Early-delayed reactions appear a few weeks to a few months after XRT, and patients may experience neurological symptoms such as l’hermitte’s syndrome or somnolence (Loeffler et al., 1990; Sheline et al., 1980). L’hermitte’s syndrome is characterized by tingling sensations in the limbs following head flexion after spinal cord irradiation. Fatigue and drowsiness following cranial irradiation characterize the somnolence syndrome. These syndromes are typically reversible. The late responses that occur several months to years following XRT are irreversible and the most serious. Various neurological deficits can result from brain XRT, including visual dysfunction (al-Mefty et al., 1990), development of seizures (Sasaki et al., 1996), neurocognitive deficits (Duffey et al., 1996) to dementia (Vigliani et al., 1999). Late effects in the irradiated spinal cord may include spasticity, weakness and hemiparesis (Schultheiss et al., 1995).
Histopathological changes to CNS tissue have been well described after XRT-induced injury. In rats, apoptosis has been reported in the spinal cord (Li et al., 1996a; Li et al., 1996b) and brain (Bellinzona et al., 1996; Shinohara et al., 1997) within 24 h of XRT. Within months of XRT, demyelination (Koehler et al., 1996; Laxmi et al., 1996), vasodilation (Kamiryo et al., 1996) and breakdown of the blood-brain barrier and blood-spinal cord barrier (Rubin et al., 1994; Stewart et al., 1995) has been observed. Months to years after XRT, hemorrhage (Kawano et al., 1996) and white matter necrosis (Barcikowska et al., 1995; Russo et al., 1999) have been reported. The pathogenesis of these reactions is currently unknown.

1.7 Target Cells of XRT-induced Injury in the CNS

The target cells for XRT-induced injury in the CNS remain unclear. Vascular endothelial cells, which form the blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB), have been postulated to be a target of XRT-induced damage. Previous studies reported breakdown of the BBB prior to the development of white matter necrosis (Rubin et al., 1994; Stewart et al., 1995). It has been hypothesized that disruption of the BBB leads to edema and ischemia that consequently contributes to tissue damage. Evidence for this hypothesis is supported by recent results from our lab (Tsao et al., 1999) that demonstrate that XRT-induced disruption of the BSCB is accompanied by an upregulation in vascular endothelial growth factor (VEGF), a potent endothelial cell mitogen and permeability factor for blood vessels, in rat spinal cord at 20 weeks following 22 Gy. The increase in VEGF mRNA and protein appear to be governed by
astrocytes and this upregulation may be prompted by the hypoxia resulting from XRT-induced damage.

Another proposed target for XRT-induced injury in the CNS are the oligodendrocytes (van der Kogel, 1986) since these cells appear to be involved in the evolution of symptoms leading to white matter necrosis. One of the earliest responses of the CNS to radiation is oligodendrocyte apoptosis (Li et al., 1996a; Li et al., 1996b), and the demyelination (Mastaglia et al., 1976) and white matter necrosis that characterize late XRT effects suggest that oligodendrocytes are injured or depleted by XRT. The apoptotic oligodendrocytes seen within 24 hours of XRT may trigger a more serious cell loss months or years following XRT. We have previously reported that although the peak apoptotic incidence in oligodendrocytes was only 0.86 - 0.92% at 8 h after 22 Gy, a 24% decrease in oligodendroglial density was observed at 24 h following irradiation (Li et al., 1996b).

The significant loss of oligodendrocytes within 24 h of XRT, and the observed demyelination and white matter necrosis months or years after XRT suggest that oligodendrocytes may be a major target for XRT-induced damage. An understanding of oligodendrocyte development, and the mechanism by which XRT induces death of these cells may lead to the genesis of novel therapeutic strategies for the modulation of CNS injury following XRT (see Chapter 4).

1.8 Oligodendrocyte Development

Oligodendrocytes differentiate from a bipotential progenitor cell called the oligodendrocyte-type II astrocyte (O-2A) cell. The O-2A cell, first described in vitro
(Raff et al., 1983), demonstrates an ability to differentiate along either an astrocyte lineage or an oligodendrocyte lineage, depending on culture conditions (Raff et al., 1983) (Figure 1.2). O-2A cells originate from the subventricular zone and migrate throughout the entire CNS (Hardy and Reynolds, 1991; Levison and Goldman, 1993). These cells have a characteristic bipolar morphology and are positive for the ganglioside A2B5 (Eisenbarth et al., 1979).

The pathway by which O-2A cells differentiate into oligodendrocytes is still under investigation. O-2A cells appear to undergo a set number of cell divisions before dividing. An internal "clock" has been postulated to time the number of divisions the cell will undergo before differentiating (Raff et al., 1985; Temple and Raff, 1986). This clock allows the O-2A cell to undergo an average of 8 divisions before differentiating (Temple and Raff, 1986). The mechanism by which this clock operates has been shown to be at least partially dependent on platelet-derived growth factor (PDGF) (Raff et al., 1988), a growth factor produced by astrocytes (Noble et al., 1988; Silberstein et al., 1996) and neurons (Ellison et al., 1996). PDGF receptors, which are present in O-2A cells, and not oligodendrocytes (Pringle et al., 1992), appear to be downregulated as the cell matures, making the cells less responsive to PDGF stimulation. It is believed that the lack of growth factor stimulation leads to differentiation into oligodendrocytes (Calver et al., 1998). Once the O-2A cell has differentiated into a mature oligodendrocyte, the expression of the ganglioside A2B5 is lost, and new cell surface markers, such as galactocerebroside (GC), (Raff et al., 1978), proteolipid protein (plp) (Griffiths et al., 1995) and Leu-7 (Schuller-Petrovic et al., 1983) are acquired.
Figure 1.2 Differentiation of oligodendrocytes in vitro. O-2A cells (A), which have a bipolar morphology and are A2B5+, give rise to either multipolar GC+ oligodendrocytes (B) or bipolar A2B5+ GFAP+ type II astrocytes (C), depending on the absence or presence of specific growth factors in fetal bovine serum (FBS). A2B5 is conjugated to Texas Red in (A) and (C), GC is conjugated to Texas Red in (B), and GFAP is conjugated to FITC (green fluorescence) in (C). All cells are counterstained with the nuclear stain DAPI (blue).
Growth factors are important not only in the timing of O-2A cell differentiation, but also appear to play a role in oligodendrocyte survival. In the CNS, many more oligodendrocytes are originally produced than will be needed in the fully formed brain (Raff et al., 1993). The most efficient way of matching neuron and oligodendrocyte cell numbers is through the competition for a limiting amount of survival factors, including PDGF, produced by the target cells. Neurons, the target cells, in this case, may regulate growth factor availability through electrical activity (Barres and Raff, 1993). One study has documented cell death in an estimated 50% of the newly formed oligodendrocytes before the birth of the organism (Barres et al., 1992). These dying oligodendrocytes showed evidence of apoptosis (Barres et al., 1992).

1.9 Apoptosis

Apoptosis is a form of cell death in which the cell activates an intrinsic death program. Cells undergoing apoptosis demonstrate characteristic morphological changes. These include cell shrinkage, membrane blebbing, nuclear condensation and fragmentation and the generation of apoptotic bodies, which are membrane-enclosed vesicles of nuclear fragments (Wyllie et al., 1980). Biochemical characteristics of apoptosis are also observed alongside these morphological hallmarks of apoptotic cells. Biochemical attributes include the cleavage of DNA into fragments of 300 kb, then into smaller fragments of 50 kb (Walker et al., 1993). In the final steps of apoptosis, DNA fragments are further cleaved into distinctive 180 - 200 base pair fragments (Wyllie et al., 1980). The process of apoptotic cell death allows the cell to be degraded in a controlled manner, avoiding the release of potentially harmful cellular contents, such as lysosomal
enzymes, into the extracellular environment. In contrast, in another form of cell death, known as necrosis, the cell swells, loses its membrane integrity, and releases its contents into the extracellular environment, often provoking an inflammatory response. In vivo, the apoptotic bodies are phagocytosed by neighbouring cells (Renvoize et al., 1998). In vitro, apoptotic cells are not phagocytosed, but commonly exhibit a kind of "secondary necrosis", in which progressive degeneration of apoptotic bodies leads to membrane permeabilization (Renvoize et al., 1998).

Apoptosis is commonly seen in developing organisms, where the apoptotic process plays an important role in eliminating cells produced in excess and regulating cell number (Raff, 1992; Raff et al., 1993). In the mature organism, the apoptotic process maintains tissue homeostasis (Raff, 1992), removes potentially harmful cells, such as self-reactive lymphocytes (Cohen, 1991) virus-infected cells (Vaux et al., 1994), and tumour cells (Williams, 1991). The apoptotic process is crucial to the normal development and health of the organism; impairment of the apoptotic mechanism in cells, whether by genetic processes or by viral infection, can promote the development of such diseases as cancer and autoimmunity. Aberrant activation of apoptosis may also contribute to a number of pathological states, including neurodegeneration in Parkinson’s disease, Alzheimer’s disease and stroke (Barinaga, 1998; Barinaga, 1998; Burke and Kholodilov, 1998; Chan et al., 1999; Thompson, 1995; Velier et al., 1999).

1.10 Morphological Apoptosis Assays

Morphological and biochemical hallmarks of the apoptotic process are recognized through several common apoptosis assays (Table 1.1). The characteristic DNA
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<th>Apoptotic Characteristic</th>
<th>Assay</th>
<th>Endpoint</th>
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<tr>
<td>Morphological</td>
<td>Electron Microscopy</td>
<td>nuclear fragmentation, nuclear condensation, cell shrinkage</td>
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<tr>
<td></td>
<td>Light Microscopy</td>
<td>nuclear fragmentation, nuclear condensation, cell shrinkage</td>
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<td>- histological stains (H&amp;E)</td>
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<td></td>
<td>Epifluorescent Microscopy</td>
<td>intensely stained nuclear fragments, nuclear condensation</td>
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<td>- DAPI, Hoescht 33342, acridine orange and ethidium bromide</td>
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<tr>
<td>Biochemical</td>
<td>Agarose Gel Electrophoresis</td>
<td>evidence of 180bp DNA fragments, the DNA “ladder” pattern</td>
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<td></td>
<td>Flow Cytometry</td>
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<td></td>
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fragmentation, nuclear condensation and cell shrinkage associated with apoptosis can be visualized with electron microscopy (Figure 1.3A). Similarly, light microscopy, using standard histochemical stains, such as hematoxylin and eosin, can be used to distinguish the morphology of apoptotic cells (Figure 1.3B). In the detection of apoptotic cells through epifluorescent microscopy, several fluorescent dyes can be used, including Hoescht 33342, 4,6 diamidino-2-phenylindole (DAPI), acridine orange (AO) and ethidium bromide (EtBr). Due to nuclear condensation in apoptotic cells, nuclear dyes stain apoptotic nuclei more intensely than non-apoptotic nuclei. In the AO/EtBr method, apoptotic cells are distinguished from necrotic and healthy cells by the intensity of the nuclear staining and the presence of condensed chromatin.

1.11 Biochemical Apoptosis Assays

One distinctive characteristic of apoptosis is the cleavage of DNA into fragments of multiples of 180 bp. The length of this fragment corresponds to the length of DNA wrapped around a nucleosome. This biochemical hallmark of apoptosis is recognized through different biochemical assays for apoptosis (Table 1.1). The DNA fragments can be subjected to agarose gel electrophoresis, which will separate the fragments according to size. The different fragment sizes produce a characteristic “ladder” pattern. Another way to detect DNA fragmentation is through the use of flow cytometry, which uses lasers to excite fluorescent dyes. When cells are stained with the fluorescent dye, propidium iodide (PI), the intensity of the emitted fluorescence is proportional to DNA content. Cells synthesizing DNA, or undergoing mitosis will have a higher DNA content and will
Figure 1.3 Morphological characteristics of apoptosis. An oligodendrocyte demonstrates nuclear fragmentation, as assessed by electron microscopy (arrow, A). Glial cells demonstrate nuclear condensation and fragmentation under light microscopy (arrows, B, H&E). 3A reproduced from Li et al., 1996a.
consequently exhibit a more intense fluorescence than non-cycling cells that are in the G₀/G₁ phase of the cell cycle. In flow cytometric analysis, apoptotic cells stained with PI will demonstrate a level of fluorescence that is less intense than non-cycling cells, since DNA has been degraded into small fragments and many of these fragments will be lost from the cell. This region of low DNA content is often referred to as the “sub-G₁ peak” (Ormerod, 1998).

DNA fragmentation can also be detected in situ using the terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay (Gavrieli et al., 1992), in which the free 3’ OH ends of the fragmented DNA are tagged with digoxigenin-deoxyribonucleotide triphosphate (dUTP) using the TdT enzyme. TUNEL-positive cells can then be detected using flow cytometry or fluorescent microscopy.

In the early stages of apoptosis, the membrane properties of the apoptotic cell change. One of these changes involves the exposure of phosphatidylserine residues to the outer leaflet of the plasma membrane (Fadok et al., 1992). Annexin V is a protein that binds preferentially to negatively charged phospholipids, such as phosphatidylserine. Binding of Annexin V can then be assessed by microscopy or by flow cytometry.

1.12 Caspases and Apoptosis

There are many pathways leading to apoptosis, but the end result of the apoptotic pathway appears similar in all cells—activation of cysteine proteases. The first intimation that cysteine proteases were important in apoptosis came with the discovery that the cysteine protease, ced-3, in the nematode Caenorhabditis elegans was required for apoptosis. Ced-3 mutations in C. elegans prevented almost all the cell death that
normally occurs during its development (Ellis and Horvitz, 1986; Yuan et al., 1993). Ced-3 was homologous to the mammalian cysteine protease, interleukin-1β-converting enzyme (ICE) (Yuan et al., 1993). Since this discovery, more than 14 mammalian homologs to ced-3 have been discovered, and all have similarities in amino acid sequence, structure and substrate specificity (Nicholson and Thornberry, 1997). Because these cysteine proteases have been shown to play such an important role in apoptosis, a new nomenclature has been assigned to them (Alnemri et al., 1996). Now known as caspases, recent research has proven that certain members of this family are important for apoptosis.

Caspases are present in the cell as proenzymes, and structurally consist of a prodomain, a large (about 20 kb) and a small (about 10 kb) subunit. Cleavage of the proenzyme leads to an active enzyme through the removal of the prodomain, and association of the large and small subunits into a heterodimer. Two heterodimers have been shown to form a tetramer (Rotonda et al., 1996; Walker et al., 1994; Wilson et al., 1994). There is an absolute requirement for an aspartic acid at the cleavage site (Talanian et al., 1997; Thornberry et al., 1997), and the four amino acids N-terminal to the critical aspartic acid residue have also been shown to be important to the specificity of cleavage (Thornberry et al., 1997). Since caspase precursors are activated by proteolytic processing at internal recognition sites that are similar from caspase to caspase, it has been suggested that caspases can process themselves and other precursor caspases to generate active enzymes (Figure 1.4). There are two proposed classes of caspases: initiator and effector caspases. Initiator caspases are postulated to activate effector caspases, which then act on specific substrates to effect apoptosis. Initiator caspases
Figure 1.4 The caspase cascade. In the caspase cascade, an apoptotic stimulus activates adaptor proteins (FADD, cytochrome c) necessary for the auto-processing of initiator caspases. The activation of initiator procaspases leads to the proteolytic cleavage and activation of effector caspases, which subsequently execute apoptosis. Caspase-8 appears to act downstream of death receptor pathways, while caspase-9 responds to a variety of cytotoxic insults. Adapted from Nunez et al., 1998.
include ced-3, caspases-1, 2, 4, 5, 8, 9, 10, 11, 12, and 13. Effector caspases include caspases-3, 6, 7, and 14.

1.12.1 The Caspase Cascade

It has been hypothesized that a sequential activation of initiator and effector caspases leads to the execution of apoptosis (Figure 1.4). This “caspase cascade” can be quite complex, requiring the availability of various co-factors and possibly involving cross-talk between different caspases to amplify the apoptotic response (Nunez et al., 1998).

The initiator pro-caspase, pro-caspase-9, has been reported to be activated in response to cytotoxic agents, including XRT (Hakem et al., 1998; Kuida et al., 1998). In contrast, pro-caspase-8 has been shown to be activated through the ligation of death receptors (Varfolomeev et al., 1998). Activation of both pro-caspase-8 and -9 require the presence of co-factors that bind to the pro-caspases through homologous domains. Pro-caspase-8 activation requires the co-factor fas-associated protein with death domain (FADD) (Boldin et al., 1996; Muzio et al., 1996) and pro-caspase-9 requires the co-factors Apaf-1, cytochrome c and ATP (Li et al., 1997). Co-factors have been postulated to activate initiator pro-caspases by causing their aggregation. The induced proximity of many pro-caspase molecules is thought to trigger auto-proteolysis and auto-activation (Nunez et al., 1998). Once activated, initiator caspases cleave and activate effector caspases. Effector caspases execute apoptosis through the cleavage of proteins modulating the structural integrity of the cell or through the inactivation of inhibitors of apoptosis. Caspase-6 has been implicated in the cleavage of nuclear lamins (Orth et al.,
1996; Takahashi et al., 1996), which leads to the collapse of the nucleus. Caspase-3 cleaves and inactivates the protein inhibitor of CAD (caspase-activated deoxyribonuclease) (Enari et al., 1998; Liu et al., 1997; Sakahira et al., 1998), the nuclease responsible for DNA cleavage during apoptosis (Liu et al., 1997).

### 1.13 Regulation of Caspases through bcl-2 Family Proteins

Bcl-2 is a proto-oncogene, which was originally discovered to be activated by chromosome translocation in human follicular lymphoma (Cleary et al., 1986; Tsujimoto et al., 1984). It was subsequently found that bcl-2 promoted the survival of cytokine-dependent hematopoietic cells in the absence of cytokine (Vaux et al., 1988). There are at least 15 mammalian homologs belonging to the bcl-2 protein family. All bcl-2 family members contain at least one of 4 structural motifs or bcl-2 homology domains, BH1 - BH4. Bcl-2-like proteins can be roughly subdivided into pro- and anti-apoptotic proteins. Pro- and anti-apoptotic members of the bcl-2 family appear to heterodimerize, and the relative ratio of pro-survival:pro-apoptotic proteins may determine cell death or survival (Korsmeyer et al., 1993; Oltvai et al., 1993).

One way anti-apoptotic Bcl-2 members, such as Bcl-xL and Bcl-2, appear to inhibit apoptosis is through disruption of caspase activation. Bcl-xL has been reported to interact with Apaf-1, thus inhibiting Apaf-1-mediated activation of caspase-9 (Hu et al., 1998; Pan et al., 1998). Apaf-1-mediated activation of caspase-9, which requires the presence of cytochrome c, can also be inhibited by Bcl-2, which has been reported to prevent cytochrome c release from mitochondria (Kluck et al., 1997; Yang et al., 1997).
The pro-apoptotic relative, Bax, has been reported to form channels in lipid bilayers in vitro (Antonsson et al., 1997; Schlesinger et al., 1997), and it has been hypothesized that Bax may initiate apoptosis by promoting the release of cytochrome c (Jurgensmeier et al., 1998) (Figure 1.5).

Pro-apoptotic members of the bcl-2 family interact with anti-apoptotic bcl-2 family members and antagonize the pro-survival function of these proteins. The pro-apoptotic Bad has been shown to bind to Bcl-xL via Bad's BH3 domain and promote apoptosis (Zha et al., 1997). After cytokine stimulation, Bad is phosphorylated, binds to 14-3-3 protein (Zha et al., 1996) and is sequestered in the cytosol in an inactive form (Figure 1.6). Stimuli which result in Bad dephosphorylation, such as calcium influx (Wang et al., 1999) and ceramide treatment (Basu et al., 1998), may result in Bad activation and apoptosis (see section 1.16), whereas stimuli that result in Bad phosphorylation may lead to survival. Recent evidence suggests that Bad inactivation can be mediated by the phosphatidyl-inositol-3-kinase (PI3K) pathway (Miho et al., 1999).

1.14 XRT-induced Apoptosis

Although it has been long established that XRT has cytotoxic effects (see sections 1.3 and 1.4), the molecular mechanism by which this occurs remains unclear. Caspase activation has been shown to occur in some cases following XRT (Hallan et al., 1997; Kurihara et al., 1998; Gong et al., 1999), but little is known about the molecular mechanism acting upstream of the caspases. Two major pathways of apoptosis have however been identified following XRT-induced damage. One pathway is activated by
Figure 1.5 Bax-mediated apoptosis. Bax monomers homodimerize and translocate to the mitochondrial membrane following an apoptotic stimulus. Bax dimers are postulated to form a pore in the mitochondrial membrane, permitting the release of such pro-apoptotic species as Apaf-1 and cytochrome c. These molecules will activate procapases, which will subsequently initiate apoptosis. Adapted from Korsmeyer, 1999.
Figure 1.6 Bad-mediated apoptosis. Inactive Bad is phosphorylated and sequestered in the cytosol by 14-3-3. Following dephosphorylation, Bad can interact with pro-survival Bcl-2 relatives, such as Bcl-X₁, and promote apoptosis. Adapted from Korsmeyer, 1999.
XRT-induced DNA damage, while the second pathway is activated by damage to cellular membranes.

1.15 The Mechanism of Nucleus-Driven Apoptosis: p53

p53 is a tumour suppressor gene which has been shown to be mutated in over 50% of human malignancies (Hollstein et al., 1991). Considered the "guardian of the genome" (Lane, 1992), p53 monitors DNA damage in the cell and prompts cell cycle arrest or apoptosis, depending upon cell type and severity of DNA damage (Chen et al., 1996). It is believed that cell cycle arrest allows for the repair of DNA damage and prevents the accumulation of genetic defects and subsequent oncogenic transformation (Kastan et al., 1991; Kastan et al., 1995). This gene has been implicated in the XRT-induced apoptotic pathway of intestinal crypt cells (Merritt et al., 1994), thymocytes (Clarke et al., 1993; Lowe et al., 1993) and cerebellar neurons in vitro (Enokido et al., 1996) and in vivo (Wood and Youle, 1995).

The human p53 protein is 393 amino acids long and is divided into different structural and functional domains (Arrowsmith and Morin, 1996; Soussi and May, 1996) (Figure 1.7). The amino terminal end of the protein constitutes the transactivation domain, and the amino acids leucine 22 and tryptophan 23 are required for the transcriptional activity of p53 (Lin et al., 1994). The central core of the p53 protein shows sequence-specific DNA-binding properties (Cho et al., 1994), and it is this core portion of the p53 protein that is most commonly mutated (Hollstein et al., 1991). The carboxy terminus of the p53 protein has been found to have regulatory properties. Sequence-specific DNA-binding was significantly enhanced by post-translational
modification to the C-terminal end of the protein, or by incubation with anti-p53 antibodies to this domain (Hupp et al., 1993), suggesting that this domain was responsible for regulating p53 activity (Hupp et al., 1995; Jayaraman and Prives, 1995; Lee et al., 1995; Selivanova et al., 1997). The regulatory domain at the C-terminus of the protein may co-operate with a proline-rich region of the protein, located between the transactivation and core domains. The association of these two domains may result in maintenance of p53 in a latent, low-affinity DNA-binding conformation (Muller-Tiemann et al., 1998). The oligomerization domain, which is also located at the carboxy end of the p53 protein, is crucial for correct assembly of the p53 protein. Studies have demonstrated that improper oligomerization causes formation of a dominant negative p53 mutant. A truncated "mini protein" has been used to disrupt the function of p53 by disrupting oligomerization (Eizenberg et al., 1995). Oligomerization normally occurs with formation of a tetramer from two homodimers (Jeffrey et al., 1995).

Under normal, unstressed conditions, the level of p53 protein is low. The half-life of the protein is short (approximately 20 minutes) due to ubiquitin-mediated proteolysis. This process has been shown to be mediated by Mdm2 protein. Mdm2 overexpression can promote rapid p53 degradation in transient assays, and mutations in either p53 or mdm2 that prevent their interaction result in increased p53 stability (Haupt et al., 1997; Kubbutat et al., 1997). Evidence suggests that Mdm2 may target p53 to the proteasome by acting as a ubiquitin ligase (Honda et al., 1997) and that Mdm2 may deliver the p53 protein to the cytosol by shuttling it from the nucleus (Roth et al., 1998).

Accumulation of p53 protein is commonly observed following DNA damage (Kastan et al., 1991). DNA strand breaks, such as those generated by XRT, have been
shown to be sufficient for p53 induction (Huang et al., 1996; Nelson and Kastan, 1994), and XRT has been reported to induce p53 accumulation in thymocytes (Clarke et al., 1993; Lowe et al., 1993) and intestinal crypt cells (Merritt et al., 1994). Subsequent to p53 accumulation, these cells underwent apoptosis. XRT-induced apoptosis observed in thymocytes and intestinal crypt cells was shown to require p53, since the absence of p53 abolished the apoptotic event (Clarke et al., 1993; Lowe et al., 1993; Merritt et al., 1994).

The mechanism by which p53 effects apoptosis following XRT is still under investigation. Following DNA damage signaling to p53, changes in p53 protein levels, post-translational modification and changes in subcellular localization of the protein may all play a role in its activation.

1.15.1 Sensing DNA Damage

The C-terminal end of p53 has been postulated to be a DNA damage recognition site. Experiments have shown that the p53 C-terminal domain shows a particular affinity for short single-stranded DNA ends (Bakalkin et al., 1995), and DNA damage may be sensed by p53 in this manner (Jayaraman and Prives, 1995). The C-terminus has also been shown to bind to DNA mismatches (Lee et al., 1995), Holliday junctions (Lee et al., 1997) and irradiated DNA (Reed et al., 1995). The protein product of the ataxia telangiectasia mutated (ATM) gene has also been implicated in sensing DNA damage. Studies indicate that p53 induction is suboptimal in ATM-/- mice (Herzog et al., 1998; Kastan et al., 1992; Khanna and Lavin, 1993), suggesting that p53 interacts with, but does not require ATM. Other kinases, such as DNA-activated protein kinase (DNA-PK),
casein kinase I (CKI), CDK-activating kinase (CAK) may also signal DNA damage to p53 by phosphorylation of specific residues (see sections 1.15.3 and 1.15.4).

1.15.2 Changes in p53 Protein Levels

Following DNA damage, p53 protein has been shown to accumulate (Fritsche et al., 1993). This accumulation may be due to both an increase in p53 mRNA translation or an increased stability of the protein. Protein synthesis inhibitors can block p53 accumulation following DNA damage (Kastan et al., 1991), and both the 5' and 3' untranslated region of the p53 gene has been implicated in the regulation of p53 protein levels (Fu and Benchimol, 1997; Mosner et al., 1995). The half-life of p53 protein has also been reported to be enhanced following DNA damage (Maki and Howley, 1997; Maltzman and Czyzyk, 1984; Price and Calderwood, 1993), and this has been associated with a decrease in Mdm2-p53 binding (Kamijo et al., 1998; Pomerantz et al., 1998; Shieh et al., 1997; Zhang et al., 1998). Post-translational modifications to the N-terminus of p53 protein may affect Mdm2-p53 interaction (see section 1.15.3, below) and consequently affect p53 stability.

1.15.3 XRT-Induced N-terminal Post-Translational Modifications

An important player in the post-translational modification of p53 appears to be ATM (Figure 1.7). ATM activity is enhanced following XRT (Banin et al., 1998; Canman et al., 1998) and phosphorylation of p53 at serine 15 is observed following XRT (Shieh et al., 1997; Siliciano et al., 1997) is markedly reduced in ATM -/- cells.
Figure 1.7 Functional domains of p53. Post-translational modification in functional domains of the p53 protein after XRT lead to changes in p53 activity. ATM has been shown to phosphorylate serine 15 in the amino-terminal transactivation domain (TA) and dephosphorylate serine 376 in the carboxy regulatory domain (CRD) following XRT. The kinases CKI and CAK are believed to phosphorylate serines in the TA domain, and the acetyltransferase, p300, has been shown to acetylate lysine 382 following XRT. No post-translational modifications have been reported in proline-rich domain (PRD), DNA-binding domain (DBD) or in the region of the nuclear localization signal (NLS). Adapted from Giaccia and Kastan, 1998.
Phosphorylation at this site is not completely abolished, however, suggesting that protein kinases other than ATM can also phosphorylate the site (Carman et al., 1998; Siliciano et al., 1997). DNA-activated protein kinase (DNA-PK) has been reported to phosphorylate serine 15 \textit{in vitro} (Meek, 1994), but the role of DNA-PK in p53 post-translational modification is controversial. Although one study suggests that DNA-PK may signal upstream of ATM (Woo et al., 1998), a later report showed that the p53 response was intact in DNA-PK \(-/-\) cells, suggesting that DNA-PK is dispensable for p53 activation (Jimenez et al., 1999). Interestingly, phosphorylation at serine 15 may contribute to the increased half-life and transcriptional activity of p53 by decreasing the ability of Mdm2 to bind to p53 (Shieh et al., 1997).

Other phosphorylation sites in the p53 N-terminus has been reported following XRT, but the kinases responsible for these post-translational modifications have not yet been identified \textit{in vivo}. Casein kinase I may be responsible for the phosphorylation of two serines within the first 20 amino acids of the amino terminus of p53 since this kinase has been shown to phosphorylate the amino-terminus of p53 \textit{in vitro} and \textit{in vivo} following topoisomerase treatment (Knippschild et al., 1997) (Figure 1.7). CDK-activating kinase has been shown to phosphorylate serine 33 (Ko et al., 1997) \textit{in vitro}, but it is unknown if this kinase also acts \textit{in vivo}.

\subsection*{1.15.4 XRT-Induced C-Terminal Post-Translational Modifications}

ATM is not only involved in N-terminal phosphorylation, as described above, but is also implicated in the dephosphorylation of serine 376, the creation of a 14-3-3 protein-binding site and enhancement of sequence-specific DNA binding (Waterman et al., 1998,
Figure 1.7). It is unlikely that ATM itself possesses phosphatase activity, but it may mediate the activation of a phosphatase that acts upon serine 376.

Another XRT-induced post-translational modification to the C-terminus of p53 that has been reported is the acetylation of lysine 382 in vitro by the acetyltransferase, p300 (Sakaguchi et al., 1998). This acetylation was accompanied by enhancement of sequence-specific DNA-binding (Figure 1.7). Importantly, this study showed that p53 that had been previously phosphorylated by DNA-PK was a better substrate for acetylation than unphosphorylated p53, demonstrating the interdependence of post-translational modifications.

The physiological consequences of post-translational modification are under investigation. It is not clear if these alterations in p53 protein following XRT lead to activation of p53 and p53-mediated events, such as apoptosis. A further complication is that different post-translational modifications may work interdependently, so that elimination of one particular modification may yield only a slight change in p53 activity (Giaccia and Kastan, 1998).

1.15.5 Subcellular Localization

p53 activity may also be controlled through its subcellular localization. In several cell types, including normal keratinocytes (Helander et al., 1993), normal fibroblasts (Rotter et al., 1983), and some subpopulations of neuroblastoma cells (Isaacs et al., 1998; Moll et al., 1995), p53 is normally sequestered in the cytoplasm, where it remains in an inactive state. After DNA damage, p53 has been observed to accumulate in the nucleus (Fritsche et al., 1993). Subsequently, the cell undergoes growth arrest or apoptosis. A
nuclear localization signal at the C-terminal end of the protein suggests that p53 may be targeted to the nucleus by this mechanism. One study further suggests that p53 may be differentially phosphorylated by compartment-specific kinases following DNA damage (Martinez et al., 1997). Following XRT in ras-transformed primary rat embryo fibroblasts, p53 is hyperphosphorylated at its C-terminal end in the cytoplasm, shifted to the nucleus and hyperphosphorylated at its N-terminal end (Martinez et al., 1997). Presumably, the hyperphosphorylated p53 protein has a different conformation that permits DNA-specific binding and transcriptional activation of genes promoting growth arrest or apoptosis.

1.15.6 Downstream Events in p53-Mediated Apoptosis

Once activated by DNA damage, p53 may induce apoptosis through the transcriptional activation of genes responsible for apoptosis or by the negative regulation of anti-apoptotic genes. p53 consensus sequences have been reported in insulin-like growth factor binding protein 3 (IGFBP-3) (Buckbinder et al., 1995), bax (Miyashita and Reed, 1995), and bcl-2 (Miyashita et al., 1994), among other genes. Wildtype, but not mutant, p53 enhanced IGFBP3 secretion and blocked mitogenic signaling by insulin-like growth factor (Buckbinder et al., 1995). The loss of a mitogenic signal may contribute to p53-dependent apoptosis (Neuberg et al., 1997). Similarly, wildtype p53 was reported to directly activate the pro-apoptotic gene, bax, in vitro (Miyashita and Reed, 1995). Mutant p53 failed to transactivate bax, and the introduction of mutations in the p53 consensus sequences of bax resulted in the loss of transcriptional activity (Miyashita and Reed, 1995). p53 was also found to negatively regulate the anti-apoptotic gene, bcl-2
(Miyashita et al., 1994). The ability of p53 to influence the ratio of pro- and anti-apoptotic members of the bcl-2 family can result in apoptosis (see section 1.12).

In summary, it is likely that p53-dependent apoptosis occurs in a complex manner, influenced in part by the apoptosis-inducing stimuli, cell type, endogenous levels of p53, availability of kinases and acetyltransferases, subcellular localization of p53 and the microenvironment.

1.16 The Mechanism of Membrane-Driven Apoptosis: Ceramide

Several studies have identified the cell membrane as an upstream target for XRT-induced damage. Definitive evidence that membranes alone could activate apoptosis was reported by Haimovitz-Friedman and colleagues. This group showed that nuclei-free preparations of endothelial cells underwent apoptosis following XRT, and that apoptosis was preceded by sphingomyelin hydrolysis and ceramide production (Haimovitz-Friedman et al., 1994).

Sphingomyelinases (SMases) are specific enzymes that catalyze the hydrolysis of membrane-bound sphingomyelin to generate phosphocholine and ceramide. Two isoforms of SMases are known, but the role of acid SMase (aSMase) in XRT-induced apoptosis is better characterized. Pulmonary endothelial cells of mice deficient in aSMase are resistant to XRT-induced apoptosis (Santana et al., 1996) and do not generate ceramide in vivo. Furthermore, lymphoblasts from patients with Niemann-Pick Disease, who have an inherited deficiency in aSMase, showed a resistance to XRT-induced apoptosis (Santana et al., 1996).
Ceramide is believed to be generated primarily through the action of SMases following cellular stress. However, in some cases, ceramide can also be produced de novo through the action of ceramide synthase. Apoptosis following daunorubicin treatment has been shown to be mediated by ceramide synthase (Bose et al., 1995).

In one pathway, ceramide generation results in the activation of the ras and raf-1 kinases (Basu et al., 1998). This leads to inactivation of the Akt kinase, which has been reported to phosphorylate Bad at serine 136 (Datta et al., 1997; del Peso et al., 1997; Miho et al., 1999; Zundel and Giaccia, 1998) preventing it from forming heterodimers with Bcl-XL, leading to cell survival (Figure 1.6). However, with the inactivation of Akt, Bad is dephosphorylated at serine 136, leading to its binding to Bcl-XL, and apoptosis (Basu et al., 1998).

In a second pathway, ceramide generation activates the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway. One simple model of SAPK/JNK signalling involves the sequential activation of the MEKK1, SEK1 and SAPK/JNK kinases, culminating in the phosphorylation of the c-Jun transcription factor (Kyriakis et al., 1994). c-Jun overexpression was shown to be sufficient to induce apoptosis in NIH 3T3 fibroblasts, and this apoptotic process was blocked with inhibitors of ICE/CED-3-like caspases, suggesting that c-Jun induces apoptosis by the activation of caspases (Bossy-Wetzel et al., 1997).

1.17 Cross-talk Between the Ceramide and p53 Pathways Following Stress

An initial study conducted in aSMase -/- and p53 -/- mice demonstrated that the p53 and ceramide pathways of XRT-induced apoptosis were likely independent (Santana
et al., 1996). Subsequent reports, using different apoptotic stimuli, have also concluded that p53 and ceramide operate independently to initiate apoptosis in neuroblastoma cells (Maurer et al., 1999), glioblastoma cells (Yount et al., 1999) and oocytes (Perez et al., 1997).

However, there is evidence that, at least in some cell types, the p53 and ceramide apoptotic pathways may converge. p53 may act downstream or upstream of ceramide, depending on the cell type and stimulus. Induction of apoptosis in macrophages by C2-ceramide was accompanied by increased p53 expression, but when the cells were pretreated with p53 antisense oligonucleotides, C2-ceramide could not initiate apoptosis (Kinscherf et al., 1998). C2-ceramide can also induce apoptosis in fibrosarcoma cells with intact p53, but apoptosis was abrogated in cells lacking p53 (Pruschy et al., 1999). Both these reports indicate that C2-ceramide operates upstream of p53. However, human leukemia cells were reported to accumulate p53 before ceramide levels increased, suggesting that, in this case, p53 was upstream of ceramide (Dbairo et al., 1998).

Furthermore, a recent study suggests that DNA double strand breaks induced by XRT could cause post-translational modification of ceramide synthase, resulting in ceramide production and apoptosis (Liao et al., 1999). This ceramide synthase-dependent apoptosis was intact in ataxia telangiectasia (AT)-derived cell lines, but the apoptotic response was abolished in cell lines with the intact ATM gene (Liao et al., 1999).
1.18 XRT-Induced Apoptosis in the CNS

Apoptosis is a common event in the developing CNS when neural pathways are being formed (Raff et al., 1993). Following low doses of XRT to the developing CNS, the apoptotic response is enhanced (Inoiye et al., 1983; Harmon et al., 1988; Hoshino et al., 1991).

As mentioned previously (section 1.7), the oligodendrocyte may be an important cell target in the adult CNS after XRT. These cells undergo apoptosis following XRT in vitro (Vrdoljak et al., 1992), and work in our lab has demonstrated that oligodendrocytes undergo XRT-induced apoptosis in the adult rat spinal cord in vivo (Li et al., 1996a; Li et al., 1996b). These studies indicated that XRT-induced apoptosis occurred predominantly in white matter of the adult rat spinal cord and that the apoptotic incidence peaked at 8 h and returned to baseline level by 24 h after XRT (Li et al., 1996a; Li et al., 1996b). This apoptotic response increased with increasing XRT doses of 1 – 30 Gy, and reached a plateau at 22 Gy (Li et al., 1996b). Morphological studies, using both light and electron microscopy suggested that the apoptotic cells were oligodendrocytes and immunohistochemical staining indicated that 96.1% of the apoptotic cells were positive for the oligodendrocyte specific marker, Leu-7 (Li et al., 1996a; Li et al., 1996b). Based on the number of apoptotic cells and oligodendrocyte density changes, it was estimated that the duration of apoptosis was approximately 1 h. Overall, a 24% decrease in oligodendrocyte density was observed 24 h after 22 Gy (Li et al., 1996b).

Subependymal cells have been reported to undergo XRT-induced apoptosis in vivo following XRT (Bellinzona et al., 1996; Shinohara et al., 1997). The maximal apoptotic response in these cells was reached at 6 h after XRT (Bellinzona et al., 1996;
Shinohara et al., 1997), and subependymal cells were observed to proliferate 2 – 3 days following XRT (Shinohara et al., 1997). Although these cells are not proposed to be a major target of XRT-induced injury, subependymal apoptosis may contribute to XRT-induced responses of the CNS, since a depletion of these undifferentiated stem cells (Chiasson et al., 1999) may lead to a decreased capacity for regeneration of other cell populations.

1.19 Role of p53 in XRT-Induced Apoptosis

The role of p53 in the apoptotic pathway following XRT in certain cell types has been well established. These studies have been done using transgenic animals that have a targeted disruption of the p53 gene. Thus, both intestinal crypt cells of p53 -/- mice and thymocytes from mice null for p53 were resistant to XRT-induced apoptosis (Merritt et al., 1994; Clarke et al., 1993; Lowe et al., 1993).

Neonatal CNS cells have also been shown to undergo p53-dependent apoptosis following XRT. In the fetal rat brain, an increase of p53 protein at 1 - 3 h following 4 Gy was observed prior to the peak of apoptosis, which was seen at 5 h (Borovitskaya et al., 1996). The olfactory bulb, cerebellum and hippocampus in the developing mouse CNS showed p53 stabilization 2 h following a single dose of 14 Gy, and apoptosis was observed in the cerebellum subsequent to p53 upregulation (Herzog et al., 1998). In contrast, the developing CNS in p53 null mice demonstrated a resistance to XRT-induced apoptosis (Herzog et al., 1998). In vivo, cerebellar neurons in mice wildtype for p53 demonstrated XRT-induced apoptosis and decreased cell density, whereas neurons in p53 null mice showed a resistance to XRT-induced apoptosis (Wood et al., 1995). Cerebellar
neurons cultured from p53 wildtype mice underwent apoptosis 24 - 72 h following XRT, but neurons cultured from p53 -/- mice were resistant to XRT-induced apoptosis (Enokido et al., 1996).

1.20 Role of p53 in Oligodendrocyte Apoptosis

Although it has been well established that oligodendrocytes (Li et al., 1996a; Li et al., 1996b; Vrdoljak et al., 1992) and subependymal cells (Bellinzona et al., 1996; Shinohara et al., 1997) undergo XRT-induced apoptosis in vitro and in vivo, the mechanism by which this occurs is unknown. Oligodendrocytes have been shown to undergo apoptosis following treatment with ceramide or ceramide analogs (Brogi et al., 1997; Casaccia-Bonnefil et al., 1996a; Casaccia-Bonnefil et al., 1996b; Larocca et al., 1997), but it is uncertain whether XRT induces a ceramide-dependent apoptotic death in these cells.

p53 has been reported to mediate XRT-induced apoptosis in the neonatal cerebellar neurons in vivo and in vitro (Wood et al., 1995; Enokido et al., 1996). Furthermore, oligodendrocytes have been reported to undergo p53-dependent apoptosis after a number of different stimuli. p53 protein expression in unstressed oligodendrocytes in culture appeared to be restricted to the cytoplasm, but following treatment with dimerized interleukin-2 (IL-2), p53 was reported to shift from the cytoplasmic to the nuclear compartment (Eizenberg et al., 1995). This shift in subcellular localization occurred within 15 mins, and preceded the onset of apoptosis, which occurred an hour following IL-2 treatment (Eizenberg et al., 1995). Similarly, nuclear accumulation of p53 was observed in an oligodendrocyte cell line in vitro following
hydrogen peroxide treatment (Uberti et al., 1999). The involvement of p53 in oligodendrocyte apoptosis in vitro following a treatment of IL-2 (Eizenberg et al., 1995) or hydrogen peroxide (Uberti et al., 1999) suggests that a p53-dependent apoptotic pathway exists in oligodendrocytes.

1.21 Hypothesis

The involvement of p53 in XRT-induced apoptosis in other cell types, including CNS cells, suggests that XRT-induced apoptosis in oligodendrocytes and subependymal cells may also occur by a p53-dependent pathway. We postulate that XRT-induced apoptosis in oligodendrocytes and subependymal cells of the adult CNS is p53-dependent. To test this hypothesis, the apoptotic incidence in the CNS of adult mice wildtype (+/+), heterozygous (+/-) and null (-/-) for p53 was compared over a 24 or 36 h time course after XRT. Immunohistochemistry was used to identify the cells undergoing apoptosis, as well as to assess the expression of p53 protein following XRT. Oligodendrocyte density was assessed before and at 24 h following XRT to determine if a significant depletion in the oligodendrocyte cell population had occurred. This in vivo work is described in Chapter 2.

As outlined above (see section 1.15.5), one means of p53 regulation is through subcellular localization of p53 protein. Cultured oligodendrocytes that have been treated with a cytotoxic dose of IL-2 dimer have demonstrated a shift of p53 protein from the cytoplasm to the nucleus (Eizenberg et al., 1995). To determine if subcellular localization of p53 was an important mechanism of p53 regulation in CNS cells, we
developed a method to culture oligodendrocytes and astrocytes, and quantitate p53 protein levels in these cells. Preliminary results of this work are described in Chapter 3.

Finally, in Chapter 4, the results of this work are discussed in the larger context of the ongoing research in our laboratory, and several experiments are proposed to extend the conclusions drawn from this thesis.
1.22 References


Chapter 2

The Role of p53 in XRT-Induced Apoptosis in the Adult CNS In Vivo\(^1\)

\(^1\)This chapter is the modified text of a manuscript submitted for publication to *Cell Death and Differentiation*. Authors of the paper are B. M. Chow, Y-Q. Li and C. S. Wong. B. M. Chow performed all experiments with the exception of the GC immunostaining (see section 2.3.5) and the plp *in situ* hybridization (see section 2.3.7), which were done by Y-Q. Li.
2.1 Abstract

Oligodendrocytes and subependymal cells in the adult CNS have been shown to undergo radiation-induced apoptosis. Here, we examined the role of p53 in radiation-induced apoptosis in the adult mouse CNS. In the spinal cord of p53 +/- mice, apoptotic glial cells were observed within 24 h after irradiation, and the apoptotic response peaked at 8 h. These apoptotic cells demonstrated the immunohistochemical phenotype of oligodendrocytes, and decreased oligodendrocyte density was observed at 24 h after 22 Gy. A similar time course of radiation-induced apoptosis was seen in subependymal cells in the adult mouse brain. Radiation-induced apoptosis was preceded by an increase in nuclear p53 expression in glial cells of the spinal cord and subependymal cells of the brain. There was no evidence of radiation-induced apoptosis in the spinal cord and subependymal region of p53 -/- animals. We conclude that the p53 pathway may be a mechanism through which DNA damage induces apoptosis in the adult CNS.

2.2 Introduction

Numerous stresses, including chemotherapeutic drugs, oxidative stress and ionizing radiation (XRT) may induce apoptosis. The mechanism of apoptosis appears to be dependent on many factors, including the nature of apoptotic stimuli, cell type and the microenvironment. XRT-induced apoptosis has been observed in a number of cell types, including thymocytes (Clarke et al., 1993; Lowe et al., 1993) intestinal crypt cells (Merritt et al., 1997; Merritt et al., 1994), pulmonary endothelial cells (Santana et al., 1996), and cerebellar neurons (Enokido et al., 1996; Kim et al., 1998; Wood and Youle, 1995).
Oligodendrocytes have been shown to undergo XRT-induced apoptosis \textit{in vitro} (Vrdoljak \textit{et al.}, 1992) and \textit{in vivo} in the rat spinal cord (Li \textit{et al.}, 1996a; Li \textit{et al.}, 1996b). Subependymal cells \textit{in vivo} have also been shown to undergo XRT-induced apoptosis (Bellinzona \textit{et al.}, 1996; Shinohara \textit{et al.}, 1997). Oligodendrocytes are post-mitotic cells that ensheath neuronal axons with concentric layers of myelin. Myelinated axons conduct much faster than bare axons and demyelination is associated with many neuropathologic conditions. Subependymal cells are cells adjacent to the ventricles of the brain. The function of these cells is largely unknown, but they have been observed to undergo proliferation in response to injury and can give rise to neuronal and non-neuronal cell populations (Goldman \textit{et al.}, 1996).

The tumour suppressor gene, p53, has been shown to play a role in XRT-induced apoptosis in thymocytes (Clarke \textit{et al.}, 1993; Lowe \textit{et al.}, 1993) and intestinal crypt cells (Merritt \textit{et al.}, 1994). A delayed p53-independent apoptosis after XRT however has been observed in intestinal epithelial cells (Merritt \textit{et al.}, 1997), and XRT-induced apoptosis in pulmonary endothelial cells was described to be p53-independent (Santana \textit{et al.}, 1996). Certain cells of the central nervous system (CNS) have been reported to undergo p53-dependent apoptosis. \textit{In vitro}, post-mitotic cerebellar neurons undergo p53-dependent apoptosis following some DNA damaging agents, including XRT (Enokido \textit{et al.}, 1996). Cultured oligodendrocytes have been shown to undergo p53-dependent apoptosis following treatment with an interleukin-2 (IL-2) dimer (Eizenberg \textit{et al.}, 1995). In the developing CNS, cerebellar granule cells underwent XRT-induced apoptosis, but XRT failed to induce apoptosis in the cerebellum of p53 null mice (Wood and Youle, 1995).
The role of p53 in XRT-induced apoptosis in the adult CNS has not been described. In this study, we show evidence for upregulation of the p53 protein in the CNS of p53 wild-type (+/+ ) animals after XRT. Adult mice that were null (+/- ) for the p53 gene demonstrated an absence of XRT-induced apoptosis in oligodendrocytes and subependymal cells. We conclude that the p53 pathway may be a mechanism through which XRT induces apoptosis in the adult CNS.

2.3 Materials and Methods

2.3.1 Animals

Adult C57BL6/J female p53 +/-, +/− and +/- mice (Jackson Laboratories), 57 to 123 days old, were used in this study. The animals were housed at 4 - 5 per cage, with food and water freely available, and with lighting between 6 AM and 6 PM in the animal colony of the Ontario Cancer Institute, an animal colony accredited by the Canadian Council of Animal Care.

2.3.2 Irradiation

Animals that were to be irradiated were anaesthetized with 3.5% halothane prior to immobilization in a plastic jig. Control animals were neither sham irradiated, nor anaesthetized. Irradiations were carried out using two Picker Gemini 100 kV X-ray units employed in a parallel, opposed configuration. Port films to confirm the accuracy of field placement were performed prior to irradiation. Animals were given a single dose of 2 Gy (to the entire brain) or 8, 22, or 30 Gy (to a 1 cm length of C2-T2 of the spinal cord), and sacrificed at various time points up to 36 hours after XRT. The doses of 2 Gy and 22 Gy
were chosen since these respective doses were previously reported to induce the greatest apoptotic response in the brain and spinal cords of the adult rat in vivo, (Li et al., 1996b; Shinohara et al., 1997). Details of irradiation and dosimetry were previously described (Wong et al., 1992). Control animals were not irradiated.

2.3.3 Histopathology

Animals were given an injection of 1 cc of 10% somnotol intraperitoneally. Following transcardiac perfusion using 10% neutral buffered formalin, the cervical portion of the spinal cord (from cervical segment 2 to thoracic segment 2, C2-T2) was dissected free of bone and fixed in formalin overnight before embedding in paraffin. Transverse sections, 4 μm thick, were cut from the middle of the spinal cord and stained with hematoxylin and eosin (H&E) or processed for immunohistochemical studies. Non-irradiated controls and animals irradiated to the whole brain were processed similarly. Coronal sections of the brain, 4 μm thick, were cut at the level of the optic chiasm, fixed, embedded, sectioned and stained in the same manner as for the spinal cord.

2.3.4 Assessment of apoptosis

Apoptosis in the brain and the spinal cord of p53 +/+ , +/- and -/- animals was assessed morphologically and biochemically using the TUNEL assay (Gavrieli et al., 1992). For the quantitative assessment of apoptosis, H&E-stained brain and spinal cord sections were examined. Criteria for an apoptotic cell included: cell shrinkage with eosinophilic cytoplasm and nuclear condensation or nuclear fragmentation, as described previously (Li et al., 1996a; Li et al., 1996b).
Apoptosis was assessed biochemically using the In Situ Death Detection Kit Fluorescein (Boehringer Mannheim), based on the method of Gavrieli et al (Gavrieli et al., 1992). Unstained paraffin sections of the brain or spinal cord were deparaffinized, rehydrated and digested with proteinase K (Sigma). 3’OH ends of nicked DNA were tagged with digoxigenin (DIG)-dUTP using terminal deoxynucleotidyl transferase (TdT). The tagged ends were labelled with an antibody conjugated to fluorescein isothiocyanate (FITC). Sections were dual stained with 4,6 diamidino-2-phenylindole (DAPI, Sigma) using methods described previously (Li and Wong, 1998). The DIG-TdT enzyme was omitted as a negative control.

The apoptotic incidence (AI) was obtained for each time point. In the spinal cord, AI was defined as the percent of apoptotic glial cells, as identified by morphology, per transverse section of the spinal cord. For the brain, it was the percent apoptotic subependymal cells in the subependyma, defined as a 4 - 5 cell-layer thick region surrounding the lateral ventricles. Ependymal cells were excluded from this analysis.

For the spinal cord experiments, three animals were used for each time point, with the exception of 0, 6, 10 and 24 h, where 6, 7, 4, and 6 animals were used respectively. In the spinal cords of +/- and -/- mice, three animals were used for all time points. For the time course of XRT-induced apoptosis in the brain, three p53 +/- or -/- animals were used at all time points, except at 24 h in the +/+ mice and 36 h in the -/- mice, when two animals at each time point were used. Two p53 +/- mice were used at both 4 and 16 h after XRT. In all cases, three sections were counted per mouse. The scoring was performed by an observer (BMC) blinded to the treatment each animal had received.
2.3.5 Characterization of cell type(s) undergoing apoptosis

To characterize the cell type(s) undergoing apoptosis in the spinal cord, antibodies against galactocerebroside (GC), Leu-7 and glial fibrillary acidic protein (GFAP), were used. GC (Raff et al., 1978) and Leu-7 (Schuller-Petrovic et al., 1983) are specific oligodendrocyte markers, and GFAP is a type of intermediate filament specific for astrocytes (Ludwin et al., 1976). Methods for immunostaining were similar to those described previously (Li et al., 1996b). For both Leu-7 and GFAP immunohistochemistry, sections were deparaffinized and endogenous peroxidase activity was blocked by H$_2$O$_2$. Briefly, for Leu-7 immunostaining, sections were sequentially incubated with anti-Leu-7 antibody (Becton Dickinson, clone HNK-1, 1:100), biotinylated anti-mouse IgM and streptavidin horseradish peroxidase. The reaction was visualised using 3-amino-9-ethylcarbazole (AEC). For GFAP immunostaining, sections were sequentially incubated with anti-GFAP antibody (DAKO), biotinylated goat anti-mouse IgM, streptavidin peroxidase complex and AEC. All slides were counterstained with hematoxylin. Three different sections from three different animals at 6 h after 22 Gy were used to quantify the percent Leu-7 positive apoptotic cells. Two different sections from two different mice at 6 h after 22 Gy were used to quantify the percent of GFAP-positive apoptotic cells. Apoptotic cells were considered positive if immunoreactivity for GFAP or Leu-7 completely surrounded the pyknotic nucleus or nuclear fragments.

The spinal cords of three animals were irradiated separately with a dose of 22 Gy for GC immunohistochemistry. These animals were sacrificed at 8 h after XRT, perfused with 2% paraformaldehyde for 10 min, and the dissected spinal cords were post-fixed for
5 days at 4°C in 2% paraformaldehyde prior to GC immunohistochemistry as described previously (Ellison and de Vellis, 1994). Cryosections were incubated with 0.3 mg/ml NaBH₄ in PBS to reduce aldehyde bonds and allow antibody access to antigen. This was followed by incubation with the primary antibody (5 μg/ml; Boehringer Mannheim), and then with FITC-conjugated goat anti-mouse IgG secondary antibody (Boehringer Mannheim) diluted 1:200 in PBS containing 0.1% bovine serum albumin. Sections were dual stained with DAPI (Sigma) to identify apoptotic nuclei. The percent GC positive apoptotic cells from 9 spinal sections was recorded. Apoptotic cells were considered GC-positive if immunoreactivity for GC completely surrounded the pyknotic nucleus or nuclear fragments counterstained with DAPI.

The primary antibodies (Leu-7, GC and GFAP) were omitted in negative controls.

2.3.6 p53 immunohistochemistry

For p53 immunohistochemistry, sections were deparaffinized, rehydrated, and blocked in 3% H₂O₂. Antigen retrieval was performed by microwaving sections in a 0.01M citrate buffer (pH 6). Sections were then sequentially incubated in anti-p53 antibody (Nova Castra, clone CM5, 1:1000), biotin anti-rabbit IgG, and streptavidin-horseradish peroxidase. The reaction was visualised with AEC and counterstained with hematoxylin. Sections of the mouse small bowel, obtained at 3 h after 8 Gy, were used as a positive control (Merritt et al., 1994). The p53 primary antibody was omitted as a negative control. Spinal cord and brain sections from p53 -/- mice were stained with the p53 antibody, as described above, to determine the specificity of staining.
Cells were considered p53 positive only if strong p53 immunoreactivity was found in the entire nucleus. The number of p53 positive cells in the spinal cord was expressed as a percent of all the glial cells in the transverse spinal cord section. The subependyma was again defined as the region of cells adjacent to the ependymal cells, 4 - 5 cell layers thick, surrounding the lateral ventricles. In the spinal cord, 3 sections from 3 different mice were counted, except in the non-irradiated animals, in which 3 sections were counted from 2 different mice. In the brain, three sections from three different mice were counted.

2.3.7 Oligodendrocyte density after XRT

To determine if apoptosis in the spinal cord was associated with a decrease in oligodendroglial cell density, proteolipid (plp) mRNA was used as a specific marker for oligodendrocytes (Griffiths et al., 1995). Transverse sections of non-irradiated and irradiated (24 h after 22 Gy) p53 +/- spinal cords were processed for plp in situ hybridization.

Dr. Griffiths donated the cDNA encoding plp. The cDNA fragment was cut out by digesting the pGEM3 plasmid containing plp cDNA with EcoRI and HindIII and subcloned into pGEM-4Z vector (Promega). The plasmid carrying a 450 base pair fragment of the plp cDNA coding sequence was linearized with EcoRI or HindIII (Boehringer Mannheim). The DIG-labelled sense and antisense RNA probes were obtained by in vitro transcription with T7 or SP6 RNA polymerase in the presence of DIG-UTP using a DIG RNA labelling kit (Boehringer Mannheim), according to manufacturer's instructions.
The *in situ* hybridization procedure was performed using a modification of a protocol in the Nonradioactive *In Situ* Hybridization Application Manual (Boehringer Mannheim). Briefly, the slides were fixed in 4% paraformaldehyde in PBS, and treated with 0.2 N HCl and proteinase K. The sections were acetylated in freshly prepared 0.5% acetic anhydride in 0.1 M triethanolamine-HCl pH 8.0. The slides were hybridized with DIG-labelled sense or anti-sense probes (100 ng/100 µl) in hybridization buffer (50% formamide, 2 × SSC, 10% dextran sulfate, 0.01% sheared salmon sperm DNA, and 0.02% SDS). Hybridization was performed at 70°C, followed by a post-hybridization wash in 50% deionized formamide in 1 × SSC at 55°C. The slides were equilibrated in 1 × SSC and blocked in blocking mixture (0.1 M Tris pH 7.5, 0.05 M NaCl, 0.2% Tween 20, 10% fetal bovine serum and 1% blocking reagent, Boehringer Mannheim). The slides were then incubated with anti-DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim), diluted 1:500 in blocking mixture. Visualization of the plp mRNA message was done by incubating sections in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Slides were counterstained with methyl green (Sigma). The sense probe was used on sections as a negative control.

Because the number of apoptotic cells returned to control level by 24 h after XRT (Li *et al.*, 1996a; Li *et al.*, 1996b), the impact of XRT-induced apoptosis was evaluated by comparing the number of plp mRNA positive cells at 24 h with non-irradiated controls. Five representative 0.016 mm² areas in the spinal cord were selected for the scoring. The cell density in white matter was defined as the mean value in dorsal, lateral, and ventral white matter, and the density in gray matter was the mean of the values for dorsal and ventral gray matter. Three sections of the spinal cord were assessed per dose.
group. The number of plp positive oligodendrocytes was counted by an observer (YQL) blinded to the treatment.

2.3.8 Statistics

Sections from the same mouse were averaged to obtain one single mean for each mouse at each time point. All data represent the mean calculated from each mouse at each time point, and the standard error of the mean (SEM) was derived from the mean value from each mouse. Using Dunn’s Test, a non-parametric test, each group of animals at each time point was compared with the group of non-irradiated wild-type animals to determine if the AI rose above baseline levels. In addition, Dunn’s test was used to determine if the AI at each time point was significantly different in each of the three different p53 genotypes.

2.4 Results

2.4.1 XRT-induced apoptosis in the spinal cord

To characterize XRT-induced apoptosis in the p53 +/- adult mouse spinal cord, the cervical segment of the spinal cord (C2 to T2) was given a single dose of 8, 22 or 30 Gy. At different time points, up to 24 h after XRT, apoptosis was assessed morphologically using H&E staining, and confirmed using the TUNEL assay. TUNEL-stained sections were counterstained with the fluorescent nuclear stain, DAPI.

There was no evidence of any gross histopathology in the spinal cord during the 24 h following XRT. The tissue architecture in irradiated tissues was similar to that in
non-irradiated tissues. All blood vessels appeared normal and there was no evidence of inflammation or edema in the irradiated spinal cord.

There was little evidence of apoptosis in the non-irradiated mouse spinal cord. The apoptotic incidence (AI) in the non-irradiated spinal cord, as quantified using morphological criteria in H&E-stained sections, was very low (0.024 ± 0.009%). Apoptotic glial cells were observed within the first 24 h after XRT (Figure 2.1A, B and C). There was no evidence of apoptosis in neurons or vascular endothelial cells. These apoptotic cells were scattered singly throughout white and gray matter.

A significant increase in AI was observed by 6 h after 22 Gy (p < 0.05) compared to non-irradiated wild-type animals (Figure 2.2). The peak apoptotic response was observed at 8 h and the AI returned to baseline levels by 16 h after 22 Gy (Figure 2.2). There was no evidence of an increase in AI beyond a single dose of 22 Gy (Figure 2.3).

The astrocyte-specific marker, glial fibrillary acidic protein (GFAP), and oligodendrocyte-specific markers, galactocerebroside (GC) and Leu-7, were used to identify the apoptotic cell type. Immunohistochemical staining on sections demonstrated that virtually all the apoptotic cells (84.6 ± 5.3%) observed at 8 h after 22 Gy were GC-positive (Figure 2.1D and E), and almost half (48.6 ± 18.6%) of the apoptotic cells were Leu-7 positive (Figure 2.1F). Apoptotic cells that exhibited little or no Leu-7 immunoreactivity in the cytoplasm were scored as Leu-7 negative apoptotic cells (Figure 2.1G). None of the apoptotic cells were positive for GFAP (Figure 2.1H). Omission of the primary antibodies resulted in no staining, confirming the specificity of the immunohistochemistry.
Figure 2.1 Adult mice wildtype for p53 were given a single dose of 22 Gy to the spinal cord or 2 Gy to the brain, and sacrificed at different times up to 36 h after XRT. Apoptosis was assessed by H&E staining or TUNEL assay. At 10 h after 22 Gy (A), one apoptotic cell (arrow) shows evidence of nuclear condensation and cell shrinkage, while a second apoptotic cell (arrowhead) shows nuclear fragmentation (H&E). At 6 h after 22 Gy in a TUNEL-stained section (B), a brightly fluorescent TUNEL positive apoptotic cell also shows morphological evidence of nuclear fragmentation, which is more clearly seen when the sections are counterstained with DAPI (arrow, C). Immunohistochemistry on spinal cord sections for the oligodendrocyte-specific marker, GC, demonstrates 2 apoptotic cells (arrows, D) completely surrounded by GC immunoreactivity at 8 h after 22 Gy. The apoptotic cells (arrows, E) demonstrate nuclear condensation and fragmentation upon counterstaining with DAPI. Immunohistochemical staining for the oligodendrocyte-specific marker, Leu-7, at 8 h after 22 Gy is observed in an apoptotic cell (arrowhead, F) that also demonstrates nuclear fragmentation. Another Leu-7 positive apoptotic cell shows nuclear condensation (arrow, F). In contrast, Leu-7-negative apoptotic cells demonstrated little or no cytoplasmic Leu-7 immunoreactivity (arrow, G). Immunohistochemistry on spinal cord sections for the astrocyte-specific marker, GFAP, demonstrates that apoptotic cells at 8 h after 22 Gy were negative for GFAP. The location of this GFAP-negative apoptotic cell (arrow, H) suggests that it is most likely a perineuronal satellite oligodendrocyte. An non-irradiated section of the subependyimal region shows no evidence of apoptosis (H&E, I). At 16 h after 2 Gy, clusters of apoptotic cells (arrows, J) show evidence of cell shrinkage, nuclear fragmentation and eosinophilic cytoplasm. At 6 h after 2 Gy, a brightly fluorescent TUNEL-positive apoptotic subependymal cell (K) shows morphological evidence of nuclear fragmentation, which is more clearly seen when the section is counterstained with DAPI (arrow, L). Original magnifications x 1000. V, ventricle; E, ependymal cells.
Figure 2.2 The time course of XRT-induced apoptosis in the spinal cords of adult p53+/+ or -/- mice after a single dose of 22 Gy was assessed using morphological criteria with H&E staining at 0, 4, 6, 8, 10, 16 and 24 h after irradiation. At some time points (0, 6, 8, 10 or 24 h), apoptosis was also assessed in p53 +/- mice. Three mice were used at each time point, except for 0, 6, 10 and 24 h in the +/- mice, where 6, 7, 4, and 6 animals were used, respectively. Vertical bars are SEM.
Figure 2.3 Dose response for XRT-induced apoptosis in the p53 +/- mouse spinal cord was assessed using morphological criteria with H&E staining at 6 h after doses of 8, 22 and 30 Gy. 6, 3, 7 and 3 animals were used at 0, 8, 22 and 30 Gy, respectively. Vertical bars are SEM.
2.4.2 XRT-induced apoptosis in the subependyma

To examine XRT-induced apoptosis in the subependymal regions, adult p53 +/- mice were given a single dose of 2 Gy to the whole brain. At different time points, up to 36 h, tissues were fixed, embedded in paraffin and sectioned at the level of the optic chiasm. Apoptosis was assessed morphologically using H&E-stained sections, or using TUNEL-stained sections, counterstained with DAPI.

The non-irradiated subependymal region of adult p53 +/- animals (Figure 2.1I) showed a low incidence of spontaneous apoptosis (0.33 ± 0.02%). Unlike the scattered distribution of apoptotic cells within the irradiated spinal cord, clusters of apoptotic cells were often observed in the irradiated subependymal region (Figure 2.1J). There was no evidence of apoptosis in the ependymal cells. As in the irradiated spinal cord, apoptotic subependymal cells also possessed the biochemical hallmarks of apoptosis, as demonstrated by positive TUNEL reactivity (Figure 2.1K and L). Peak AI was reached at 4 h after 2 Gy (11.5 ± 1.0%), p < 0.05 compared to non-irradiated animals. By 24 h, AI decreased to baseline levels (Figure 2.4).

2.4.3 Absence of XRT-induced apoptosis in p53 -/- CNS

To examine the role of p53 in XRT-induced apoptosis in the adult CNS, the apoptotic response in the brain and spinal cord of adult mice heterozygous (+/-) and null (-/-) for the p53 gene were compared to that in the wild type animals. In p53 -/- spinal cord, there was no evidence of XRT-induced apoptosis. Values for AI at the different
Figure 2.4 The time course of XRT-induced apoptosis in the subependymal region of adult p53+/+ or +/- mice after a single dose of 2 Gy was assessed using morphological criteria with H&E staining at 0, 4, 16, 24 and 36 h after irradiation. At some time points (4 and 16 h after 2 Gy), apoptosis was also assessed in the subependymal regions of p53 +/- mice. Three animals each were used at each time point, except at 24 h in the p53 +/+ mice, 36 h in the p53 -/-, and 4 and 16 h in the p53 +/- mice, where 2 animals each were used. Vertical bars are SEM.
time points after XRT were not significantly different compared to the corresponding AI in the non-irradiated p53 +/+ animals (Figure 2.2).

Although there was a suggestion of an intermediate response in the spinal cord of p53 +/- animals, AI values for the different time points after XRT did not rise significantly above the AI observed for the non-irradiated p53 +/+ animals (Figure 2.2).

There was also no evidence of XRT-induced apoptosis in the subependymal region of adult p53 +/- animals (Figure 2.4). The apoptotic response in the p53 +/- animals at 4 h after 2 Gy was not significantly different from the apoptotic response in the p53 +/- animals, but was significantly different from the AI found in the wildtype animals. At 16 h after 2 Gy, the AI in p53 +/- animals was significantly different from that of the p53 +/- animals, but not significantly different from that of p53 +/+ animals. Thus, the apoptotic response in p53 +/- animals appeared to be an intermediate-delayed response compared to that found in p53 +/+ and +/- animals.

2.4.4. p53 immunohistochemistry

To determine if the p53 protein is upregulated in the adult CNS following XRT, spinal cord and brain sections from the irradiated and non-irradiated wildtype animals were processed for p53 immunohistochemistry. In the non-irradiated spinal cord, very few glial cells of the spinal cord demonstrated p53 immunoreactivity (Figure 2.5A, B and C). However, by 4 h following a single dose of 22 Gy to the spinal cord, there was a significant increase in the percent glial cells that demonstrated nuclear immunostaining for p53 (Table 2.1, Figure 2.5D). Glial cells in both white (Figure 2.5E) and gray matter
Figure 2.5 Upregulation of p53 protein after XRT in the CNS of p53 +/+ animals by immunohistochemistry. There are virtually no p53 immunopositive cells in the non-irradiated spinal cord (A. original magnifications X 100; B. white matter, X 1000; C. gray matter, X 1000). At 4 h after a single dose of 22 Gy, many more p53 positive cells are observed (D. original magnifications X 100; E. white matter, X 1000; F. gray matter, X 1000). Some p53 positive cells appear to be glial cells (E), while others have morphological characteristics of neurons (arrows, F). The non-irradiated brain shows p53 immunoreactivity in ependymal cells (G. original magnification X 250), but no evidence of p53 immunoreactivity in the subependymal region (H. original magnification X 1000). At 4 h after 2 Gy, many p53 positive cells are seen in the subependymal region (I. original magnifications X 250; J. X 1000). Cells of the choroid plexus are p53 positive before and after irradiation (arrow, I). p53 positive cells are intensely stained with a red-brown chromogen. V. ventricle.
Table 1: Percent nuclei in subependyma and spinal cord showing p53 immunoreactivity after XRT

<table>
<thead>
<tr>
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<th>Mean ± SEM (%)</th>
<th>Apoptosis (%)</th>
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<tr>
<td><strong>Subependyma</strong></td>
<td></td>
<td></td>
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<tr>
<td>Non-irradiated</td>
<td>0.23 ± 0.13</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>4 h after 2 Gy</td>
<td>15.7 ± 2.8</td>
<td>11.46 ± 0.97</td>
</tr>
<tr>
<td><strong>Spinal Cord</strong></td>
<td></td>
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<tr>
<td>Non-irradiated</td>
<td>0.07 ± 0.14</td>
<td>0.024 ± 0.009</td>
</tr>
<tr>
<td>4 h after 22 Gy</td>
<td>21.2 ± 2.5</td>
<td>0.061 ± 0.012</td>
</tr>
<tr>
<td>8 h after 22 Gy</td>
<td>12.9 ± 2.4</td>
<td>0.44 ± 0.08</td>
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(Figure 2.5F) showed nuclear p53 immunoreactivity. In gray matter, there were also some neurons that demonstrated p53 immunoreactivity after XRT (Figure 2.5F).

In the non-irradiated brain, cells in the choroid plexus showed moderate cytoplasmic p53 immunoreactivity, which was non-specific, while the ependymal cells surrounding the ventricles were weakly p53 positive (Figure 2.5G). Subependymal cells did not demonstrate any p53 immunoreactivity (Table 2.1, Figure 2.5H). At 4 h after 2 Gy, there was an increase in the percentage of p53 immunopositive cells in the subependymal region (Table 2.1, Figure 2.5I and J). Most of the p53 immunoreactivity appeared to be located in the nucleus of subependymal cells (Figure 2.5J). In the choroid plexus, the intensity and subcellular localization of p53 staining remained unchanged following XRT (Figure 2.5J).

Sections of the irradiated small bowel demonstrated p53 immunoreactivity only in the crypt cells, as previously reported (Merritt et al., 1994), indicating specific staining for p53. Omission of the p53 primary antibody resulted in no specific staining of the secondary antibody. p53 immunostaining on sections from p53 -/- animals demonstrated no staining in the spinal cord, and only the choroid plexus was stained in the brain sections, suggesting that p53 staining in the choroid plexus is non-specific.

2.4.5 Oligodendrocyte cell density

Since the number of apoptotic oligodendrocytes returned to baseline by 24 h after XRT, oligodendrocyte density was quantified before and at 24 h after XRT to determine if XRT-induced apoptosis is associated with a depletion of the oligodendrocyte population in the spinal cord. Oligodendrocytes were identified using in situ
hybridization for mRNA of a major myelin-associated protein (Figure 2.6). There was a significant ($p = 0.023$) decrease in the density of plp mRNA positive cells in gray matter at 24 h after 22 Gy, but the decrease in white matter was not significant ($p = 0.17$, Table 2.2) compared to non-irradiated age-matched controls.

### 2.5 Discussion

In this study, we used p53 +/+ , +/− and −/− mice to investigate the role of the p53 tumour suppressor gene in XRT-induced apoptosis in the adult CNS. An apoptotic response was observed in oligodendrocytes and subependymal cells in the adult mouse CNS similar to that reported previously in the adult rat CNS (Bellinzona et al., 1996; Li et al., 1996a; Li et al., 1996b; Shinohara et al., 1997). The lack of an apoptotic response in p53 −/− mice following XRT suggests that p53 is required for XRT-induced apoptosis in the adult mouse CNS. To our knowledge, this is the first study that describes the involvement of p53 in XRT-induced apoptosis in the adult CNS in vivo.

The time course of XRT-induced apoptosis in the spinal cord and the subependymal region of p53 +/+ mice was similar to results previously observed in the adult rat CNS (Li et al., 1996a; Li et al., 1996b; Shinohara et al., 1997). The peak apoptotic response in the subependymal region of wild-type mice was earlier than that observed in the rat, and the peak AI appeared lower in the mouse compared to the rat. These differences could be explained by species-specific responses to XRT, as well as differences in the definition of the subependymal region. In the present study, a dose-response for XRT-induced apoptosis in the mouse spinal cord was observed. This was again similar to the results reported previously in the rat spinal cord (Li et al., 1996b).
Figure 2.6 In situ hybridization for plp mRNA was performed on spinal cord sections from non-irradiated and irradiated (24 h after 22 Gy) p53 +/- animals in order to determine if oligodendrocyte cell density changed following XRT. GM, gray matter; WM, white matter. Original magnification x 1000.
<table>
<thead>
<tr>
<th></th>
<th>0.023</th>
<th>0.17</th>
<th>0.065</th>
<th>0.22</th>
<th>0.85</th>
<th>0.018</th>
<th>0.54</th>
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<tr>
<td>24 h - 22 Gy</td>
<td>11.8±1.7</td>
<td>5.9±0.2</td>
<td>7.9±0.2</td>
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<td></td>
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<tr>
<td>unXRT</td>
<td>13.1±1.3</td>
<td>8.0±0.3</td>
<td>8.0±0.8</td>
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<tbody>
<tr>
<td>Dorsal</td>
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<tr>
<td>Lateral</td>
<td></td>
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<tr>
<td>Ventral</td>
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Number of oligodenrocyes / 0.106 mm²

Hybridization

Table 2: Oligodenrocye density change in adult mouse spinal cord after XRT using pip in situ
Immunohistochemistry results using Leu-7, GC and GFAP were in agreement with those in our rat spinal cord studies (Li et al., 1996b), suggesting that the apoptotic cells observed after XRT in the mouse spinal cord are likely to be mature oligodendrocytes. Differentiated oligodendrocytes cultured from the neonatal rat brain have also been shown to undergo XRT-induced apoptosis (Vrdoljak et al., 1992).

In a previous study (Li et al., 1996b), we showed that even a very low AI in the rat spinal cord was associated with a significant decrease in oligodendrocyte density at 24 h after XRT. In the present study, apoptosis was associated with a decrease in oligodendrocyte density. The lack of statistical significance for the decrease in white matter in the present study could simply be due to the smaller number of oligodendrocytes in the mouse sections compared to that in the rat.

In the developing mouse CNS, upregulation of p53 coincided with apoptosis after XRT (Herzog et al., 1998). In E17-18 rat brain, upregulation of p53 protein was reported at 1-3 h after 4 Gy (Borovitskaya et al., 1996). To assess if XRT-induced apoptosis in the adult mouse CNS is associated with p53 protein upregulation, we performed p53 immunohistochemistry on irradiated and non-irradiated spinal cord and brain sections. We observed a significant increase in p53 expression in glial cells and subependymal cells following XRT. Consistent with the dependence of XRT-induced apoptosis on p53, p53 accumulation was observed at 4 h in the spinal cord, preceding the peak AI observed at 8 h after XRT. A similar pattern of increased p53 expression prior to apoptosis has been observed in intestinal crypt cells (Merritt et al., 1994) and thymocytes following XRT (Clarke et al., 1993; Lowe et al., 1993).
In the spinal cord, we have not identified the cell type(s) that showed p53 upregulation, but oligodendrocytes are candidate cells. Cultured oligodendrocytes demonstrated nuclear accumulation of p53 after an apoptosis-inducing treatment of dimerized IL-2 (Eizenberg et al., 1995). Translocation of p53 from the cytosolic to the nuclear compartment was observed recently in oligodendroglia-like cells following hydrogen peroxide treatment (Uberti et al., 1999). Accumulation of p53 is associated with transcriptional activation of pro-apoptotic genes downstream of p53, such as insulin-like growth factor binding protein-3 and bax (Buckbinder et al., 1995; Miyashita and Reed, 1995). Accumulation of p53 triggered by DNA strand breaks can be induced by a number of agents, including XRT. Free radicals have been implicated as the causative agent in p53 upregulation and subsequent apoptosis of cerebellar neurons following XRT (Wood and Youle, 1995). The present results are thus consistent with the mechanism, whereby DNA damage, free radical production and p53 nuclear accumulation lead to apoptosis in oligodendrocytes and subependymal cells following XRT.

A very low level of apoptosis was observed in the non-irradiated p53 -/- mouse CNS, implying that the mechanism of spontaneous apoptosis may be p53-independent. A low physiological incidence of p53-independent apoptosis has also been observed in neurons (Wood and Youle, 1995) and thymocytes (Clarke et al., 1993; Lowe et al., 1993). A low level of p53-independent apoptosis is consistent with the general absence of developmental abnormalities in the CNS of p53 -/- mice (Donehower et al., 1992), although an increased incidence of neural tube defects was reported in p53 -/- mice (Sah et al., 1995).
In thymocytes and intestinal crypt cells, the apoptotic response following XRT in p53 +/- mice was intermediate to that found in p53 +/- and +/- mice (Lowe et al., 1993; Merritt et al., 1994). In our study, mice heterozygous for p53 showed a delayed intermediate apoptotic response in the subependymal region, implying that there is a gene-dose effect of p53. There was no definite evidence for an intermediate response in the spinal cords of p53 +/- mice. However, the low AI observed may make it difficult to distinguish an intermediate response in the p53 +/- spinal cord.

The mechanism by which cells of the developing and adult CNS undergo apoptosis is likely to depend on the nature of the apoptosis-inducing stimulus as well as the cell type. Oligodendrocytes in vitro undergo p53-dependent apoptosis following IL-2 (Eizenberg et al., 1995). Post-mitotic neurons cultured from neonatal mouse brain undergo p53-dependent apoptosis following XRT (Enokido et al., 1996). However, other pathways of stress-induced apoptosis do exist in the CNS. Activation of c-Jun N-terminal kinase in primary cultures of rat astrocytes, oligodendrocytes and an oligodendrocyte progenitor cell line, CG4, in response to cytokines and other stress inducers has been described (Zhang et al., 1996). Oligodendrocytes also undergo apoptotic death in response to ceramide treatment (Casaccia-Bonnefil et al., 1996a; Casaccia-Bonnefil et al., 1996b; Larocca et al., 1997). Thus, oligodendrocytes and neurons die of apoptosis after a variety of stress-induced stimuli and insults. The role of p53 in these apoptotic pathways has remained uncertain. Although p53 may play a role in some apoptotic pathways, clearly other mechanisms of apoptosis exist in the CNS.

XRT-induced apoptosis in the developing CNS has been shown to be p53-dependent. Postnatal cerebellar granule neurons were described to undergo p53-
dependent XRT-induced apoptosis \textit{in vivo} (Wood and Youle, 1995). Cerebellar neurons cultured from postnatal p53 \textit{\textminus/-} mice were also resistant to XRT-induced apoptosis (Enokido \textit{et al}., 1996). In this study, we showed that XRT induced apoptosis in oligodendrocytes and subependymal cells in the adult CNS, and that this process was p53-dependent. Oligodendrocytes are post-mitotic cells, whereas subependymal cells are proliferating cells in the adult CNS (Shinohara \textit{et al}., 1997). Taken together, these results suggest that apoptosis in both proliferating and post-mitotic cells in the CNS induced by XRT damage is mediated by the p53 pathway.
2.6 References


Chapter 3

The Association Between p53 Subcellular Localization and XRT-Induced Apoptosis in Astrocytes

Preliminary Work In Vitro
3.1 Abstract

Oligodendrocytes, a major cell type in the central nervous system (CNS), have been shown to undergo radiation (XRT)-induced apoptosis in vitro and in vivo. Other central nervous system (CNS) cells, such as astrocytes, demonstrate a resistance to XRT-induced apoptosis. We have recently shown that XRT-induced apoptosis in oligodendrocytes and subependymal cells requires the tumour suppressor gene p53. p53 is a transcription factor that activates genes which promote cell cycle arrest or apoptosis, depending on the cell type and severity of DNA damage. In some cell types, p53 is found in the cytoplasm in a latent form, and upon activation, translocates to the nucleus. We hypothesize that resistance to XRT-induced apoptosis is associated with cytoplasmic localization of p53, and that susceptibility to XRT-induced apoptosis requires nuclear translocation of p53. Here, we established primary cultures of astrocytes and oligodendrocytes to determine the subcellular localization of p53 following XRT. We also developed an objective method to assess p53 intensity and its subcellular localization, using confocal microscopy. Preliminary results suggest that resistance to XRT-induced apoptosis in astrocytes is associated with no apparent subcellular shift of p53 protein from the cytoplasmic to the nuclear compartment after XRT.

3.2 Introduction

We have previously demonstrated that certain cells in the CNS undergo XRT-induced apoptosis in vivo (Li et al., 1996b; Li et al., 1996b). The apoptotic cells were identified as oligodendrocytes (Li et al., 1996b; Li et al., 1996b) and subependymal cells (Chapter 2). Although these CNS cell types in p53 +/- mice underwent XRT-induced
apoptosis, there was a resistance to XRT-induced apoptosis in the CNS of p53 -/- mice, (Chapter 2). Furthermore, immunohistochemical techniques demonstrated an increase in nuclear expression of p53 in irradiated CNS tissues compared with non-irradiated tissues (Chapter 2). These results suggest that XRT-induced apoptosis in oligodendrocyte and subependymal cells of the adult CNS is mediated by p53. Consistent with a previous report, our results (Chapter 2) showed that another glial cell type, the astrocyte, was resistant to XRT-induced apoptosis (Gobbel et al., 1998).

3.2.1 p53 Subcellular Localization

The p53 tumour supressor gene acts in the nucleus as a transcription factor. The mechanism by which p53 exerts its actions is unclear. Some studies have indicated that its function may be regulated through its subcellular localization (Martinez et al., 1997a; Martinez et al., 1997b; Ostermeyer et al., 1996; Takahashi et al., 1993). Subcellular localization should be distinguished from p53 nuclear accumulation, which has also been reported in intestinal crypt cells and thymocytes, as well as glial cells (Clarke et al., 1993; Lowe et al., 1993; Merritt et al., 1994, Chapter 2). Subcellular localization is the translocation of p53 protein from one cellular compartment to another. An accumulation of p53, however, is the increase in p53 protein in an intracellular compartment, which may or may not result from a translocation of protein from a different subcellular structure. Similarly, the detection of a subcellular shift of localization of p53 may also be accompanied by the accumulation of protein.

When DNA damage occurs, p53 may transcriptionally activate pro-apoptotic genes, such as bax (Miyashita and Reed, 1995) and IGF-BP3 (Buckbinder et al., 1995),
or growth arrest genes, such as p21 (Chin et al., 1997; Kim, 1997). In some cell types, p53 is found in the cytoplasm of the cell in a latent form. Upon activation, p53 translocates to the nucleus (Martinez et al., 1997b). It is suggested that when p53 is sequestered in the cytoplasm its function is inactivated (Moll et al., 1996). In a recent study, it was proposed that p53 may be retained by an intermediate filament network (Klotzsche et al., 1998). Through an as yet unknown mechanism, p53 may then be transported to the nucleus. p53 possesses a nuclear localization signal in its carboxy terminus, which may signal the protein to become targeted to the nucleus upon specific stimulation (Shaulsky et al., 1991; Ostermeyer et al., 1996).

In support of the hypothesis that p53 is regulated through its subcellular localization, glioma cell lines that normally expressed cytoplasmic p53 demonstrated a shift in subcellular localization of p53 protein to the nucleus following XRT. This process could be blocked by the free radical scavenger WR1065, suggesting that nuclear accumulation of p53 is mediated by free radicals (Martinez et al., 1997b). Similarly, cytoplasmic sequestration of p53 protein has been reported to lead to p53 inactivation (Takahashi and Suzuki, 1994). Although in many instances nuclear accumulation of p53 precedes p53 activation, most notably in breast cancer (Suzuki et al., 1992; Takahashi and Suzuki, 1993) or neuroblastoma cells (Isaacs et al., 1998; Martinez et al., 1997b; Ostermeyer et al., 1996) in vitro, p53 nuclear translocation does not always correspond to p53 activation. For example, the breast cancer cell line, MCF-7, underwent p53-dependent apoptosis without demonstrating p53 nuclear accumulation (Amellem et al., 1997).
In Chapter 2, we observed p53 nuclear accumulation following XRT in the adult mouse CNS in vivo. It is currently unclear which cell type(s) demonstrate increased nuclear p53 expression following XRT. The initial in vitro studies proposed in this chapter will extend these in vivo findings by determining which glial cell type undergoes XRT-induced apoptosis in vitro, and if this apoptosis is associated with an accumulation of p53, a subcellular shift in p53 or both. To accomplish these goals, we first established primary cultures of oligodendrocytes and astrocytes, and developed a method to quantify p53 intensity in different subcellular compartments. This latter technique involved the use of the confocal microscope.

3.2.2 Confocal Microscopy

Epifluorescence or immunohistochemistry can be used to discern a shift in subcellular localization of proteins and macromolecules (Eizenberg et al., 1995; Uberti et al., 1999). However, criteria for such a shift is often based upon subjective measures, which may vary from study to study. A quantitative measure of the intensity of staining within different intracellular compartments would contribute greatly to the analysis and interpretation of data. One way in which objective analysis and quantification of the intensity of staining can be achieved is through image analysis of a digitized image of the stained cells.

Confocal microscopy produces sharper images with better spatial resolution compared with epifluorescent microscopy, and the use of a confocal microscope is particularly well-suited for studies requiring quantitative analysis. The main difference between confocal microscopy and epifluorescent microscopy is in the detection of the
image. In epifluorescent microscopy, the fluorochromes over the entire volume of the cell are excited and all the emitted light is detected simultaneously by the viewer. Emitted light from out-of-focus points of the cell can then contribute to the final image, leading to a blurry representation (Figure 3.1A). In confocal microscopy, a pinhole placed in front of the detector allows only light from one focal plane to form an image, leading to a much crisper image (Figure 3.1B). However, much information about the pattern of staining can be lost because the intensity of staining may vary from one focal plane to another. To solve this problem, a series of images at different depths of the cell can be taken, and a three-dimensional (3-D) composite of the cell can be constructed (see section 3.3.6).

3.2.3 Hypothesis

We postulate that the susceptibility of CNS cells to undergo XRT-induced apoptosis is related to the subcellular localization of p53 protein, and that radioresistant cells, such as astrocytes, will demonstrate cytoplasmic retention of p53 following XRT. To test this hypothesis, astrocytes and oligodendrocytes were established in vitro from neonatal rat brain. Indirect immunofluorescence was performed to detect p53 at different time points following XRT. Slides were imaged using an epifluorescent microscope and a scanning laser confocal microscope. A method to quantify the subcellular shift in localization of p53 protein was established using confocal microscope images. This technique will also determine if there is an accumulation of p53 protein following XRT. Preliminary results suggest the absence of a subcellular shift of p53 protein in cultured astrocytes following XRT.
Figure 3.1 A comparison of epifluorescent and confocal microscopy. In conventional epifluorescent microscopy (A), a light source is directed to the sample by a dichroic mirror. A lens focuses the light on the sample, and all of the emitted light is passed through the dichroic mirror and through an emission filter before reaching the image plane. Note that unfocused light (dashed lines) contributes to the final image, decreasing the spatial resolution. In confocal microscopy (B), a scanning laser light source can excite discrete volumes of the sample, and after passage through an emission filter, the light is passed through a small pinhole, so that only light focused at the image plane is directed to the detector, leading to a final image with improved spatial resolution.
3.3 Materials and Methods

3.3.1 Animals

Neonatal Fisher rats (Jackson Laboratories) were used in this study. A maximum of 1 pregnant animal was housed per cage, with food and water freely available, and with lighting between 6 AM and 6 PM in the animal colony of the Ontario Cancer Institute, an animal colony accredited by the Canadian Council of Animal Care. Postnatal pups aged 1 - 3 days old were used to establish primary glial cultures.

3.3.2 Primary Cell Cultures of Oligodendrocytes and Astrocytes

A number of different techniques were used to establish primary cell cultures of oligodendrocytes from the neonatal rat brain (see Appendix 1). The glial co-culture and "shaking" technique of McCarthy and de Vellis (McCarthy and de Vellis, 1980), described below, proved to be the most successful.

Primary cell cultures of astrocytes and oligodendrocytes were prepared from neonatal rat cerebral hemispheres in a total of 28 separate experiments. Briefly, postnatal rats, aged 1 - 3 days, were decapitated and the cerebral cortex was removed and dissected free of meningeal material. Twenty-five to thirty cortexes were pooled together for one experiment. The tissue was chopped with a scalpel and the pieces were placed in a solution of 15% FBS-DMEM. The tissue was titurated with a 10 ml glass pipet until tissue had been further broken down. After tissue dissociation, the entire suspension was centrifuged at 700 rpm for 5 minutes. The cell pellet was resuspended in 15% FBS-DMEM and the cells were seeded into 8 to 12 T-75 culture flasks. The media was changed on day three, and replaced with 10% FBS-DMEM. On day 5, a monolayer of
astrocytes formed, and by day 7 or 8, phase-bright cells grew on top of the astrocyte monolayer. From these glial cell co-cultures, astrocytes, oligodendrocytes or O-2A cells (Appendix 1) could be isolated.

Isolation of astrocytes and oligodendrocytes from astrocyte-oligodendroglial co-culture

Following the stratification of oligodendrocytes on top of astrocytes, the flasks were shaken for 1 h on an orbital shaker at 200 rpm at 37°C to remove microglia cells. The media was removed and discarded, and fresh 10% FBS-DMEM was added and the flasks were returned to the shaker for 17 - 18 h at 200 rpm and 37°C. The media, containing the cells of the oligodendroglial lineage, was removed, filtered through a 20 μm Nitex mesh (B & SH Thompson & Co. Ltd.), and centrifuged at 1000 rpm for 5 min. This non-adherent cell population was not composed purely of oligodendrocytes, and required further enrichment (see below). Astrocytes, the remaining adherent cells, were pure enough to be used without further enrichment. Astrocytes were trypsinized and seeded in 4-well chamber slides (Lab-Tek) at approximately 5x10^4 cells per well. Astrocytes were fed 10% FBS-DMEM every other day and were used before 5 days in vitro.

Enrichment of oligodendrocytes from an isolated heterogenous cell population

After shaking overnight on an orbital shaker, the media, containing cells of the oligodendroglial lineage, was removed and filtered through a 20 μm Nitex mesh and centrifuged at 1000 rpm for 5 minutes. The filtered cells were resuspended in 10% FBS-DMEM and seeded into 1 or 2 T-75 culture flasks for 1 - 2 hours. In this differential
adherence step, the more adherent astrocytes will adhere to the flasks within this time interval. At the end of this step, the less adherent oligodendrocytes were poured off, centrifuged at 1000 rpm for 5 min, and then resuspended in chemically defined media (Bottenstein and Sato, 1979). Our media formulation consisted of: 50 μg/ml transferrin, 100 μg/ml bovine serum albumin, 60 ng/ml progesterone, 5 ng/ml sodium selenite, 10 ng/ml tri-iodo thyronine, 5 μg/ml insulin, 60 μg/ml N-acetyl choline, 2 mg/ml glucose, 6.5 ng/ml biotin, 2.5 mM glutamine, made up in 1:1 Ham's F12 and DMEM. Oligodendrocytes were seeded into 4-well chamber slides (Lab-Tek) that had been precoated with poly-D-lysine (Sigma, 5 μg/ml) at a density of approximately $5 \times 10^4$ cells per well. In these experiments, oligodendrocytes were used at 1 or 2 days after seeding.

3.3.3 Irradiation

Cells, plated in 4-well chamber slides, were irradiated with a single dose of 10 Gy using a Gamma Cell 40 Exactor machine (Nordion), at a dose rate of 1.07 Gy/min. Cells were fixed and stained (see below) at various time points up to 24 h after XRT.

3.3.4 Immunostaining

For oligodendrocytes

Cells were washed twice with PBS at the appropriate interval after irradiation and fixed with 4% paraformaldehyde for 15 min. Cells were then sequentially incubated in mouse anti-galactocerebroside IgG (Boehringer Mannheim, 10 μg/ml) for 60 min, and Texas-Red goat anti-mouse IgG (1:50) for 30 min. Cells were counterstained with 4,6 diamidino-2-phenylindole (DAPI; 0.1 ng /ml; Sigma) for 10 min, and mounted with the
fluorescent mounting media, Vectashield (Vector). A glass coverslip was placed on top of each slide.

**For astrocytes**

The cells were washed, permeabilized with 100% ice-cold methanol for 5 min at -20°C, then with 100% ice-cold acetone for 2 min at 4°C. Following permeabilization, cells were incubated with rabbit anti-p53 IgG antibody (Santa Cruz, FL-393, 1:30) at 37°C for 30 min, FITC goat anti-rabbit IgG (BioKan, 150) for 30 min, polyclonal rabbit anti-GFAP IgG antibody (DAKO, 1:100) for 30 min and Texas Red goat anti-rabbit IgG antibody (BioKan, 1:100) for 30 min. DAPI staining proceeded as described above for oligodendrocytes.

### 3.3.5 Assessment of Apoptosis

Apoptosis in primary cell cultures of astrocytes and oligodendrocytes was assessed morphologically using the fluorescent nuclear stain, DAPI. Criteria for apoptosis included nuclear condensation or nuclear fragmentation. Cultured astrocytes were assessed for apoptosis before and at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, and 24 h after a single dose of 10 Gy. Cultured oligodendrocytes were assessed for apoptosis before and at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10 and 24 h after a single dose of 15 Gy. The time course of these experiments for astrocytes and oligodendrocytes were scheduled in such a way that cells were irradiated at various times and fixed and stained at the same time, thus reducing the experimental variability in the staining procedure. One report observed a decrease in cell viability with increasing time in culture for oligodendrocytes (Vrdoljak et
al., 1992). To avoid this problem, non-irradiated control astrocytes and oligodendrocytes were kept in culture the entire time course of the experiment (24 h) and fixed and stained at the same time as the irradiated cells. Thus, any differences between the AI of irradiated and non-irradiated cells can be attributed to XRT treatment alone, and are not due to differences in the amount of time cells have been in culture. Between 162 - 534 nuclei were counted in the astrocyte cultures, and 392 - 749 nuclei were counted for the oligodendrocyte cultures, except at 24 h after 15 Gy. Only 49 nuclei were counted at this time point, as there was a very low cell density, presumably due to detachment of cells from the slide.

3.3.6 Confocal Microscopy

For the purposes of this study, images were acquired in the following manner. Three arbitrary fields of view, each consisting of approximately 20 cells, were imaged. Each 3-D field of view consisted of about 10 two-dimensional (2-D) images, taken at 1 μm intervals (Figure 3.2). A single 2-D image actually consisted of three superimposed component images. If the 2-D image were to be resolved into its three components, each one would consist of cells stained with one particular marker only. For example, one could view the same 2-D image for the cells stained for p53-FITC alone, GFAP-Texas Red alone, or DAPI alone (Figure 3.2). The separation of each marker made the quantitation and subcellular localization of p53 expression possible. Slides were imaged with a Zeiss 510 Laser Scanning Confocal Microscope within a week of staining. For comparison, some slides were also imaged using epifluorescent microscopy.
Figure 3.2 Technique for determining subcellular p53 intensity by confocal microscopy. A monolayer of about 20 astrocytes comprises one 3-D field of view (A). Each 3-D field of view is constructed from 2-D images, from 10 different focal planes imaged at 1 μm intervals (B). Considered individually, each 2-D image can be deconstructed into its three component parts, which represent three different fluorescent stains conjugated to different primary antibodies (C). The red fluorescent stain (GFAP conjugated to Texas Red) is representative of the cytoplasm of the astrocyte. The blue fluorescent stain (DAPI) specifically stains DNA. The green fluorescent stain (p53 conjugated to FITC) describes the subcellular localization of the p53 protein.
3.3.7 Image Analysis and Quantification of p53 Intensity

In order to quantify the extent of p53 nuclear translocation in astrocytes, it was necessary to accurately distinguish between the cytoplasmic and nuclear compartments. The nuclear compartment could easily be defined by the region stained by the DNA-specific stain, DAPI. GFAP is a type of intermediate filament which is found only in astrocytes, and this marker was used to define the dimensions of the cytosol.

Using the Microcomputer Imaging Device (MCID, v. 2) analysis system, each 2-D image was separated into its three different components: p53-FITC, GFAP-Texas Red and DAPI (Figure 3.3). The general strategy for quantitation of p53 expression was to use the DAPI and GFAP-Texas Red image components in order to define the boundaries of the nucleus and the cytoplasm, respectively. In the DAPI image component, each nucleus was demarcated by setting a “nuclear region” over the area of DAPI staining (Figure 3.4). Similarly, in the GFAP-Texas Red image component, a “cytoplasmic region” was drawn over the areas of GFAP immunoreactivity (Figure 3.4). The component images were linked in such a way that the areas of the nuclear regions, which were drawn first, were mutually exclusive from the cytoplasmic regions. Once the regions were drawn, the intensity of p53 staining in the third component image, the p53-FITC image, was calculated, using the MCID software, based on the mean optical intensity in the cytoplasmic and nuclear regions of the image. This calculation involved taking the intensity from each pixel of the region, and deriving a mean for each region. This mean was normalized to the area of each nuclear or cytoplasmic region. Since p53 intensity varied from 2-D image to 2-D image from within one field of view, it was
Figure 3.3 Image analysis of p53 subcellular localization. (A) A 2-D image of a monolayer of cultured astrocytes, imaged at a 1 μm-thick focal plane, is a composite of three separate stains: GFAP (conjugated to Texas Red), p53 (conjugated to FITC) and DAPI (blue fluorescent stain). When separated into its component parts, the 2-D image is composed of individual stains for the nucleus (DAPI, B), the cytoplasm (GFAP, C) and p53 (D).
Figure 3.4 p53 subcellular localization is quantified using specific cytoplasmic and nuclear markers. Once a 2-D image is separated into its component parts, the nuclear stain, DAPI, can be used to demarcate different nuclear regions (yellow lines, A). These nuclear regions are recapitulated on the p53 component (B), and the calculation of p53 intensity is made only within the outlined nuclear regions in the p53 component. Similarly, specific cytoplasmic regions are defined in the GFAP component (white lines, C) and these regions are re-drawn in the p53 component (D). Because the nuclear regions have been marked prior to the cytoplasmic regions, the nuclear and cytoplasmic regions are mutually exclusive. The p53 intensity is then calculated in only the defined cytoplasmic regions.
necessary to combine data from all the 2-D images from one field of view to accurately assess p53 intensity in the volume of the cell (Figure 3.5).

The analysis of p53 expression could not be calculated at the level of the individual cell, since astrocytes grow in small colonies. Although individual nuclei were distinct, the cytoplasm of one astrocyte could not be distinguished from the cytoplasm of a second astrocyte. Thus it was necessary to analyze p53 expression at the level of fields of view.

3.3.8 Statistics

To determine if p53 subcellular localization varied significantly between any of the time points, data was analyzed using the Kruskal-Wallis test, the non-parametric equivalent to a one-way ANOVA. Differences between specific time points were determined using Dunn’s test, a non-parametric method for detecting significant differences between pairs of data.

3.4 Results

3.4.1 Time Course of XRT-induced Apoptosis

In cultured non-irradiated oligodendrocytes, the apoptotic incidence (AI) was low. The number of apoptotic cells rose sharply at 4 h after 15 Gy and remained elevated at 24 h (Figure 3.6). This was consistent with previous results (Vrdoljak et al., 1992). In contrast, cultured astrocytes showed resistance to XRT-induced apoptosis (Figure 3.6). The AI did not rise above baseline levels throughout the 24 h time interval after 10 Gy. This was again in agreement with results reported previously (Gobbel et al., 1998).
Figure 3.5 Differential p53 expression at a single focal plane. A schematic diagram of a monolayer of astrocytes demonstrates how p53 expression (shaded areas) can vary at different focal planes, and how one focal plane is not representative of the total p53 expression within a cell volume. Differences in the way the cell sits on the slide, the shape of the cell, and the p53 expression within each individual cell can affect the calculated p53 intensity within each 2-D image. It is therefore necessary to consider the several 2-D images from a field of view to accurately assess p53 expression.
Figure 3.6  Time course of XRT-induced apoptosis in cultured oligodendrocytes and astrocytes. Between 162–534 astrocytes and 392–749 oligodendrocytes were counted at each time point in a single experiment. At 24 h after 15 Gy in the oligodendrocytes, 49 cells were counted. Apoptosis was assessed using morphological characteristics in DAPI-stained cells.
3.4.2 Epifluorescent vs Confocal Microscopy

Using the epifluorescent microscope, it was difficult to discern if the intensity of p53 expression was stronger in the nuclear or cytoplasmic compartments (Figure 3.7). During the 24 h time course, there appeared to be a stronger expression of p53 in the nucleus, but because the criteria for determining the intensity of p53 expression was subjective, this analysis may have been flawed. As a result, we established a method to quantify p53 expression in the nuclear and cytoplasmic compartments, as described in section 3.3.6 and 3.3.7.

3.4.3 Scoring Criteria for p53 Subcellular Localization

In this study, p53 subcellular localization was determined before XRT and 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, and 24 h following a single dose of 10 Gy. Subcellular localization was expressed as the ratio of intensity of nuclear to cytoplasmic stain, normalized to nuclear and cytoplasmic area.

3.4.4 Determination of p53 Subcellular Localization Using Confocal Microscopy

In non-irradiated rat astrocyte cultures, the intensity of p53 expression was slightly higher in the cytoplasm (Table 1). Following XRT, the p53 intensity there was no accumulation of p53 protein in either the nuclear or the cytoplasmic regions. In fact, the p53 intensity appeared to decrease slightly (Table 1). The ratio of nuclear:cytoplasmic p53 intensity in the non-irradiated astrocyte cultures was nearly equal, with a slightly higher intensity in the cytoplasm. Throughout most of the 24 h time course, the ratio of p53 intensity in the nuclear:cytoplasmic compartments remained close to 1. Only at 2 h after 10 Gy was the distribution of p53 protein significantly higher in
Figure 3.7 Astrocytes visualized under epifluorescent microscopy. Astrocytes stained with p53-FITC (green fluorescence, A) were visualized under the epifluorescent microscope. Although these cells were counterstained with the nuclear stain, DAPI (blue fluorescence, B), it was difficult to distinguish between nuclear and cytoplasmic regions and to discern differences in p53 subcellular localization.
<table>
<thead>
<tr>
<th>Time after XRT</th>
<th>Nuclear Intensity</th>
<th>Cytoplastic Intensity</th>
<th>Nuclear Intensity: Cytoplastic Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00±0.09</td>
<td>2.4±0.2</td>
<td>8.2±0.8</td>
<td>10 Gy</td>
</tr>
<tr>
<td>0.89±0.33</td>
<td>1.9±0.2</td>
<td>8.2±0.8</td>
<td>10 Gy</td>
</tr>
<tr>
<td>0.96±0.35</td>
<td>2.0±0.2</td>
<td>5.4±0.9</td>
<td>10 Gy</td>
</tr>
<tr>
<td>1.00±0.27</td>
<td>2.0±0.2</td>
<td>4.0±0.9</td>
<td>10 Gy</td>
</tr>
<tr>
<td>2.0±0.26</td>
<td>2.1±0.2</td>
<td>3.1±0.9</td>
<td>10 Gy</td>
</tr>
<tr>
<td>3.0±0.25</td>
<td>2.2±0.2</td>
<td>2.0±0.9</td>
<td>10 Gy</td>
</tr>
<tr>
<td>4.0±0.24</td>
<td>3.0±0.2</td>
<td>1.9±0.9</td>
<td>10 Gy</td>
</tr>
<tr>
<td>5.0±0.23</td>
<td>4.0±0.2</td>
<td>1.8±0.9</td>
<td>10 Gy</td>
</tr>
<tr>
<td>6.0±0.22</td>
<td>5.0±0.2</td>
<td>1.7±0.9</td>
<td>10 Gy</td>
</tr>
<tr>
<td>7.0±0.21</td>
<td>6.0±0.2</td>
<td>1.6±0.9</td>
<td>10 Gy</td>
</tr>
<tr>
<td>8.0±0.20</td>
<td>7.0±0.2</td>
<td>1.5±0.9</td>
<td>10 Gy</td>
</tr>
<tr>
<td>9.0±0.20</td>
<td>8.0±0.2</td>
<td>1.4±0.9</td>
<td>10 Gy</td>
</tr>
</tbody>
</table>

**Table I:** Subcellular localization of p53 protein in astrocytes following XRT.
the nucleus (0.05>p>0.01). Each individual cytoplasmic region within a field of view demonstrated a similar range of p53 intensities (Figure 3.8), implying that individual astrocytes have a similar cytoplasmic distribution of p53 from cell to cell. However, individual nuclear regions demonstrated a larger range of p53 intensities (Figure 3.8).

3.5 Discussion

Regulation of the function of the p53 tumour suppressor gene can be controlled at many levels, including the subcellular localization of p53 protein. Nuclear exclusion of p53 has been associated with inactivation of p53 (Martinez et al., 1997a; Takahashi and Suzuki, 1994), while subcellular shift of p53 from the cytoplasm to the nucleus has been shown to correlate with apoptosis in a number of cells, including oligodendrocytes after IL-2 treatment (Eizenberg et al., 1995), an oligodendroglial cell line after H2O2 treatment (Uberti et al., 1999), cells of the mouse testis after heat stress (Yin et al., 1997), and keratinocytes after ultraviolet XRT (Cotton and Spandau, 1997). One recent study suggests that association of the p53 protein to an intermediate filament network by an as yet unidentified protein may lead to p53 cytoplasmic retention and abrogation of p53 activities (Klotzsche et al., 1998).

Here, we have assessed if p53 subcellular localization and p53-dependent apoptosis are associated in primary cell cultures of astrocytes. In agreement with previously published results, astrocytes were resistant to XRT-induced apoptosis (Gobbel et al., 1998). Again, consistent with previous studies, oligodendrocytes were sensitive to XRT-induced apoptosis (Vrdoljak et al., 1992).
Figure 3.8 The range of p53 intensity in individual nuclear and cytoplasmic regions at a single focal plane. The range of p53 intensity/μm² in astrocytes was small in the cytoplasm, and larger in the nucleus. Each data point represents one defined nuclear or cytoplasmic region. This representative range of data was obtained from a 2-D image of astrocytes at 2 h following 10 Gy.
In cultured astrocytes, there was no evidence for accumulation of p53 in either the nuclear or the cytoplasmic compartment following XRT. Similarly, the ratio of nuclear:cytoplasmic p53 intensity remained unchanged for much of the 24 h time course following XRT. Only at 2 h after 10 Gy was a statistically significant shift in subcellular localization of p53 protein observed. However, this did not translate to p53 activation, as inferred by XRT-induced apoptosis. Several interpretations of these results should be considered. First, the range of values for nuclear:cytoplasmic p53 intensity was quite small (0.87 to 1.09; see Table 1). The data was derived from only three fields of view, with each field of view consisting of approximately 20 astrocytes. Thus, data was gathered for at least 60 cells for each time point. Because most astrocytes in vitro grow closely in small colonies, it was impossible to assign a particular cytoplasmic region to each nucleus. In order to solve this problem, the average intensity per unit area was calculated for each field of view, rather than each cell. Although the data at each time point represents at least 60 different cells, values were calculated for each field of view, thereby reducing the sample size to 3, and increasing the chance that a difference between nuclear:cytoplasmic p53 intensity at different time points is falsely attributed with statistical significance. This experiment must be repeated to determine if the variability in p53 subcellular localization seen here is due to a true shift in subcellular localization, or to variability in experimental technique.

If, after repetition of this experiment, there is a significant nuclear accumulation of p53 in astrocytes, several explanations can account for the apparent lack of p53 activation in these cells. First, nuclear accumulation of p53 can be dissociated from apoptosis, as is the case for MCF-7 cells exposed to hypoxia (Amellem et al., 1997).
Alternatively, a small shift in subcellular localization of p53 protein may activate p53-dependent downstream events other than apoptosis, such as growth arrest. Finally, astrocytes may be in a phase of the cell cycle that makes them less sensitive to apoptosis-inducing stimuli.

Since the range of values of nuclear:cytoplasmic localization of p53 was very small over this 24 h time course, the shift reported at 2 h was most likely due to variability in experimental technique and probably does not represent a true nuclear translocation of p53. To confirm that this is the case, this experiment should be repeated.

Although individual cytoplasmic regions for one 2-D image at a specific focal plane demonstrated little variation in intensity, the p53 intensities in different nuclear regions varied considerably. As discussed earlier, variations of p53 intensities in each 2-D image is consistent with differences in the way the cell sits on the slide, cell shape and p53 expression (Figure 3.5). Thus, to determine the range of cell-to-cell variability in p53 expression in each nucleus, it would be necessary to calculate p53 intensity over the volume of the nucleus. The small variation of cytoplasmic p53 intensity in one focal plane is likely due to the different shape of the cytoplasm compared with the nucleus. The nucleus was typically spherical in shape, while the cytoplasm was more elongated and flattened. This flattened volume was imaged in fewer 2-D images, possibly resulting in less variability in p53 intensity in this subcellular structure.

In conclusion, we demonstrate that p53 protein in cultured astrocytes, which are resistant to XRT-induced apoptosis, remains nearly equally distributed between nuclear and cytoplasmic compartments. Definitive proof of an association between sensitivity to XRT-induced apoptosis and p53 nuclear translocation remains to be shown. Further
investigation into the subcellular localization and apoptotic response in cells sensitive to XRT-induced apoptosis, such as oligodendrocytes, may reveal more insights into the subcellular regulation of p53 and the consequences for apoptosis.
3.6 References


Chapter 4

Discussion of Results and Future Work
4.1 Abstract

This thesis focused on the molecular mechanism of apoptosis in CNS cells following XRT. In Chapter 2, we established that XRT-induced apoptosis in oligodendrocytes and subependymal cells of the adult mouse CNS was p53-dependent. In preliminary studies to determine the subcellular localization of p53 in CNS cells (Chapter 3), we reported that cultured astrocytes do not demonstrate p53 nuclear translocation following XRT. The mechanism of early apoptotic death observed in oligodendrocytes and subependymal cells may be relevant to the development of therapeutic strategies to modulate the early and/or late CNS reactions to XRT-induced injury. The future studies proposed in this chapter will determine the contribution of apoptosis to the development of a compensatory proliferative response and late XRT-induced injury in vivo. Other studies, to be conducted in vitro, will investigate the role of the cytoskeleton and the role of ceramide in p53 regulation following XRT.

4.2 Summary of Results

In Chapter 2, we demonstrated that XRT-induced apoptosis in p53 +/- mice reached a peak at 8 h after 22 Gy in the spinal cord, and at 4 h after 2 Gy in the subependymal region of the brain. Immunohistochemical studies demonstrated that the majority of the apoptotic cells in the spinal cord were oligodendrocytes, but that none of the apoptotic cells were astrocytes. The low incidence of apoptosis (0.44%) observed in oligodendrocytes following XRT led to a decrease in oligodendrocyte density, which was significant in only certain areas of the white or grey matter.
In the non-irradiated CNS of p53 +/- animals, we observed that very few glial cells of the spinal cord and subependymal cells of the brain expressed p53 protein, but following XRT, there was a dramatic increase in the proportion of both glial and subependymal cells that expressed p53. Irradiated p53 -/- animals, however, were resistant to XRT-induced apoptosis. In the spinal cord of mice heterozygous for p53, there was a suggestion of an intermediate response, although the apoptotic response was not significantly different from that observed in p53 +/- mice. Similarly, subependymal cells of p53 +/- mice showed a suggestion of a delayed or intermediate response. Taken together, these results suggest that p53 is required for XRT-induced apoptosis in the adult mouse CNS.

In Chapter 3, we described a method that we have developed to determine quantitatively the relative p53 expression levels in the nuclear and cytoplasmic compartments of a cell. Our preliminary data suggest that astrocytes in vitro have a near-equal distribution of p53 protein between their nuclear and cytosolic compartments. We suggest that the lack of p53 nuclear accumulation in these cells could explain their resistance to XRT-induced apoptosis.

Understanding the mechanism by which oligodendrocytes and subependymal cells undergo apoptosis may contribute to our understanding of the pathogenesis of early and late radiation-induced injuries. As discussed in Chapter 1, XRT can induce acute, sub-acute and late damage in the CNS. Briefly, the symptoms of the acute effects include headache, nausea, vomiting and fatigue. Sub-acute effects include L’hermitte’s syndrome and somnolence syndrome, and late effects can lead to serious neurological damage, including cognitive dysfunction, memory loss and/or paralysis. The early XRT-
induced apoptotic death we described in oligodendrocytes and subependymal cells may contribute both to the acute effects of XRT and the development of late XRT-induced damage. Oligodendrocyte death may contribute to the demyelination observed following XRT. Subependymal cells have been shown to possess characteristics of stem cells (Chiasson et al., 1999), and it has been suggested that the loss of these cells after XRT-induced injury may prevent the repopulation of glial cells following CNS injury (Hopewell and Cavanagh, 1972). Growth factors have been shown to stimulate subependymal cell proliferation and cause their differentiation into astrocytes and oligodendrocytes (Craig et al., 1996).

In our model of the pathogenesis of late XRT-induced injury, we postulate that early XRT-induced apoptosis triggers a compensatory cellular proliferation, which is followed by mitotic-linked cell death of the newly-divided cells due to sustained radiation damage. This leads to a critical loss of oligodendrocytes, which could result in demyelination and, eventually, white matter necrosis. In this chapter, the results from this thesis will be discussed in the context of this hypothesis and future studies will be suggested to test several aspects of our model (section 4.4). This will be followed by a discussion of the molecular regulation of XRT-induced apoptosis in the CNS (section 4.5).

4.3 Discussion of In Vivo Studies

In Chapter 2, we reported that even at the peak of apoptosis, only 0.44% of glial cells in the spinal cord were observed to undergo XRT-induced apoptosis. Yet, this apparently small percentage of cell death led to a decrease in oligodendrocyte density
within 24 h following XRT. This is in agreement with previous studies from our lab, which showed that although the peak AI in the rat spinal cord following 22 Gy was 0.86 - 0.92% (Li et al., 1996a), this led to a 24% decrease in oligodendrocyte density within 24 h (Li et al., 1996b). Since the duration of apoptosis is brief, lasting only about an hour (Barres et al., 1992; Li et al., 1996b), it is likely that a number of apoptotic cells remain unobserved between sampled time points. Thus, it is quite probable that the number of apoptotic cells is underestimated.

The progenitor cells from which oligodendrocytes are derived, O-2A cells (Raff et al., 1983), are numerous at the perinatal stage, but decline in number after birth of the organism. Two types of O-2A cells have been shown to exist at different stages of the development of the CNS. O-2A cells in perinatal animals (O-2A\textsubscript{perinatal}) have a relatively short cycle time of 18 h (Noble et al., 1988) and migrate at the rate of 21 \textmu m / h \textit{in vitro} (Small et al., 1987). In contrast, O-2A cells in the adult CNS (O-2A\textsubscript{adult}) have a cell cycle of 65 h and a migratory rate of 4 \textmu m / h (Wolswijk and Noble, 1989). Thus, if O-2A\textsubscript{adult} cells were to replace oligodendrocytes lost through apoptosis, the lengthy cell cycle times and migration rates of O-2A\textsubscript{adult} cells may result in a long time interval to repopulation.

In the non-irradiated rat spinal cord, only 0.13% of glial cells incorporate the thymidine analog, bromodeoxyuridine (BrdU), demonstrating a low proliferative rate (Li and Wong, 1998), but we have recently shown that proliferation in glial cells does occur following XRT-induced apoptosis of oligodendrocytes (Li and Wong, 1998). The peak of proliferation was observed at 2 weeks following 8 Gy, and proliferating cells appeared to belong to the cells of the oligodendrogial lineage (Li and Wong, 1998). Proliferation has also been observed in the adult dog brain following XRT (Fike et al., 1995). It is
currently unclear if O-2A<sub>adult</sub> cells are responsible for repopulation of the apoptotic oligodendrocytes in the irradiated spinal cord. The relatively long latency to the peak of proliferative activity after XRT in the spinal cord is however consistent with repopulating cells being O-2A<sub>adult</sub> cells.

In contrast to oligodendrocytes, subependymal cells are mitotically active. Almost half (43%) of subependymal cells are proliferating in the non-irradiated rat brain (Shinohara et al., 1997). Following XRT, however, the number of proliferating cells declines. Over half of the apoptotic nuclei observed after XRT also demonstrated incorporation of the thymidine analog, bromodeoxyuridine (BrdU), suggesting that most of the cell death in the subependymal region occurred in actively dividing cells (Shinohara et al., 1997).

Although XRT-induced apoptosis occurs in post-mitotic cells, such as oligodendrocytes (Li et al., 1996a; Li et al., 1996b), proliferating cells, such as subependymal cells appear to be more sensitive to XRT. The peak apoptotic response in subependymal cells reached 25.8% at 6 h after 2 Gy (Shinohara et al., 1997), while the maximal apoptotic response in oligodendrocytes was 0.86 - 0.92% at 22 Gy (Li et al., 1996a; Li et al., 1996b). The distinct proliferative status of these cells may, in part, explain their different susceptibilities to XRT-induced apoptosis.

Although a compensatory proliferative mechanism seems to repopulate the oligodendrocytes lost through apoptosis, these proliferating cells may have sustained radiation damage that may not yet be apparent. In order to determine the role of early XRT-induced apoptosis of oligodendrocytes in the development of late XRT-induced
injury, we have already initiated one study in the laboratory (section 4.4.1) and proposed others (section 4.4.2).

4.4 Proposed Future Work In Vivo

As described above, we hypothesize that early XRT-induced apoptosis may contribute to the development of late XRT-induced injury, including loss of oligodendrocytes, demyelination and white matter necrosis. In this model, we suggest that XRT-induced apoptosis initially leads to a compensatory cell proliferation, followed by mitotic-linked death of the proliferating cells, and subsequently, a critical loss of the oligodendroglial population. In Chapter 2, we demonstrated that XRT-induced apoptosis in oligodendrocytes requires p53. These radiation-apoptosis resistant p53 +/- mice can therefore be used to determine the role of XRT-induced apoptosis in the subacute and late responses to XRT.

4.4.1 Role of XRT-induced Apoptosis in Proliferation

We have previously demonstrated that XRT-induced apoptosis in oligodendrocytes is followed by proliferation of glial cells (Li and Wong, 1998). The proliferation may be a response to the XRT alone, or it may be a compensatory response to the loss of oligodendrocytes due to XRT-induced apoptosis. Proliferation of cells following injury to the CNS has been previously observed; ependymal cells in the adult spinal cord are normally quiescent, but demonstrated upregulation of the proliferative marker, Ki-67 following spinal cord compression (Namiki and Tator, 1999).
We hypothesize that XRT-induced apoptosis in oligodendrocytes triggers a proliferative response of O-2A progenitor cells, and that this response is absent in p53 -/- animals which are resistant to XRT-induced apoptosis. To test this hypothesis, we will irradiate the spinal cords of adult mice that are +/- and -/- for the p53 gene with a dose of 8 Gy. This dose has been shown to elicit the maximal proliferative response at 2 weeks after XRT (Li and Wong, 1998). Following XRT, mice will be injected intraperitoneally with four doses of 400 mg/kg BrdU, every 12 h for 48 h prior to sacrifice at various time points following XRT. The irradiated spinal cords will be fixed in 10% neutral-buffered formalin, embedded in paraffin and cut into 4 μm thick transverse sections. The spinal cord sections will then be stained with an anti-BrdU antibody, and the time course of proliferation in p53 +/- and -/- mice will be compared.

Preliminary Work

Twenty-one adult p53 +/- mice were irradiated with 8 Gy from C2-T2, and three mice each were sacrificed at 7, 10, 14, 17.5, 21 and 28 days after XRT. Three control mice were not irradiated. The irradiated spinal cords were fixed in formalin, embedded into paraffin blocks and cut into 4 μm thick sections.

A few test sections were stained with the mouse anti-BrdU antibody (clone IU-4, Caltag, used at a concentration of 1:500), but the background staining was high, probably due to the primary antibody adhering non-specifically to mouse tissue. A new product from Vector Laboratories is now available (Mouse-on-Mouse Immunodetection Kit, cat # PK-2200), which blocks non-specific staining from mouse antibodies in mouse tissue, which should decrease background levels in future studies.
Ongoing Work

The spinal cords of adult mice, wildtype for p53, will be irradiated with 8 Gy from C2 - T2 and injected with BrdU and sacrificed in the same manner as described above for the p53 -/- mice. The spinal cord tissue will be fixed, sectioned and stained in the same manner as described above.

If XRT-induced apoptosis does indeed trigger cellular proliferation in glial cells, we would expect that abolishing the apoptotic event in irradiated animals would also abolish proliferation. Thus, if our hypothesis is correct, p53 -/- animals, which are resistant to XRT-induced apoptosis, should demonstrate no significant proliferation following XRT, beyond what is normally seen in non-irradiated wildtype animals.

4.4.2 The Importance of XRT-induced Apoptosis in the Development of Late XRT-induced Injury

Ultimately, we hope that elucidation of the mechanisms leading to late XRT-induced injury will permit the development of novel strategies to modulate the severity of late CNS radiation damage, including white matter necrosis (WMN). As outlined above, it is possible that XRT-induced apoptosis in oligodendrocytes may contribute to the development of late CNS injury by initiating proliferation of a population of radiation-damaged cells, which will ultimately undergo mitotic-linked cell death. Thus, modulation of the apoptotic mechanism may lessen the severity of late radiation-induced injury.
To test this hypothesis, we will use p53 +/+ and +/- mice to monitor the development of demyelination and radiation necrosis following XRT. Adult rats irradiated at the cervical segment of the spinal cord develop forelimb paralysis and white matter necrosis within 7 months following XRT (van der Kogel, 1991; Wong et al., 1992). However, adult p53 -/- mice have an increased susceptibility to tumour development, and in one study, 74% of p53 -/- animals developed spontaneous tumours by 6 months of age with the average time to the appearance of the tumour being 20 weeks (Donehower et al., 1992). Thus, using adult p53 +/+ and +/- animals in this study is not feasible due to the long latency to white matter necrosis and the relatively short lifespan of the p53 -/- animals.

One strategy to overcome this problem is to use neonatal animals. We have recent data (Y-Q Li, personal communication) indicating that neonatal rats (1 - 2 weeks old) develop white matter necrosis within 3 - 4 weeks after XRT. We expect that p53 +/+ neonatal mice will also demonstrate a decreased latency to white matter necrosis. If early XRT-induced apoptosis does prove to be important in the development of white matter necrosis, we expect the neonatal p53 +/+ animals to develop white matter necrosis prior to p53 -/- animals. Alternatively, we may observe that a higher dose of XRT in p53 -/- animals will be required to produce the same effects as those seen in p53 +/+ animals.

A second strategy to determine the importance of XRT-induced apoptosis in the development of late radiation injury is a comparison of the late histopathologic injury in adult p53 +/+ animals to that in +/- animals. In Chapter 2, we outlined results indicating that p53 +/- adult animals demonstrated an apoptotic response that was not significantly different than that in p53 -/- animals, but that was significantly decreased compared with
the p53 +/+ animals. In one report, approximately 2% of p53 heterozygous animals developed spontaneous tumours at about 9 months of age (Donehower et al., 1992).

Because the heterozygous animals show an abrogated apoptotic response, the late histopathologic changes in these animals could be different from those in wildtype animals.

Another endpoint to investigate is the clonogenic survival of p53 -/- and p53 +/+ cells. Although we have shown that the apoptotic event itself leads to a significant decrease in oligodendrocyte density (Li et al., 1996b), another relevant issue is the clonogenic survival of glial cells following XRT. If, after XRT-induced injury, glial cells lose their ability to regenerate themselves, this could compound the already significant cell loss observed 24 h following XRT. Clonogenic assays could be performed on glial cells cultured from p53 +/+ and p53 -/- animals.

If XRT-induced apoptosis were important in the development of late XRT-induced damage, we would expect that abolishing the apoptotic event would abrogate the proliferative response seen weeks after XRT, and also diminish the severity of late XRT-induced injury. If the responses of p53 -/- and p53 +/+ animals were the same following XRT, this does not necessarily mean that p53 does not play a role in the development of acute and late XRT-induced injuries. In this case, a similar response of p53 wildtype and null mice may signify that the apoptotic event itself does not have an appreciable impact on the development of XRT-induced injury.

Our hypothesis that oligodendrocytes play an important role in the development of XRT-induced injury does not exclude the possibility that XRT-induced damage could be incurred by other means. For example, as discussed in Chapter 1, vascular endothelial
cells are also implicated in XRT-induced injury, and vascular damage has been reported in patients prior to the development of white matter necrosis. It is possible that this vascular damage could lead to hypoxia and upregulation of growth factors, such as VEGF, which could also contribute to the development of late XRT-induced injury. Although our model suggests that oligodendrocytes are important targets for XRT-induced damage, it is likely that other cells have a role in the pathogenesis of XRT-induced injury.

4.5 Discussion of In Vitro Work

Little is known about the mechanism by which p53 activates the apoptotic pathway in irradiated CNS cells. An improved understanding of the mechanism by which p53 is regulated and activated in irradiated CNS cells may provide novel strategies for therapeutic treatment of XRT-induced damage. Some studies have reported that nuclear translocation of p53 in oligodendrocytes (Eizenberg et al., 1995) and oligodendroglia-like cells (Uberti et al., 1999) is associated with apoptosis.

To determine if p53 regulation in XRT-induced apoptosis of CNS is also dependent on p53 subcellular localization, we have conducted preliminary studies with astrocytes. In agreement with previous work (Gobbel et al., 1998), we have found these cells to be resistant to XRT-induced apoptosis. Using confocal microscopy and an objective measure of p53 intensity in the nuclear and cytoplasmic compartments, initial results suggest that p53 localization in cultured astrocytes remains unchanged throughout a 24 h time period following a single dose of 10 Gy (Chapter 3). Future work will
concentrate on the association between subcellular localization of p53 and the ability of different cell types in the CNS to undergo XRT-induced apoptosis.

Another pathway by which cells may undergo XRT-induced apoptosis is the sphingomyelinase pathway. This pathway, as discussed in Chapter 1, is activated through membrane damage. The production of the second messenger, ceramide, has been shown to induce apoptosis in vascular endothelial cells (Haimovitz-Friedman et al., 1994), neuroblastoma cells (Lievremont et al., 1999), neurons (Taniwaki et al., 1999) and oligodendrocytes (Casaccia-Bonnefil et al., 1996b; Casaccia-Bonnefil et al., 1996a; Larocca et al., 1997). Recent work in our laboratory demonstrated that XRT induced a rapid increase in ceramide levels in cultured oligodendrocytes, which was subsequently followed by apoptosis (F. Lu, personal communication). The involvement of both p53 and ceramide in XRT-induced apoptosis of oligodendrocytes may seem paradoxical. It has been reported that the p53 and sphingomyelinase pathways were independent (Santana et al., 1996). However, it would now appear that in certain cells, the two pathways may interact. In one study, XRT induced ceramide generation in cells with intact p53 function, but not in cells lacking functional p53, indicating p53 acts upstream of ceramide (Dbaibo et al., 1998). Another study, however, reports the opposite. p53 +/+ fibrosarcoma cells treated with neutral sphingomyelinase or C2-ceramide demonstrated reduced proliferation, while p53 -/- cells given the same treatment showed no difference in proliferation compared with untreated cells, indicating that p53 signals downstream of ceramide (Pruschy et al., 1999). The molecular mechanism by which the p53 and ceramide pathways interact is under investigation, although the recent observation that ceramide induced by DNA double strand breaks can post-translationally
modify ATM, a kinase involved in the p53 apoptotic pathway, suggests one way these two apparently independent apoptotic pathways intersect (Liao et al., 1999). Part of the future work proposed here will determine how these two pathways interact in oligodendrocytes.

4.6 Proposed Future Work In Vitro

4.6.1 Association between p53 Subcellular Localization and XRT-induced Apoptosis

We hypothesize that the lack of p53 nuclear accumulation in astrocytes may contribute to the resistance of these cells to XRT-induced apoptosis, and that cells susceptible to XRT-induced apoptosis demonstrate p53 nuclear accumulation. In order to determine if p53 nuclear accumulation is associated with susceptibility of cells to undergo XRT-induced apoptosis, three cell types in the CNS, differing in their response to XRT, will be irradiated, and the subcellular localization of p53 protein determined using indirect immunofluorescence and confocal microscopy. Astrocytes (Gobbel et al., 1998; Chapter 3) and O-2A cells (Vrdoljak et al., 1992) in vitro are resistant to XRT-induced apoptosis, while oligodendrocytes are susceptible to XRT-induced apoptosis in vitro (Vrdoljak et al., 1992; Chapter 3) and in vivo (Li et al., 1996a; Li et al., 1996b; Chapter 2). We hypothesize that both astrocytes and O-2A cells will not demonstrate p53 nuclear accumulation post-XRT, while oligodendrocytes will.

Consistent with previous reports (Gobbel et al., 1998), we showed, in a single experiment, that astrocytes derived from primary cell cultures of the rat cerebral hemispheres, were resistant to XRT-induced apoptosis (Chapter 3). Preliminary results
indicated that these cells demonstrated nearly equal p53 expression in the nuclear and cytoplasmic compartments throughout a 24 h time course after 10 Gy.

Another radiation apoptosis resistant cell type is the O-2A cell, which was successfully grown as primary cell cultures from neonatal rat cerebral hemispheres as described in the Appendix 1. O-2A cells in vitro have been previously reported to be resistant to XRT-induced apoptosis up to a single dose of 20 Gy (Vrdoljak et al., 1992). It is currently unknown whether these cells normally express p53, and if so, in which subcellular compartment. In order to determine if there is an association between the propensity of O-2A cells to undergo XRT-induced apoptosis and the subcellular localization of p53 in these cells, O-2A cells will be cultured from neonatal rat cerebral hemispheres (as described in the Appendix 1), given a single dose of 10 Gy, and fixed and stained for the O-2A cell marker, A2B5, and p53, similar to the method previously described for astrocytes in Chapter 3. The intensity of p53 expression in the nuclear and cytoplasmic compartments will then be analyzed as previously described for astrocytes (Chapter 3). This experiment will be performed at least three times in order to distinguish between variability in experimental technique and true variability in p53 subcellular localization.

Oligodendrocytes are known to undergo XRT-induced apoptosis in vivo (Li et al., 1996a; Li et al., 1996b, Chapter 2) and in vitro (Vrdoljak et al., 1992, Chapter 3). In order to determine if XRT-induced apoptosis in oligodendrocytes is also associated with p53 nuclear accumulation, these cells will be cultured from the neonatal rat cerebral hemispheres (as described in Chapter 3), irradiated with 10 Gy and fixed and stained for GC and p53 at various time points after XRT. The intensity of p53 expression in the
nuclear and cytoplasmic compartments will then be analyzed as previously described for astrocytes in Chapter 3.

In all the experiments described above, a number of different p53 antibodies will be used, including pAb421 (Ab-1, Oncogene), pAb240 (Ab-3, Oncogene) and Ab-6 (Oncogene). Ab-1 and Ab-3 recognizes C-terminal p53 epitopes, and Ab-6 recognizes N-terminal p53 epitopes. It has been reported that p53 may undergo a change in conformation following XRT (Spivak and Kolman, 1998), and a single antibody used in these studies may not detect the subcellular shift of p53 if the epitope which the antibody detects has been hidden.

4.6.2 Involvement of Cytoskeletal Elements in p53 Subcellular Localization

The subcellular localization of the p53 protein may direct its function. One way in which the subcellular localization of p53 may be regulated is through anchorage of the protein to the cytoskeleton. Rat glioma cells, which are rich in intermediate filaments, were shown to retain p53 by binding p53 protein indirectly to the intermediate filaments of the cytoskeleton (Klotzsche et al., 1998). Astrocytes are rich in the intermediate filament, glial fibrillary acidic protein (GFAP) (Yang et al., 1994), and O-2A cells are rich in the intermediate filament, nestin (Gallo and Armstrong, 1995). Both cell types are resistant to XRT-induced apoptosis. In contrast, nestin expression is downregulated in XRT apoptosis sensitive oligodendrocytes (Gallo and Armstrong, 1995) and the major cytoskeletal element in mature oligodendrocytes appears to be microtubules (Lunn et al., 1997). Should cytoplasmic p53 retention be important in p53 subcellular localization, an understanding of how p53 is retained in the cytosol may become important in the
modulation of p53 function. We hypothesize that p53 may be anchored to intermediate filaments in the cytoplasm, preventing p53 nuclear translocation and activation following XRT. To determine the importance of an intact cytoskeletal network in p53 subcellular localization, we will use different drugs to disrupt the major cytoskeletal elements in astrocytes, O-2A cells and oligodendrocytes. Cytochalasin D can be used to disrupt intermediate filaments, and vinblastine can be used to disrupt microtubules (Alberts et al., 1995). Co-localization of the disrupted cytoskeletal components with p53, as determined using indirect immunofluorescence and confocal microscopy, would imply that p53 is associated with the cytoskeletal element, either directly or indirectly. Higher resolution studies using the electron microscope could visualize if p53 bound directly to the cytoskeleton or indirectly via another protein. Mice deficient for intermediate filaments have been described (Colucci-Guyon et al., 1994), and these animals could be used to examine the association between the astrocyte cytoskeleton and p53 subcellular localization.

4.6.3 The role of ceramide in p53-dependent XRT-induced apoptosis of oligodendrocytes

In Chapter 2, we observed an absence of XRT-induced apoptosis in p53 -/- mice. If ceramide signaling occurred downstream of p53, we would have expected to observe XRT-induced apoptosis in p53 -/- mice. As this was not the case, we hypothesize that p53 acts downstream of ceramide. Ideally, we would like to obtain sphingomyelinase-deficient mice and determine if XRT-induced apoptosis is abrogated in CNS cells of these mice. One strategy is to use acid sphingomyelinase deficient mice (aSMase -/-)
(Santana et al., 1996), which will soon be available through Jackson Laboratories (E. Schuchman, personal communication). Alternatively, CNS cells cultured from p53 -/- mice can be used to assess their ceramide response following XRT. However, oligodendrocytes are difficult to grow from mice (see Appendix 1). Thus, to obtain p53-deficient oligodendrocytes, p53 function can be inactivated through transfection of p53 +/- oligodendrocytes with a retrovirus encoding a truncated p53 protein. In previous studies, this so-called p53 mini-protein (p53DD) prevented p53 tetramerization and consequently, abolished p53 activity (Eizenberg et al., 1995). p53DD-oligodendrocytes will be irradiated and the ceramide levels following XRT will be determined. If ceramide signals upstream of p53, we would expect to see upregulation of ceramide, but not apoptosis in p53DD-oligodendrocytes. We will also treat p53DD- and p53-intact oligodendrocytes with exogenous ceramide to determine if the absence of p53 abolishes apoptosis. If ceramide does signal in a p53-dependent manner, we would expect to see apoptosis only in oligodendrocytes with intact p53 function.

4.7 Therapeutic Strategies

In this thesis, I have shown that apoptosis in the adult mouse CNS is an early response to XRT. It is currently unclear if this apoptotic event is relevant to the development of late XRT-induced injuries. However, the studies proposed in this chapter should ascertain if apoptosis is indeed a significant occurrence in the pathogenesis of late XRT-induced injuries. If so, the manipulation of the p53-dependent apoptotic pathway may lead to an alleviation of neurological symptoms suffered after XRT therapy. For example, should p53 prove to be activated in oligodendrocytes by subcellular
localization, preventing this nuclear shift would abolish apoptosis, and perhaps lessen the severity of late XRT-induced injuries, such as demyelination and white matter necrosis. An exciting possibility is that p53 may be anchored to the cytoskeleton, and injection of soluble cytoskeletal components into the CNS prior to XRT may prevent p53 translocation and p53-dependent apoptosis. Investigation in the mechanism of XRT-induced p53-dependent apoptosis in the adult CNS, along with long-term studies examining the role of apoptosis in the development of late XRT-induced damage will widen the scope of potential therapies.

4.8 Conclusion

We have shown that p53 is required for XRT-induced apoptosis in oligodendrocytes and subependymal cells of the adult CNS. Future work in this area will determine the relevance of XRT-induced apoptosis in the development of acute, subacute and late XRT injury in the CNS. A second area of interest is the molecular mechanism whereby p53 may be activated in irradiated CNS cells. These studies offer the potential of modulating the effect of XRT on the CNS, a major dose-limiting organ in clinical radiotherapy.
4.9 References


Appendix 1

Methods of Primary Cell Culture of Oligodendrocytes
5.1 Introduction

Primary cell cultures of glial cells allow for the study of molecular mechanisms that cannot be investigated in vivo. For example, in Chapter 2 we showed that oligodendrocytes and subependymal cells in vivo require p53 for XRT-induced apoptosis using a transgenic mouse model. The molecular mechanism by which p53 exerts its apoptotic effects can only be studied in vitro, and require the establishment of a primary cell culture technique for astrocytes, oligodendrocytes and O-2A cells (see Chapter 4).

Primary culture of oligodendrocytes can be difficult due to the significant cell loss during the culturing protocol, the strict requirements of these cells for specific growth factors (Barres et al., 1993) and the necessity of sustaining these cells in a serum-free media, which will prevent the differentiation of O-2A cells into the astrocyte lineage (Raff et al., 1983). A number of techniques have been previously described to culture oligodendrocytes. The general method for primary cell culture of oligodendrocytes can be divided into three steps: the isolation of oligodendrocytes and other glial cells from the CNS, the enrichment of oligodendrocytes from a heterogeneous population, and the maintenance of oligodendrocytes in culture for several days. Each successive step in the method leads to a smaller population of viable cells.

The isolation of oligodendrocytes can be achieved through two different methods. In one technique, known as the "shaking" method, a heterogeneous population of glial cells is cultured and the less-adherent oligodendrocytes are removed from the co-culture by shaking (McCarthy and de Vellis, 1980). Another procedure involves enzymatic digestion of optic nerve tissue (Barres et al., 1992).
Once glial cells are removed from CNS tissue, enrichment of the oligodendrocyte population can be achieved through differential adherence (Nutt et al., 1995), immunopanning (Barres et al., 1992) and cell sorting (Abney et al., 1983; Trotter and Schachner, 1989). In the first method, adherent astrocytes attach to an uncoated petri dish, while less adherent oligodendrocytes do not. In immunopanning, oligodendrocytes are selectively anchored to a petri dish, which is coated with an oligodendrocyte-specific antibody. In the last method, glial cells are labeled with an oligodendrocyte-specific antibody conjugated to a fluorochrome. Using fluorescence-activated cell sorting (FACS), cells positive for the oligodendrocyte marker will be recognized by their emitted fluorescence, and will be pooled together.

Once the cell population has been enriched for oligodendrocytes, the cultured cells must be grown in a chemically defined, serum-free media, since oligodendrocyte progenitor cells, O-2A cells, differentiate along the astrocyte lineage in vitro in the presence of serum (Raff et al., 1983). This special media formulation is commonly referred to as SATO media, and is based upon a formulation described by Bottenstein (Bottenstein and Sato, 1979). The cells are seeded on plastic chamber slides, which have been precoated with poly-D-lysine. Poly-D-lysine has been shown to promote cellular adherence (Yavin and Yavin, 1980).

A variation of these techniques (described below) were used to culture and isolate oligodendrocytes from different CNS tissues of mice and rats of various ages. The successful primary cell culture of oligodendrocytes will allow future investigations into the molecular mechanism of XRT-induced apoptosis in these cells (see Chapter 4).
5.2 Materials and Methods

5.2.1 Animals

Neonatal and adult Fisher or Sprague-Dawley rats (Jackson Laboratories) and neonatal and adult CD1 mice (Ontario Cancer Institute) were used in this study. A maximum of 1 pregnant rat or mouse, 2 adult rats, or 5 adult mice were housed per cage, with food and water freely available, and with lighting between 6 AM and 6 PM in the animal colony of the Ontario Cancer Institute, an animal colony accredited by the Canadian Council of Animal Care. Postnatal pups were used at 1 - 25 days of age, and adult animals were at least 4 weeks old.

5.2.2 Primary Cell Culture of Oligodendrocytes From the Corpus Callosum and Optic Nerves

A total of 34 separate experiments were performed in an attempt to establish a primary cell culture of oligodendrocytes from the corpus callosum. Of these 34 experiments, 25 involved the use of neonatal Fisher rats (4 - 12 days old), one involved the use of adult Fisher rats (> 4 weeks old), five involved the use of neonatal mice (5 - 12 days old), and 3 involved the use of adult CD1 mice (62 - 99 days old).

A total of 4 separate experiments were performed to establish a primary cell culture of oligodendrocytes from the optic nerves. Two of these experiments were performed with adult CD1 mice (65 and 99 days old) and two were performed on neonatal Sprague-Dawley rats (5 days old).

In general, six separate steps were carried out in order to culture oligodendrocytes from the corpus callosum or optic nerves. 1) The animal was sacrificed and the tissue of
interest was removed and chopped with a scalpel. 2) The tissue was further broken down by the use of different enzymes. 3) The tissue was broken down into a single cell suspension by drawing the tissue through 25 and 27 gauge needles until no tissue clumps remained. 4) The heterogeneous cell population was enriched for oligodendrocytes using one of three methods: differential adherence, immunopanning or cell sorting. 5) Cells were seeded into T-80, T-25 or 4-well chamber slides that had been incubated in poly-D-lysine for at least 30 mins at room temperature. 6) Cells were maintained in a chemically defined, serum-free medium, modified from Bottenstein (Bottenstein and Sato, 1979), containing different concentrations of growth factors. The media was changed every other day, and growth factors were added every 22 – 24 h. Cells were kept at 37°C, in 3% CO₂. This method for culturing oligodendrocytes from the corpus callosum or the optic nerve remained invariant at steps 1, 3 and 5. Variations on the technique are described below.

**Enzymatic Digestion**

The enzymatic digestions were carried out with 1.33% collagenase at 37°C for 45 minutes, then with papain solution (5% papain, 0.13% DNase, 2 mg L-cysteine, pH 7.4) for 1 h at 37°C. The dissociated tissue was then treated with papain inhibitor (0.15% ovomucoid, 0.08% globulin-free bovine serum albumin, 0.004% DNase in DMEM). Alternatively, collagenase was used in conjunction with 0.025% trypsin and trypsin inhibitor (0.052% soybean trypsin inhibitor, 0.004% DNase, 0.3% bovine serum albumin fraction V in DMEM) in the place of papain and papain inhibitor. In some cases, only papain and papain inhibitor was used to dissociate the tissue. In other experiments,
papain was inhibited with a both a low concentration (0.15% BSA, 0.15% ovomucoid, 0.004% DNAse in DMEM, pH 7.4) and a high concentration (1% BSA, 1% ovomucoid in DMEM, pH 7.4) of ovomucoid solution.

*Enrichment of Oligodendrocytes from a Heterogeneous Cell Population*

The cells derived from either the corpus callosum or the optic nerves were purified in a number of different ways. In differential adherence, cells were allowed to adhere to a petri dish for 30 minutes at 37°C. The more adherent astrocytes stuck to the dish, while the less adherent oligodendrocytes were be poured off.

Another way to purify oligodendrocytes is through immunopanning. In this technique, a petri dish was coated with a variety of antibodies in order to specifically deplete or enrich a heterogenous population of cells. For example, microglial cells, which are known to adhere to Fc Receptors, were depleted on a plate precoated with 10 µg / ml IgG in 0.05M Tris buffer (pH 9.8). After an incubation period of about 30 minutes at room temperature, the non-adherent cells were poured off. Similarly, dishes were coated with the Ran-2 antibody, an astrocyte-specific antibody (Bartlett et al., 1980), and the cell population was depleted of astrocytes. The cell population could also be enriched for cells by selectively adhering cells to the dishes. For example, dishes were pre-coated with the antibodies GC (10 µg/ml in 0.05M Tris buffer, pH 9.8) and A2B5 in order to enrich cell populations for mature oligodendrocytes and O-2A cells, respectively. After a 30 minute incubation at room temperature, these cells were trypsinized off the dish and used for further experiments. Both the Ran-2 and the A2B5 antibodies were harvested from hybridoma cell lines that secreted the antibodies.
Lastly, the heterogeneous cell population derived from the corpus callosum or the optic nerves could be purified by cell sorting. After the tissue was dissociated into a single cell suspension, cells were labelled with A2B5 antibody, derived from A2B5 hybridoma cells, for 30 minutes at room temperature. The cells were then sorted into A2B5+ and A2B5- cell populations, using a fluorescence-activated cell sorter (FACS).

**Culture conditions**

After an oligodendrocyte population was isolated and/or purified, the cells were suspended in one of two formulations of a chemically defined serum-free media, known as SATO media (Bottenstein and Sato, 1979). SATO-1 was adapted from a formulation modified by Nutt (Nutt et al., 1995) and consists of: 2 mM glutamine, 50 µg/ml transferrin, 5 µg/ml insulin, 30 nM selenium, 30 nM triiodothyronine in DMEM. SATO-2 was adapted from a formulation modified by Barres (Barres et al., 1992) and consisted of: 100 µg/ml transferrin, 100 µg/ml bovine serum albumin, 60 ng/ml progesterone, 16 µg/ml putrescine, 40 ng/ml selenium, 40 ng/ml thyroxine, 30 ng/ml triiodothyronine, 5 µg/ml insulin, 63 µg/ml N-acetyl choline in DMEM. After seeding the cells in culture flasks precoated with Poly-D-lysine, 10 - 20 µg/ml of bFGF and PDGF-AA were added to each flask. These growth factors were added every 22 - 26 hours. In some cases, the media was additionally supplemented with up to 5% FBS.
5.2.3 Primary Cell Culture of Oligodendrocytes From the Cerebral Hemispheres

Preparation of mixed astrocyte-oligodendroglia co-cultures

Cells were cultured from the cerebral cortex of neonatal rats in 28 separate experiments. Primary cell cultures of astrocytes, oligodendrocytes and O-2A cells were prepared from neonatal rat cerebral hemispheres according to the method of McCarthy (McCarthy and de Vellis, 1980). The general method involved establishing glial cell co-cultures, followed by isolation of various cell populations from the co-culture. Briefly, the rats were decapitated and the cerebral cortex was removed and dissected free of meningeal material. Twenty-five to thirty cortexes were pooled together for one experiment. The tissue was either enzymatically dissociated or mechanically dissociated. Enzymatic dissociation was performed with 0.025% trypsin for 30 mins at 37°C, followed by treatment with 0.004% DNAse for 5 mins at room temperature. Alternatively, the tissue was chopped with a scalpel and the tissue pieces were placed in a solution of 15% FBS-DMEM. The tissue was titurated with a 10 ml glass pipet until tissue had been further broken down. After tissue dissociation, the entire suspension was centrifuged at 700 rpm for 5 minutes. The cell pellet was resuspended in 15% FBS-DMEM and the cells were seeded into 8 to 12 T-75 culture flasks. The media was changed on day three, and replaced with 10% FBS-DMEM. On day 5, a monolayer of astrocytes formed, and by day 7 or 8, phase-bright cells grew on top of the astrocyte monolayer.
Isolation of oligodendrocytes from astrocyte-oligodendroglial co-culture

Following the stratification of oligodendrocytes on top of astrocytes, the flasks were shaken for 1 h on an orbital shaker at 200 rpm at 37°C to remove microglia cells. The media was removed and discarded, and fresh 10% FBS-DMEM was added and the flasks were returned to the shaker for 17-18 h at 200 rpm and 37°C. The media, containing the cells of the oligodendroglial lineage, was removed, filtered through a 20 μm Nitex mesh (B & SH Thompson & Co. Ltd.), and centrifuged at 1000 rpm for 5 minutes. This non-adherent cell population was not composed purely of oligodendrocytes, and required further enrichment (see below).

Enrichment of oligodendrocytes from a heterogeneous cell population

After shaking overnight on an orbital shaker, the media, containing cells of the oligodendroglial lineage, was removed and filtered through a 20 μm Nitex mesh and centrifuged at 1000 rpm for 5 mins. The filtered cells were resuspended in 10% FBS-DMEM and seeded into 1 or 2 T-75 culture flasks for 1-2 hours. In this differential adherence step, the more adherent astrocytes will adhere to the flasks within this time interval. At the end of this step, the less adherent oligodendrocytes were poured off, centrifuged at 1000 rpm for 5 mins, and then resuspended in chemically defined media (modified from (Bottenstein and Sato, 1979)). Our media formulation (SATO-3) consisted of: 50 μg/ml transferrin, 100 μg/ml bovine serum albumin, 60 ng/ml progesterone, 5 ng/ml sodium selenite, 10 ng/ml tri-iodo thyronine, 5 μg/ml insulin, 60 μg/ml N-acetyl choline, 2 mg/ml glucose, 6.5 ng/ml biotin, 2.5 mM glutamine, made up in 1:1 Ham’s F12 and DMEM. Oligodendrocytes were seeded into 4-well chamber slides
(Lab-Tek), which had been precoated with poly-D-lysine (Sigma, 5 μg/ml), at a density of approximately 5x10^4 cells per well. In these experiments, oligodendrocytes were used at 1 or 2 days after seeding.

**Isolation of O-2A cells from astrocyte-oligodendroglial co-cultures**

In order to isolate O-2A cells from astrocyte-oligodendroglial co-cultures, the method previously described by Behar (Behar et al., 1988) was used. Briefly, after shaking the co-cultures overnight, the media was removed and filtered through a 20 um Nitex mesh. Next, the filtered and centrifuged cells were resuspended in about 5 ml of anti-A2B5 antibody for 15 minutes. The antibody was prepared from A2B5 hybridoma cells (ATCC) which secrete the anti-A2B5 antibody (Eisenbarth et al., 1979). The unbound antibody was washed out by centrifugation at 1000 rpm, 5 minutes and the cells were resuspended in 1% FBS-DMEM-25mM HEPES. This step was repeated twice more to thoroughly wash out any unbound antibody. The A2B5+ cells were then seeded into 100 mm tissue culture dishes, 10^6 cells per dish. The cells were allowed to adhere for 7 minutes at room temperature. The A2B5+ cells were less adherent, so at the end of this incubation, the A2B5+ cells were poured off. The cells were filtered once more through a 20 μm Nitex mesh, and centrifuged at 1000 rpm for 5 minutes. The A2B5+ cells were resuspended in 10% FBS-DMEM and 5x10^4 cells were seeded into each well of a 4 well chamber slide, which had been previously coated with poly-D-lysine (Sigma 5 μg/ml). Twenty hours after seeding, this media was removed and replaced with a chemically defined media containing: 50 μg/ml transferrin, 30nM sodium selenite, 30nM tri-iodo-thyronine, 5 μg/ml bovine insulin, 0.5% FBS, made up in DMEM, and
supplemented with 5μg/ml PDGF-AA. The PDGF-AA was added fresh to the media just before every media change, every two days. These A2B5+ cells were used at 3-4 days after the initial seeding.

5.3 Immunostaining of oligodendrocytes and O-2A cells

Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 mins and sequentially incubated in mouse anti-galactocerebroside IgG (Boehringer Mannheim, 10 μg/ml) for 60 mins or mouse anti-A2B5 IgM antibody supernatant, neat, for 30 mins, Texas-Red goat anti-mouse IgM (1:50, for A2B5+ cells) or IgG (1:50, for GC+ cells) for 30 mins, and DAPI (0.1 ng /ml) for 10 mins. Slides were mounted in Vectashield and protected with a glass coverslip.

5.4 Results

5.4.1 Optic Nerve

Cells cultured from the optic nerves of adult mice or neonatal rats typically did not survive for longer than two days. One day after seeding, the cells remained shrunken and did not bear any processes. Two days after seeding, the majority of the cells had detached from the plate.

5.4.2 Corpus Callosum

Cells cultured from the corpus callosum of adult or neonatal rats were viable the following day in 13/25 experiments. Some cells demonstrated a bipolar morphology (Figure 5.1), while other cells were multi-process bearing cells (Figure 5.1). In spite of
Figure 5.1 Morphological characteristics of primary glial cultures. Cells cultured from the corpus callosum of neonatal rats demonstrated both bipolar (large arrow) and multi-process (small arrows) morphology, but many cells did not survive the culture process (small arrowheads).

Figure 5.2 Viability of primary glial cultures. Cells cultured from the corpus callosum of neonatal rats thrived at 9 days in vitro, but could not be identified as cells of the oligodendroglial lineage.
different media formulations (SATO-1 or SATO-2) and the daily addition of growth factors, most cells did not survive in vitro longer than three days. In 2 experiments, cells of the corpus callosum did survive up to 9 days in vitro (Figure 5.2). These cells grew to a high density and had many processes, but were negative for A2B5, O4 and GC as visualized under the epifluorescent microscope.

5.4.3 *Mouse Cultures*

Cells cultured from the corpus callosum of adult or neonatal mice generally did not survive as long as cells cultured from adult or neonatal rats. These cells typically required the addition of growth factors and 5% FBS in order to remain viable. Although this enriched culture environment increased cell viability, the morphology of the surviving cells was suggestive of astrocytes, rather than oligodendrocytes or O-2A cells.

5.4.4 *Enzymatic Digestion*

There appeared to be no difference in enzymatically digesting the optic nerve or corpus callosum tissues with collagenase alone, collagenase and papain, collagenase and trypsin, or papain alone. Generally, each of these methods resulted in cell death within four days.

5.4.5 *Oligodendrocyte Enrichment*

Enrichment of the oligodendrocyte cell population resulted in fewer viable cells and often did not result in a pure oligodendrocyte population. Flow cytometric analysis demonstrated that 35% of the cells that had been subjected to immunopanning were
A2B5+ cells, while 17 - 35% of the cells that were not immunopanned were A2B5+ cells (Figure 5.3). One attempt to sort the cells by fluorescence activated cell sorting (FACS) resulted in complete cell loss in the sorted population the following day (Figure 5.4).

5.4.6 Cerebral Hemispheres

Similar to the experiments performed with corpus callosum tissue, cell viability was generally good the day following seeding of the cells. However, viability of cells varied widely. Although some experiments demonstrated that cell viability could be maintained up to 10 days in vitro, these results were inconsistent and most cells had to be used within 3 days of seeding. Indirect immunofluorescence for GC, and A2B5 (Figure 5.5) and immunohistochemistry for CNPase demonstrated that at least 75% of the cells cultured from the postnatal rat cerebral hemispheres were of the oligodendroglial lineage.

5.5 Conclusions

Oligodendrocytes were grown from three different sites (optic nerve, corpus callosum and cerebral hemispheres) in the adult or neonatal mouse or rat CNS. Oligodendrocytes established from neonatal and adult mice required enrichment with FBS in addition to growth factor addition. While these factors improved cell viability, cells had a flat, multi-process morphology reminiscent of astrocytes. Oligodendrocytes were more difficult to culture from mice compared with rats (Barres, personal communication) and the extra factors needed to increase cell viability probably influenced cell differentiation into type II astrocytes, rather than along the oligodendroglial lineage.
Figure 5.3 A comparison of the effectiveness of oligodendrocyte enrichment after immunopanning. Cells that had not been subjected to immuno-panning had a similar proportion of A2B5+ cells (upper right quadrant, A) compared with cells that were subjected to immunopanning (upper right quadrant, B). The y-axis is the intensity of the fluorescent antibody (FITC) conjugated to A2B5. The x-axis represents the size of the cells.
Figure 5.4 Morphology of cells after enrichment by cell sorting. Cells cultured from the corpus callosum of the neonatal rat did not survive 24 h after enriching for A2B5+ cells by fluorescence-activated cell sorting (FACS) (arrowheads).

Figure 5.5 Identification of oligodendroglial cells by indirect immunofluorescence. Cells cultured from neonatal rat cerebral hemispheres and purified by the "shaking" method were positively identified as A2B5+ O-2A cells (A) and GC+ oligodendrocytes (B) by indirect immunofluorescent staining. Both A2B5 and GC are conjugated to the fluorochrome Texas Red.
Viable cell cultures could be established from the corpus callosum or the cerebral hemispheres of neonatal rats. Using immunofluorescent staining for oligodendroglial cell markers, cultures established from the corpus callosum could not be identified as oligodendroglial cells. Under light microscopy, these cells had many processes and were possibly type II astrocytes. Alternatively, the immunofluorescent technique, which was being perfected in parallel with the oligodendrocyte cell culture technique, could have failed to detect oligodendrocytes.

 Cultures derived from neonatal rat cerebral hemispheres were not consistently viable, but immunofluorescent staining could positively identify these cells as those of the oligodendroglial lineage. Consequently, oligodendrocytes were cultured from the cerebral hemispheres of neonatal rats in further experiments.
5.6 References


Appendix 2

Abbreviations
6.0 Abbreviations

AEC – 3-amino-9-ethylcarbazole
AI – apoptotic incidence
AO – acridine orange
AT – ataxia teleangiectasia
ATM – ataxia teleangiectasia mutated
BBB – blood brain barrier
BrdU – bromodeoxyuridine
BSCB – blood spinal cord barrier
C2-T2 – cervical spinal segment 2 to thoracic spinal segment 2
CAD – caspase-activated deoxyribonuclease
CAK – CDK-activating kinase
CKI – casein kinase I
CNS – central nervous system
DAPI – 4, 6 diamidino-2-phenylindole
DIG – dioxigenin
DMEM – Dulbecco’s modified Eagle’s medium
DNA-PK – DNA-activated protein kinase
dUTP – deoxyribonucleotide triphosphate
EtBr – ethidium bromide
FACS – fluorescence-activated cell sorting
FADD – fas-associated protein with death domain
FBS – fetal bovine serum
FITC – fluorescein isothiocyanate
GC – galactocerebroside
GFAP – glial fibrillary acidic protein
H&E – hematoxylin and eosin
ICE – interleukin-1β-converting enzyme
IGFBP-3 – insulin-like growth factor binding protein 3
IL-2 – interleukin-2
MCID – Microcomputer Imaging Device
O-2A – oligodendrocyte type II astrocyte
PBS – phosphate buffered saline
PDGF – platelet derived growth factor
PI – propidium iodide
PI3K – phosphatidylinositol-3-kinase
plp – proteolipid protein
SAPK/JNK – stress-activated protein kinase/c-Jun N-terminal kinase
SEM – standard error of the mean
SMase – sphingomyelinas
aSMase – acid sphingomyelinas
TdT – terminal deoxynucleotidyl transferase
TUNEL – terminal deoxynucleotidyl transferase deoxyribonucleotide triphosphate nick end labelling
VEGF – vascular endothelial growth factor
WMN – white matter necrosis
XRT – radiation