Aus dem Medizinischen Zentrum für Pathologie
der Philipps-Universität Marburg
Abteilung der Neuropathologie
Geschäftsführender Direktor: Professor Dr. med. H.D. Mennel

# Functional Analysis of the Molecular Response to Ionising Radiation in Malignant Human Glial Tumours *in vitro*

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Alison Clova Kraus, geboren Stark
aus Aberdeen, Schottland
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Referent: Professor Dr. med. H.D. Mennel

Korreferent: Professor Dr. med. E. Weihe

For Sigurd

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# 1. ABBREVIATIONS

All units of measurement are abbreviated according to the Standard International units (SI units). In the following text, genes are referred to in italic capitals and their gene products in standard small letters, with the exception of p53, where the convention of referring to both gene and gene product as „p53“ is followed.

| | |
|---|---|
| A | adenosine |
| *ATM* | Ataxia telangiectasia mutated gene |
| ATP | adenosine triphosphate |
| bp | base pairs |
| C | cytosine |
| Cdk | cyclin dependent kinase |
| cDNA | complementary DNA |
| CGH | comparative genomic *in situ* hybridisation |
| CNS | central nervous system |
| DMEM | Dulbecco’s Minimal Essential Medium |
| DNA | deoxyribonucleic acid |
| dNTPs | 2‘-deoxynucleoside 5‘-triphosphate set |
| *EGFR* | epidermal growth factor receptor gene |
| et al | and others |
| EtBr | ethidium bromide |
| FCS | foetal calf serum |
| G | guanosine |
| *GADD 45* | growth arrest and DNA damage inducible gene |
| GBM | Glioblastoma multiforme |
| Gy | Grays |
| h | hours |
| Hepes | N-(2-hydroxyethyl)-piperazine-N’-2-ethansulphonic acid |
| HRP | horse radish peroxidase |
| kb | kilobase pairs |
| kD | kilodaltons |

| | |
|---|---|
| LOH | loss of heterozygosity |
| *MDM2* | murine double minute 2 gene |
| min | minutes |
| M-MLV RT | Moloney Murine Leukaemia Virus reverse transcriptase |
| NaCl | sodium chloride |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PMSF | phenylmethylsulphonyl fluoride |
| Pu | purine |
| PVDF | Polyvinylidene difluoride |
| Py | pyridamine |
| *RB* | retinoblastoma gene |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RT-PCR | reverse transcription PCR |
| SDS | sodiumdodecylsulphate |
| SSCP | single stranded conformation polymorphism |
| T | thiamine |
| TAE | tris-acetate-EDTA |
| TBS | tris buffered saline |
| TE | tris-EDTA |
| TNE | tris-sodium-EDTA |
| tris | tris(hydroxymethyl)aminomethane |
| U | unit |
| UV | ultraviolet |
| WHO | World Health Organisation |
| wt | wild type |

# 2. INTRODUCTION

## 2.1 The therapeutic challenge of Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most malignant of the glial tumours, classified by the WHO as grade IV. GBM alone accounts for 15-23 % of all intracranial tumours (VandenBerg 1992). Despite so many advances in oncology in recent years, the prognosis of patients with GBM remains distressingly poor. Operative resection forms the basis of treatment but, since it is not feasible to resect effectively beyond the limits of macroscopically visible tumour without drastically compromising neurological function, improvements in operative techniques over the past twenty years have had little effect on the survival of patients with GBM. To date no chemotherapeutic regime has been shown to have significant beneficial effects.

Adjuvant radiotherapy, however, does provide a marginal improvement in the prognosis, increasing the average survival time to fourteen months (Burger and Green 1987). While this represents only an extra two to three months survival on average to the patient, it is nonetheless a benefit. The limited efficacy of radiation treatment is believed to arise from the poor apoptotic response to ionising radiation in the tumour cells (Kerr et al. 1994). Gene therapy strategies for GBM are in the experimental stage of development and, although there are good grounds for optimism, their introduction as routine treatment for GBM patients is still some way off. Thus, radiation treatment remains the main stay of adjuvant therapy in GBM.

The search for novel therapeutic strategies in GBM begs a better understanding of GBM tumorigenesis. In particular, clearer insight into the mechanisms involved in the cellular response to ionising radiation is essential, if we are to combat the radioresistant nature of these tumours.

## 2.2 Subclassification of Glioblastoma multiforme

### 2.2.1 The WHO classification of glial tumours

GBM is the most malignant form of the astrocytic glial tumours, which as a group account for more than 50% of all CNS neoplasms. According to the histopathological classification by the WHO, glial tumours are graded from grade I to IV (Kleihues et al. 1994). The grade I astrocytic tumours, which include pilocytic astrocytomas, are well circumscribed and are regarded as benign. Malignant astrocytomas, which are diffusely infitrating in character, comprise a spectrum of increasing neoplasia from grade II astrocytoma to grade III anaplastic astrocytoma to grade IV GBM, whereby cellularity and pleomorphism increase from grade II to IV. The presence of endothelial proliferation and necrosis mandates the diagnosis of grade IV GBM. The WHO grading system discloses the tendency of grade II and III astrocytomas to progress malignantly, a significant proportion ultimately attaining status as grade IV. Typical patient survival times range from more than five years for grade II astrocytomas, two to five years for anaplastic astrocytomas and approximately one year for GBMs (Kleihues and Cavanee 1997).

Mixed gliomas, bearing both astrocytic and oligodendroglial features, form an exception to the general pattern of progression from grade II through to grade IV in glial tumours. Oligoastrocytomas seldom progress further than grade III, and oligodendrogliomas only extremely rarely progress to GBM (Reifenberger et al. 1996).

### 2.2.2 Clinical subgroups of Glioblastoma multiforme

A distinction between different GBMs on clinical grounds has long been recognised. Approximately 60% of GBM arise *de novo*, without history of progression from a grade II or III glial tumour, and display a peak incidence in the fifth and sixth decades (Codd and Kurland 1985). In contrast, approximately 30% of GBMs show progression from astrocytoma or anaplastic astrocytoma. This clinical entity, referred to as secondary GBM, tends to occur in younger patients, with an incidence peaking in the fourth decade, and bears a slightly more favourable prognosis (Winger et al. 1989). Histologically, *de novo* and secondary GBMs cannot be distinguished from one another (Burger and Green 1987), both exhibiting tumour necrosis, vascular endothelial

proliferation and cellular atypia.

Paediatric GBMs constitute a minor subgroup, with only 4% of GBM presenting before the age of twenty years (Sure et al. 1997). Likewise, the giant cell GBM, a histological subvariant, is a rarity with circa 1% incidence (Meyer-Puttlitz et al. 1997).

### 2.2.3 Genetic subgroups of Glioblastoma multiforme

Genetic studies of GBM tissue have revealed a vast array of mutations, gene amplifications and chromosomal rearrangements, reflecting a pronounced genetic instability. Certain specific genetic alterations, however, occur with high frequency and have led to the tentative genetic subclassification of GBM.

Amplification of the *EGFR* gene, located at 7p13, occurs in 40-50% of GBM (Wong et al. 1987, Leenstra et al. 1994). The gene amplification, constituting an increased copy number of circa 10 to 50, invariably leads to an overexpression of the EGF receptor (Schlegel et al. 1994). Approximately a third of GBMs with *EGFR* amplification also display rearrangements of the EGF receptor, which seem to arise from errors during the amplification process. Such rearrangements result in the expression of truncated EGF receptors, which are deficient in their extracellular domain and are consequently constitutively active (Moscatello et al. 1995).

Loss of genetic material on chromosome 10 occurs in 60-85% of GBMs (Fults et al. 1990). In these GBMs cytogenetic studies demonstrate monosomy for chromosome 10 and loss of heterozygosity is observed at multiple chromosome 10 markers (Schlegel et al. 1996). GBMs with *EGFR* amplification always show loss of chromosome 10, suggesting that deletion of chromosome 10 both precedes and is a prerequisite for *EGFR* amplification (Schrock et al. 1994, von Deimling et al. 1992).

This raises the possibility that a tumour suppressor gene, of importance in the tumorigenesis of GBM with *EGFR* amplification, may be located on chromosome 10. Recently a candidate tumour suppressor gene, located at 10q23, has been identified in GBM cell lines (Li et al. 1997, Steck et al. 1997) and in 23% of GBM tumour specimens (Teng et al. 1997). Sequence analysis of this gene has revealed a protein tyrosine phosphatase domain and homolgy to the cytoskeletal protein tensin. Thus, it has been named *PTEN* (Phosphatase and Tensin homolog deleted on chromosome ten), but is also referred to as *MMAC1* or *TEP1* in the literature. The functional significance of the gene product is not yet certain, although its tyrosine phosphatase activity has led

to speculation that it may oppose the signalling cascade of receptor tyrosine kinases, such as the EGF receptor. A recent study in GBMs, however, has not been able to demonstrate any association between *PTEN* deletion and *EGFR* amplification (Liu et al. 1997).

Loss of chromosome 10 and *EGFR* amplification are changes which occur predominantly in older patients exclusively in GBM, and are demonstrable in neither grade II nor grade III astrocytomas (Leenstra et al. 1994). This genetic group, therefore, seems to represent the clinical *de novo* group of GBM.

A second genetic subgroup of GBMs displays mutations in the tumour suppressor gene p53, accounting for 30-40% of GBM (Ohgaki et al. 1995, Louis 1994). Notably, amplification of the *EGFR* almost never occurs in GBMs with p53 mutations (Rasheed et al. 1994), thus creating two mutually exclusive genetic groups.

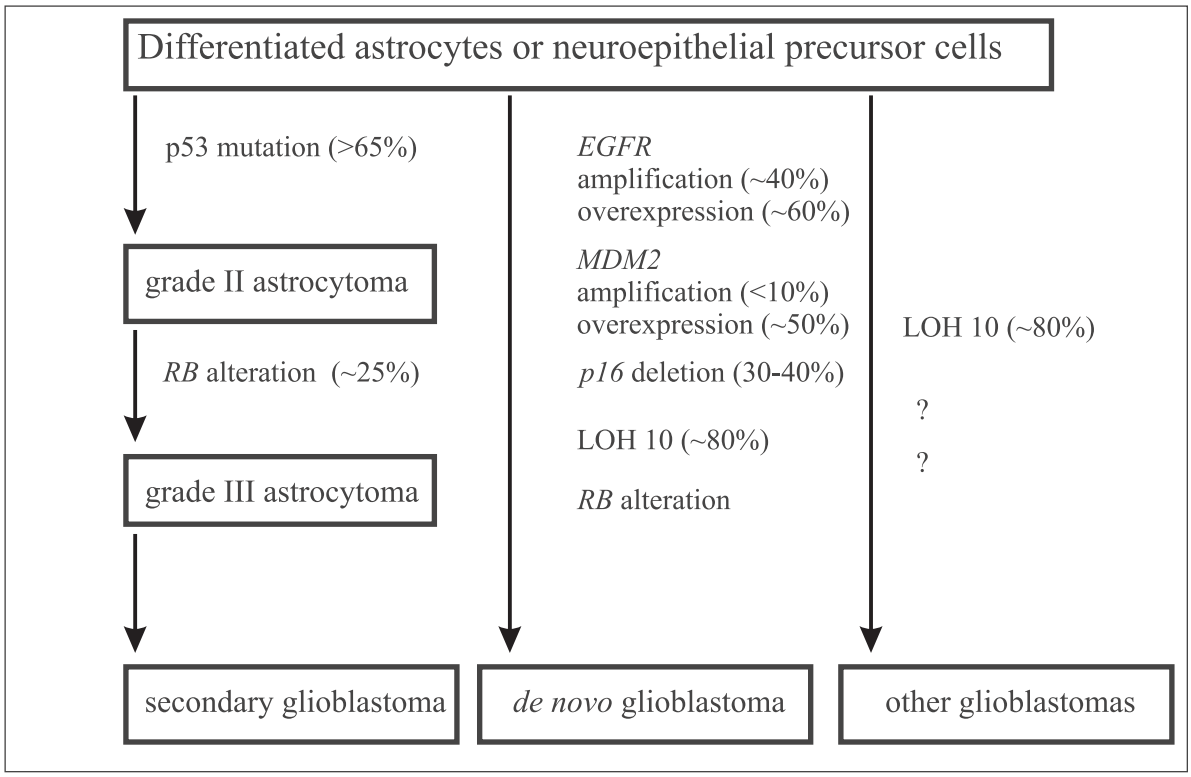
Mutation in p53 is the earliest genetic alteration detectable in glial tumours, occurring in grade II astrocytomas with an incidence equivalent to that seen in GBM (Campomenosi et al. 1996). Sidransky et al showed that subpopulations of cells in grade II and III glial tumours contained the same p53 mutation as was subsequently demonstrated after their progression to GBM, suggesting that clonal expansion of p53-mutated cells occurs (Sidransky et al. 1992). These findings indicate that aberrations in p53 are critical in triggering tumorigenesis in grade II or III astrocytomas but, since the incidence of p53 mutation after progression remains unchanged, other factors contribute to their malignant progression.

Thus, the genetic subgroup of p53-mutated GBMs correlates to the clinically defined secondary GBM, which arises from malignant progression from grade II or III astrocytomas.

This system of subclassification, summarised in Figure 2.2.3, certainly provides an attractive model for the further study of GBM, particularly given the alliegance between clinical and genetic subgroups. However, it undoubtedly oversimplifies a more complex picture and omits approximately one third of GBMs, which exhibit neither p53 mutation nor *EGFR* amplification.

As the next section will highlight, the presence of a p53 mutation readily explains the genetic instability and radioresistance seen in secondary GBMs. *De novo* GBMs, however, are equally radioresistant (Baxendine-Jones et al. 1997), a fact not easily explicable on the grounds of their known genetic alterations. An intriguing finding,

**Figure 2.2.3** Genetic pathways operative in the evolution of de novo




modified from Kleihues and Cavanee 1997

however, in approximately 30% of *de novo* GBMs is an accumulation of wild type p53 protein (Anker et al. 1993, Rubio et al. 1993). Although the functional significance of this stabilised p53 protein is not understood, it raises the possibility that p53 functions abnormally in at least some *de novo* GBMs, despite the presence of an intact p53 gene. If this were the case, a functional defect in p53 or its pathway might account for the genetic instability and radioresistance of *de novo* GBMs.

The project described in this thesis investigates the hypothesis that the presence of wt p53 is not synonymous with normal p53 function in GBMs.

## 2.3 The p53 pathway

### 2.3.1 Outline of the p53 pathway

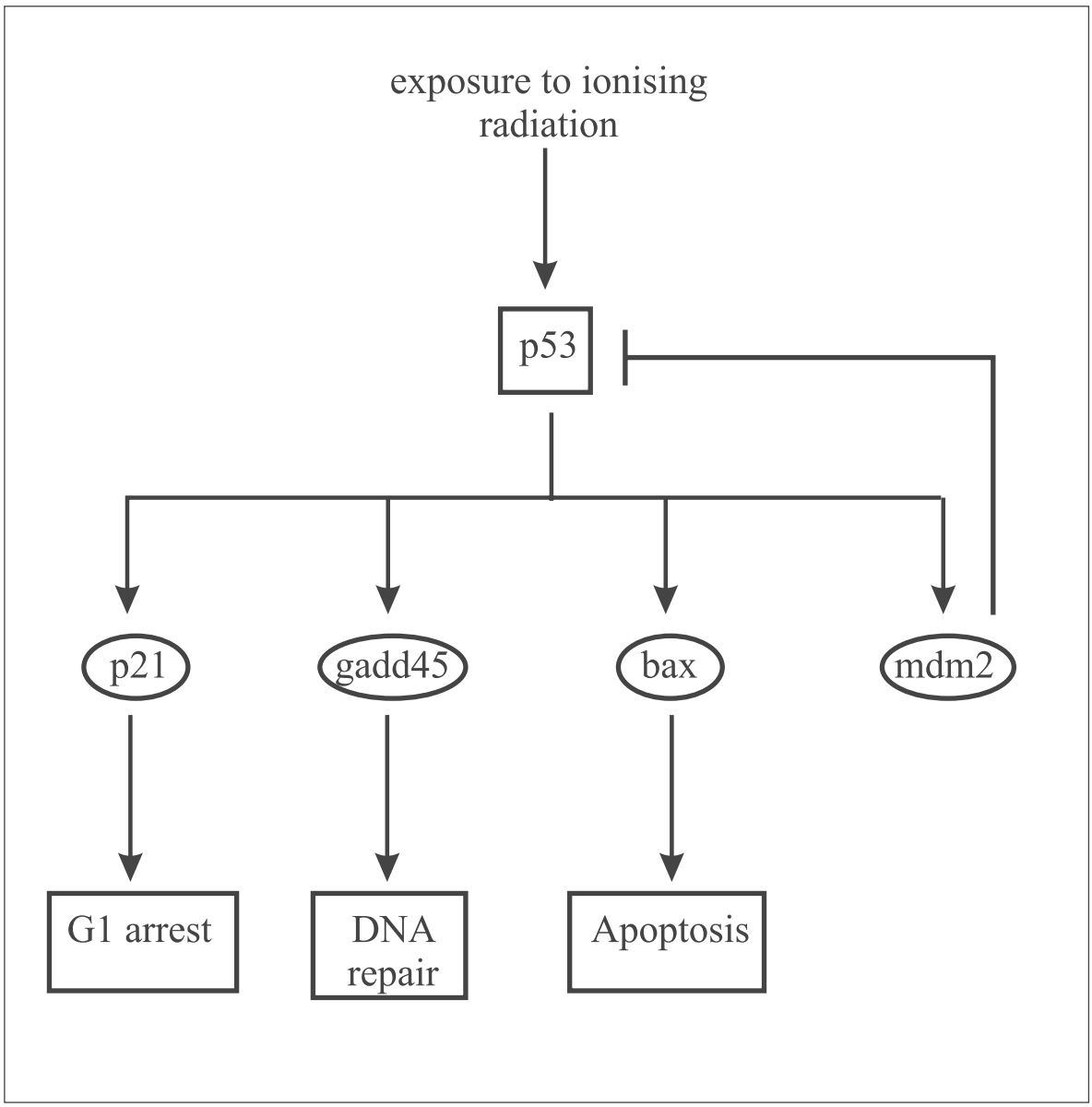
The tumour suppressor gene p53 plays a critical role in maintaining the genetic integrity of cells. p53 has been heralded as the 'guardian of the genome' because of its protective role in instigating DNA repair or apoptosis in response to genotoxic stress. A 'loss of function' mutation, leading to marked genetic instability and radioresistance, is found in more than half of human tumours (Sidransky and Hollstein 1996).

DNA damage in a cell activates wt p53 protein, which is then transported to the cell nucleus, where it becomes phosphorylated at its amino terminus and then transcriptionally activates an array of cell cycle and apoptosis regulators, including p21, gadd45, mdm2 and bax (Martinez et al. 1997), as illustrated in Figure 2.3.1.

Activation of p21 by p53 invokes cell cycle arrest in G1, via inhibition by p21 of retinoblastoma (Rb) protein phosphorylation. Activated p21 also binds and inhibits PCNA, preventing the activation of DNA polymerase $\delta$ and thus halting DNA replication. The DNA repair apparatus is activated when p53-induced gadd45 complexes to PCNA (Vairapandi et al. 1996). Transcriptional activation of mdm2 by p53 is a delayed process. By directly binding and degrading the p53 protein (Haupt et al. 1997), mdm2 serves a negative feedback loop, permitting entry of the cell into S phase once DNA repair is complete.

p53-dependent apoptosis is at least partially determined by the balance between bax and bcl-2 and other members of the bcl-2 family. Whereas bax, an inducer of

**Figure 2.3.1** Ionising radiation activates p53 which induces its downstream effectors p21 and gadd45 are responsible for cell cycle arrest and DNA repair, bax induction promotes apoptosis, while mdm2 induction inhibits p53 function.

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apoptosis, is transcriptionally activated by p53, the apoptosis inhibitor bcl-2 is transcriptionally repressed. The activation of p53, therefore, results in a shift towards apoptosis.

Clearly the outcome of DNA damage and p53 activation is either G1 arrest with DNA repair, or apoptosis. The mechanism by which p53 decides between these two outcomes, however, is unclear. It has been suggested that limited DNA damage leads to a transient and moderate activation of p53 and thus into the repair route, whereas considerable DNA damage results in prolonged, pronounced p53 activation and initiates apoptosis (Enoch and Norbury 1995). This would ensure that cells with irreparable DNA damage are directed into apoptosis and eliminated from the cell population.

Since cells deficient in ATM, an upstream regulator of p53, are able to undergo apoptosis but not G1 arrest and DNA repair (Meyn 1995), it is probable that ATM influences the switch into repair, but not into apoptosis. Other p53 regulators, capable of switching the cell into apoptosis, may exist but are as yet unidentified. Since misdirection into the repair pathway of cells normally bound for apoptosis is a possible tumorigenic mechanism, the elucidation of this switch mechanism is of great interest.

### 2.3.2 Historical perspective of p53

The cellular protein p53 was first described in 1979 when a 53kD polypeptide was observed in extracts of cells transformed by the DNA tumour virus SV40 (Lane and Crawford 1979, Linzer and Levine 1979). While this protein was barely detectable in normal cells, it was strongly expressed not only in SV40-transformed cells but also in a variety of other tumour-derived cells (DeLeo et al. 1979, Rotter et al. 1980).

On the grounds of its elevated expression in cancer cells, p53 was postulated as an oncogene. Expression plasmids encoding cloned mouse p53 were shown to exert oncogenic effects both *in vitro* and *in vivo* (Eliyahu et al. 1984), seemingly clinching p53's role as an oncogene. When normal cellular p53 was cloned, however, it became clear that the protein used in early experiments was a mutant form. Furthermore, wt p53 effectively inhibited neoplastic transformation by various cellular and viral oncogenes (Eliyahu et al. 1989), thus casting grave doubts on the 'proto-oncogene' theory.

p53 became accepted as a tumour suppressor gene when it was shown to satisfy the 'two hit hypothesis', whereby a p53 mutation associates with deletion of the second p53 allele on chromosome 17 in colorectal carcinoma (Baker et al. 1989). This

hypothesis remains valid today, postulating that a first event of mutation in one allele, either hereditary or spontaneous, must be followed by the loss of the remaining wt allele before the neoplastic transformation can take place. In other words, the normal wt 'tumour suppressing' function of the gene is lost in cancer.

Ironically, the categorisation of p53 has proved more complex and the early transformation experiments supporting an oncogenic role have regained credence. While p53 predominantly functions as a tumour suppressor gene, some mutations probably also lead to a dominant negative effect on the remaining wt allele. Thus, certain p53 mutant forms do also satisfy the criteria for an oncogene.

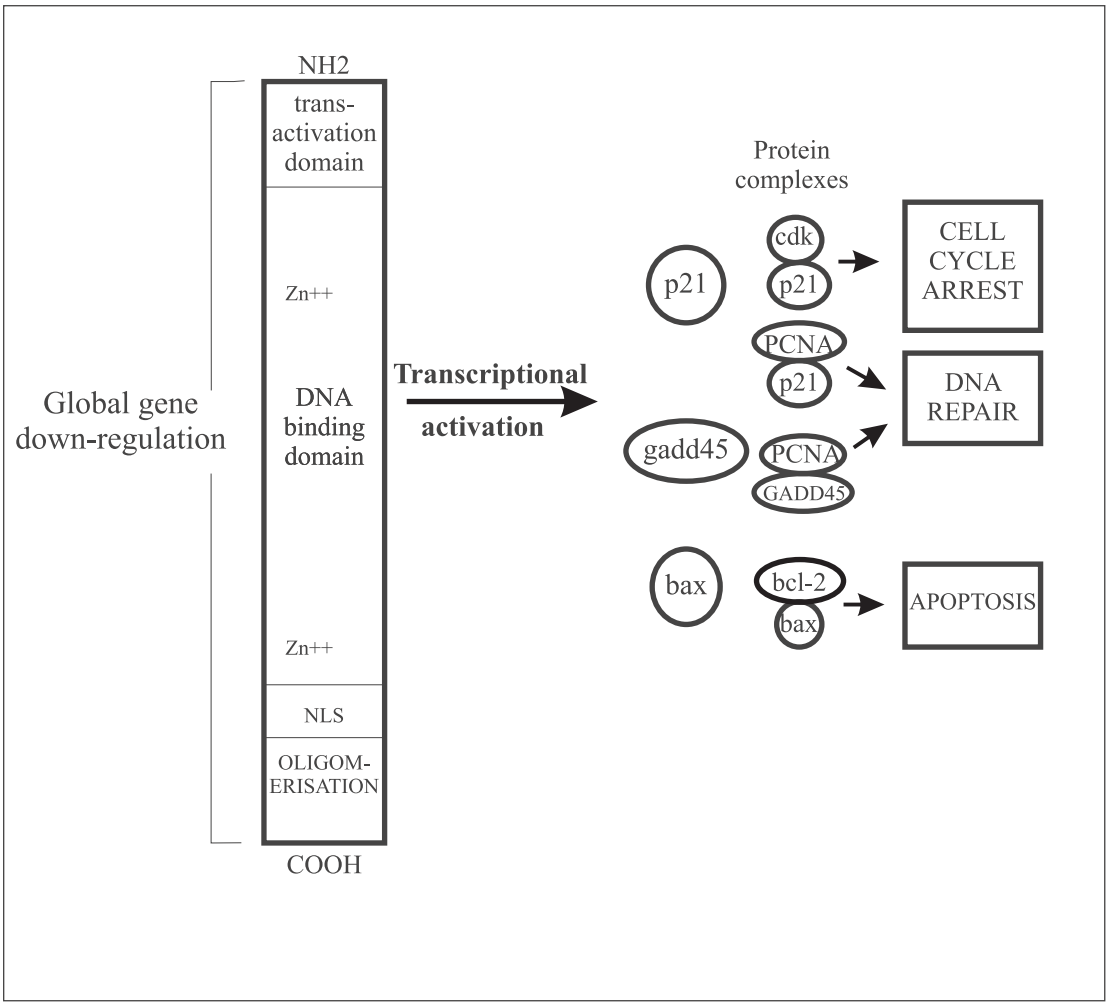
### 2.3.3 The topology of p53

p53 protein is the product of a 20kb gene on the short arm of human chromosome 17. Convention in this particular case refers to both the gene, *TP53*, and the protein as 'p53', largely on the historical grounds that the protein was discovered well before the gene.

The protein comprises four functional domains as shown in Figure 2.3.3. A transcriptional activation domain resides in the acidic amino terminus (Unger et al. 1993). A DNA-binding domain, which is independently folded and contains a zinc ion, spans residues 102 to 292. This domain binds specifically to any DNA matching the concensus sequence 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' (Pavletich et al. 1993). p53 exists as a tetramer, whose spontaneous formation is dependent on the oligomerisation domain at residues 319 to 360. This segment consists of one α helix and one ß strand, which assemble into primary dimers consisting of two antiparallel α helices linked by a ß sheet. Helix-helix interactions lead to the formation of a tetramer from two dimers (Clore et al. 1994). The basic carboxy terminus of p53 has been shown to be alternatively spliced in normal mouse epidermal cells, and may provide a negative regulatory effect on the DNA-binding domain (Kulesz-Martin et al. 1994).

p53 acts as a transcription factor, capable of transactivating specific genes to effect growth suppression in response to genotoxic stress. Since this function is not disrupted by foreign replacement of the oligomerisation and transactivation domains (Pietenpol et al. 1994), the DNA-binding domain of p53 appears to be critical to its role as a tumour suppressor. Targets of the DNA-binding domain include promoters or

**Figure 2.3.3** The p53 protein and its biological effects. p53 is a zinc-binding transcription factor which binds to a DNA sequence motif in the promotors of its specific target genes.



introns of specific genes controlling growth, as well as single stranded DNA and DNA helicases (Oberosler et al. 1993). Thus, p53 itself may be able directly to bind to and influence repair of damaged DNA, besides transcriptionally activating specific genes.

The transactivation domain complexes specifically with a number of regulatory proteins including the TATA-box binding protein TBP, CCAAT-specific transcription factor and transcription factors such as Sp-1 and WT-1. These interactions probably contribute to a global repression of many genes within a cell containing activated p53. Association between the transactivation domain and the DNA replication factor RPA inhibits the binding of RPA to single stranded DNA, which is a critical step in initiating DNA replication (Dutta et al. 1993). Such properties, however, are not essential for tumour suppression by p53.

The paramount importance of the DNA-binding domain is further emphasised by the finding that more than 90% of missense mutations in p53, including all the 'hotspots' of mutation, reside in this region (Hollstein et al. 1996). Furthermore, all reported p53 mutations in glial tumours lie within this region, which encompasses exons 5 to 8 (Kleihues and Cavanee 1997). The two most common sites of mutation are residues 248 and 273, where mutations lead to defective DNA binding and loss of p53's ability to act as a transcription factor. Another class of mutations within the DNA-binding domain disrupts the integrity of its conformational structure, inhibiting the accessibility of the region to its specific-binding DNA sequences (Cho et al. 1994). Thus, effective DNA binding and an intact conformational architecture are both fundamental to the suppression of tumours by p53.

### 2.3.4 Mechanisms of p53 activation

Any endangerment to the genomic integrity of the cell instigates a protective cellular response, in which p53 is pivotal. DNA damage may occur spontaneously during the normal cell cycle, may be induced by exogenous agents such as ionising radiation, UV light or genotoxic chemicals, or may result from cellular stresses such as hypoxia, which generates genotoxic free radicals. Ionising radiation, for example, produces strand breaks and base damage in the DNA. Were such defects allowed to pass through the stage of DNA synthesis and into the G2 phase of the cell cycle, the repair machinery would no longer be able to distinguish intact from aberrant DNA sequences and the damage would be incorporated into dividing cells. p53 acts to prevent this.

In a normal cell p53 protein is barely detectable as a result of its short half-life of approximately 30 minutes. DNA damage, however, causes a rapid increase in the cellular p53 concentration, with protein accumulating in the nucleus (Kastan et al. 1991). The speed of this response indicates that it occurs as a result of post-translational modification. An extension in the half-life up to 8 hours is primarily responsible for the stabilisation of p53. Increased translational efficiency of p53 mRNA may also contribute to the increased p53 levels. Although increased transcription of p53 mRNA plays no part in the immediate protein stabilisation, it may occur on prolonged genotoxic stress.

The mechanism by which damaged DNA activates p53 is not thoroughly understood and probably comprises multiple pathways. Given the ability of p53 to bind single stranded DNA with high affinity, direct activation may occur. However, DNA damage also activates signal transduction pathway(s), resulting in post-translational stabilisation of p53.

The study of cells from patients with the cancer-predisposing syndrome ataxia telangiectasia (AT) has brought some light to this question. AT cells, in which the gene *ATM* is homozygously mutated, lack G1 arrest and show impaired DNA repair in response to ionising radiation. Furthermore, these cells display significantly reduced and delayed stabilisation of p53 after irradiation (Kastan et al. 1992). These findings suggest that the gene product ATM is involved in the upstream regulation of p53 and is essential for p53-dependent G1 arrest. ATM itself is constitutively expressed in normal cells and is thought to undergo post-translational modification(s) in response to irradiation-induced genomic damage (Brown et al. 1997).

Interestingly, p53-dependent apoptosis is not blocked in irradiated AT cells, indicating that ATM is not essential for apoptosis. Similarly, while irradiation-induced p21 activation and subsequent G1 arrest were abrogated in the thymus of ATM deficient mice, the induction of bax and apoptosis was completely normal (Barlow et al. 1997). This raises the possibility that several distinct pathways exist upstream of p53, perhaps providing a regulatory switch between the option of cell cycle arrest with DNA repair and the alternative outcome of apoptosis when a cell undergoes genotoxic stress.

ATM has been shown to bear homology to phosphatidyl-3 kinase, but p53 is not subject to this phosphorylating activity (Jung et al. 1997). Recent studies have identified c-Abl tyrosine kinase as a downstream target of phosphorylation and activation by ATM kinase in the cellular response to ionising radiation (Shafman et al. 1997). Ionising

radiation has been shown to trigger a direct physical association between c-Abl and p53 (Yuan et al. 1996). However, although both events are dependent on ATM, c-Abl activation by ionising radiation appears to be independent of p53 stabilisation (Baskaran et al. 1997). p53 and c-Abl may represent two distinct targets of ATM. The signal transduction pathway connecting ATM to p53 activation is thus far from clear.

Another possible pathway of p53 activation may involve the generation of reactive oxygen species, occurring during oxidative stress. Since sulphydryl oxidation disrupts the structure and conformation of p53, its function is sensitive to the redox conditions (Hainaut and Milner 1993). Oxidative stress also leads to activation of the MAP kinase cascade. The carboxy terminus of the p53 molecule possesses sites susceptible to phosphorylation by MAP kinase. Since this region of p53 is itself capable of influencing the activity of the DNA-binding domain, phosphorylation here may represent a point of control of p53 activity.

### 2.3.5 Termination of the p53 signal

During embryogenesis the inhibition of apoptosis is crucial in order to maintain viability and, therefore, inhibition of p53 is essential. It has been shown using knock-out mice that mdm2 is responsible for this negative regulation of p53; the *MDM2*-null genotype results in embryonic lethality, whereas embryogenesis occurs normally when *MDM2* and p53 are simultaneously deleted (Jones et al. 1995). Thus, the p53 pathway exerts negative regulation on itself via the p53- inducible mdm2 protein, which forms a complex with p53 protein, potently inhibiting p53's ability to transactivate its specific target genes (Momand et al. 1992).

Until recently it was not understood exactly how this mechanism operated, but experimental manipulation of the structure of mdm2 has clarified this question. The mdm2 protein possesses a p53 binding domain localised to its amino terminus at codons 19 to 102, an acidic domain, a central zinc-finger motif and a carboxy terminus RING finger zinc binding motif (Chen et al. 1993). Transfection studies, using either full length mdm-2 or a truncated form deprived of its p53 binding domain, demonstrated that full length mdm2 promotes a rapid reduction in p53 protein detectable by Western blotting, without altering mRNA expression, whereas the truncated form is unable to influence p53 protein levels (Haupt et al. 1997). This suggests that the termination of the p53 signal depends on p53 protein degradation induced by mdm2 binding to the p53

protein. This is thought to be effected by the ubiquitin-proteasome pathway.

The p53 binding domain of mdm2 also interacts with the E2F1 transcription factor (Martin et al. 1995) and the Rb protein (Xiao et al. 1995), stimulating the transcriptional activity of E2F1 to generate transcripts required in the S phase of the cell cycle.

In contrast to p53-inducible genes responsible for arresting the cell cycle or inducing apoptosis, whose mRNA levels rise within 2 hours of ionising radiation, induction by p53 of *MDM2* is delayed (Perry et al. 1993). This provides a time window, allowing p53 to achieve DNA repair or resort to apoptosis before mdm2 switches its signal off. Furthermore, recent evidence suggests that DNA-dependent protein kinase (DNA-PK), induced directly by DNA damage, phosphorylates both p53 and mdm2, thereby inhibiting mdm2's ability to complex and degrade p53 (Shieh et al. 1997, Mayo et al. 1997). Once DNA damage is effectively repaired, DNA-PK is no longer induced, therefore releasing the inhibitory effect on mdm2 and allowing termination of the p53 signal. When the route to apoptosis is taken, mdm2 is cleaved by a caspase-3 (CPP32)-like protease (Erhardt et al. 1997, Chen et al. 1997) and subsequently cannot be detected in apoptotic cells. Thus it seems likely that degradation of mdm2 by apoptotic proteases ensures that it is unable to oppose apoptosis.

In summary, mdm2 acts in favour of cell proliferation. After a time window in which p53 checks the cell cycle and effects either DNA repair or apoptosis, mdm2 is activated by and then degrades p53, constituting a negative feedback loop. Concurrently, mdm2 stimulates the transcriptional activation of genes essential for the S phase as a result of its binding to E2F1. This allows the cell to re-enter the cell cycle and progress into S phase. Alternatively, when apoptosis is initiated, the negative regulatory effects of mdm2 are prevented by its degradation by proteasomal components of the apoptotic cascade. Therefore, one would predict that *MDM2* functions as an oncogene and, indeed, stable transfection of the amplified *MDM2* gene into normal NIH3T3 cells results in a transformed phenotype (Fakharzadeh et al. 1991).

The *MDM2* gene is amplified and overexpressed in approximately 10% of GBMs (Biernat et al. 1997), particularly in those GBMs with intact wt p53. *MDM2* amplification leads to maximal inhibition of p53's tumour suppressive effects and unleashes the pro-proliferative effects of mdm2, representing a tumorigenic mechanism by which wt p53 function is deregulated. The *MDM2* amplification seen in *de novo* GBMs cannot alone account, however, for the deregulation of p53 since it is observed in a maximal 10% of cases.

Interestingly, RT-PCR performed on a range of human tumour cells, including ovarian and bladder carcinomas, has shown that the mdm2 transcripts can be alternatively spliced (Sigalas et al. 1996). Characterisation of these splice variants revealed the partial or total absence of the p53 binding domain, disrupting not only p53 binding but also stimulation of the transcriptional activity of E2F1. Although the full length mdm2 is certainly oncogenic, whether these splice variants also have an oncogenic capacity is not certain. Sigalas et al found that the presence of splice variants correlated significantly with poor tumour differentiation and that transfection of cloned splices into NIH 3T3 cells resulted in transformation. In contrast, studies in which p53-/ Rb- human osteosarcoma (SaOS-2) cells were transfected with mdm2 and truncated forms, identified the minimum essential transforming region of mdm2 as the p53 binding domain (Dubs Poterszman et al. 1995). Thus, spliced mdm2 transcripts, which lack this binding domain, may lose their transforming potential. Furthermore, the growth advantage conferred by mdm2 in SaOS-2 cells was inhibited by the co-transfection of wt p53. This raises the interesting possibility of a reciprocal relationship between p53 and mdm2; while mdm2 is crucial in terminating the tumour suppressor's signal, p53 may inhibit the oncogenic activity of mdm2. Both of these activities are dependent on the p53 binding domain in mdm2.

## 2.4 Cell Cycle Regulation

### 2.4.1 Cell cycle checkpoints

The cell cycle was first described by the radiation biologists Howard and Pelc in 1953. They described a M phase of mitosis, when cytokinesis and chromosomal segregation were easily visualised by light microscopy. Using auto-radiographic methods, they were able to define a period of DNA synthesis, the S phase. The period between M and S phases they termed G1, the first gap phase, and that between S and M, G2, the second gap phase. The majority of differentiated mammalian cells, however, exist in a quiescent state, G0, and re-enter G1 only when undergoing proliferation.

The fundamental task of the cell cycle is to ensure that DNA is faithfully duplicated during S phase and that two identical sets of chromosomes are divided into two daughter cells during M phase. Any threat to the integrity of the genetic information

must be countered before replication and mitosis proceed, since replication renders mutations unrecognisable and thus irreparable, and mitosis in the presence of unrepaired strand breaks can lead to gross chromosomal aberrations in the daughter nuclei.

Cell insults causing DNA damage may be extrinsic, such as irradiation or chemical mutagens, or of intrinsic origin, for example oxidative stress. In general, the DNA damage constitutes either a modification of the nitrogenous bases or of the phosphodiester backbone. The cell possesses a complex repair machinery capable of restoring the DNA to its intact state. Lesions to the bases are removed by excision repair pathways, and the resultant 'excision gap' is filled in by a repair polymerase and ligated. Breakage of phosphodiester bonds results in nicks or double stranded breaks in the DNA. These broken DNA ends are highly recombinogenic and are necessarily repaired by nonhomologous end joining or the addition of new telomeres (Paulovich et al. 1997). The damage evoked by gamma irradiation occurs predominantly in the form of double stranded DNA breaks (Lu and Lane 1993).

Clearly, if the cell is to avoid permitting the perpetuation of mutations and chromosomal aberrations, the effective repair of DNA damage is of utmost importance. In order to afford sufficient time for repair, however, a delay in the progression of the cell cycle is essential. To this end, multiple 'checkpoints' exist, capable of detecting DNA damage and halting the progression of the cell cycle. The DNA damage checkpoints operate at three stages of the cell cycle, one at the G1/S transition referred to as G1 arrest, one that monitors progression through S phase and one at the G2/M boundary. The positioning of these checkpoints immediately before and during the vulnerable S and M phases prevents the cell from either replicating damaged DNA templates, or erroneously segregating chromosomes.

The consequences of checkpoint failure are clearly grave and it is no surprise that defective checkpoints play a significant role in tumorigenesis. The following section will describe in some detail the important components of the cell cycle checkpoints. In particular the response to gamma irradiation, which triggers arrest at all three checkpoints (Elledge 1996), will be discussed.

### 2.4.2 G1 Arrest

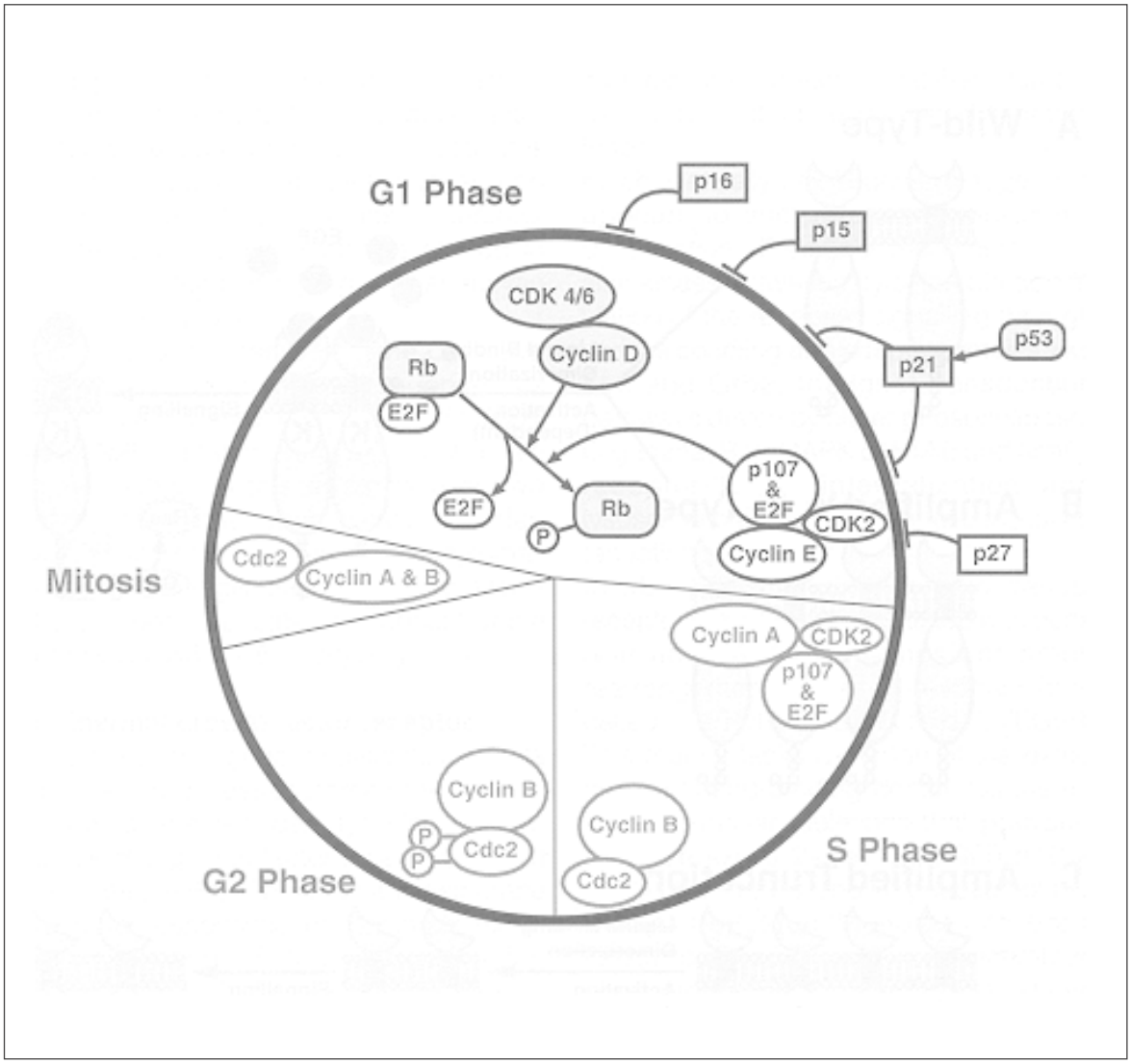
As discussed previously, the tumour suppressor gene p53 has been shown to play a pivotal role in bringing about an arrest at the G1/S transition (Kastan et al. 1991, Yount, et al. 1996). Activation of p53 by DNA damage, mediated at least in part by ATM, leads to the transcriptional activation of p21, an inhibitor of the cyclin dependent kinases (Cdk).

The Cdk family is regarded as the 'engine of the cell cycle', responsible for driving the cell through the various phases of the cycle when bound to its respective cyclin. p21 specifically inhibits the kinase activity of the Cdk2/cyclinE and Cdk4/cyclin D complexes which are consequently prevented from phosphorylating their substrate, the Rb protein (Dulic et al. 1994). As illustrated in Figure 2.4, in its hypophosphorylated state Rb binds the E2F family of transcription factors, thus preventing their transcription of multiple genes essential for the passage into S phase. Only once Rb is phosphorylated is E2F released and capable of initiating transcription of these genes.

Since G1 arrest can be induced by the overexpression of p21 (el-Deiry et al. 1993) and, furthermore, is abrogated in irradiated p21-deficient cells (Brugarolas et al. 1995), p21 seems to be the key effector of the G1 arrest in response to DNA damage. However, the question of whether p21 can be induced independently of p53 is not fully answered. While some groups show that p21 is solely dependent on p53 for its induction, for example in human peripheral neuroepithelial tumour cell lines (Isaacs et al. 1997), other groups maintain that p21 can be induced independently of p53. For example p21 is induced under conditions of oxidative stress in p53-null SaOS-2 cells (Russo et al. 1995) but whether this can effect a G1 arrest remains unanswered. To date all evidence suggests that G1 arrest and induction of p21 after irradiation are both strictly dependent on the presence of wild type p53 (el-Deiry et al. 1994).

In response to stimuli other than DNA damage, additional gene products do influence the G1/S checkpoint. p16 and p15, members of the INK4 family which also includes $p19^{ARF}$, have an inhibitory effect on Cdk4 and Cdk6, whose kinase activity is induced in response to growth factor stimulation during G1 (Sherr 1994). Although p15 has been shown to be induced by transforming growth factor (TGF) ß, resulting in growth inhibition (Hannon and Beach 1994), the mechanism of regulation of the

**Figure 2.4** Phase G1 of the cell cycle provides a target for mutations in GBM. The diagram illustrates the proteins essential for passage through the cell cycle. Those shown in circles are accelerators through the checkpoints and those in squares are inhibitors of cell cycle progression.




modified from Hunter and Pines 1994

INK4 family is far from clear. It does not, however, appear to be directly related to the response to DNA damage.

Genetic alterations in some of these regulatory components of the G1 checkpoint are observed in GBMs. A deletion of *RB* occurs in approximately 30% of GBMs, but is not observed in grade II or III astrocytomas. Furthermore, *RB* deletion is found in both 2° and *de novo* GBMs, not associating exclusively with one or the other group (Henson et al. 1994). The observation that *RB* is deleted in secondary GBMs, but not in grade II or III tumours suggests that changes in Rb function are critical in progression to GBM in this group.

An amplification of *CDK4* occurs in approximately 3% of GBMs, resulting in facilitated progression into S phase (He et al. 1994). Since both *CDK4* and *MDM2* are located at the chromosome site 12q13, *CDK4* amplification may purely be a spin off from the more frequent occurrence of *MDM2* amplification. While both events have the effect of deregulating the control of G1 arrest by p53, overexpressed mdm2 may well have additional consequences on p53-mediated apoptosis. As previously mentioned, however, this still only accounts for 10% of GBMs, leaving the mechanism of radioresistance in the vast majority of *de novo* GBMs unexplained.

Complementary to deletions in *RB* in GBM, genetic changes in the *INK4* genes are also observed. *p16*, *p15* and $p19^{ARF}$ reside in chromosome 9p, which has been shown to be homozygously deleted in approximately one-half of GBMs (Schlegel et al. 1996). This is mirrored by a loss of *p16* in 57% of GBMs (Ueki et al. 1996). It has been recently shown that loss of p16 function associates with *EGFR* amplification in *de novo* GBMs (Biernat et al. 1997). It is not clear, however, how such a regulatory defect would hinder G1 arrest in response to irradiation, since p16 inhibits CDK activity in parallel to p21 and is not thought to constitute part of the response pathway to DNA damage. Furthermore, it is not clear why GBM cells with loss of p16 function are not eliminated by apoptosis. This suggests that, besides disruption of cell cycle regulators such as p16 and Rb, an obstacle to apoptosis coexists in *de novo* GBMs.

Interestingly, p21 is never found to be mutated in GBMs, a finding common to all tumour types (Koopmann et al. 1995). Why this should be is unclear; perhaps loss of p21 function proves fatal to the cell?

### 2.4.3 S phase checkpoint

The S phase checkpoint is rather less well defined than the other two checkpoints and relatively little is reported in the literature regarding its control. It is responsible, however, for a rapid reduction of circa 50% in DNA synthesis immediately following irradiation (Larner et al. 1994).

It is clear that cyclin A function is required for progression through the S phase. Cyclin A associates with and activates Cdk2 during the later stages of S phase. The kinase activity of Cdk2 during S phase appears to be able to inhibit E2F, thus switching off the synthesis of the array of gene products required for DNA synthesis (Hunter and Pines 1994) and presumably permitting entry into G2.

The mechanism by which cyclin A may be inhibited to allow a checkpoint in S phase is undetermined. Intriguingly, a deregulation of cyclin A synthesis may be involved in the anchorage-independent growth seen in transformed cells, since fibroblasts expressing increased levels of cyclin A gain the capacity to grow in suspension (Guadagno et al. 1993). This suggests that cell surface adhesion molecules may normally exert some control over the S phase checkpoint.

Although gamma irradiation certainly does result in an arrest at the S checkpoint, the signal transduction pathway from DNA damage to S phase arrest is not known. There is, however, definitive evidence in fibroblasts, treated with bleomycin to induce DNA strand breaks, that neither p53 nor p21 influence arrest in S phase, since unimpaired S arrest persisted despite stable expression of mutant p53 with abrogated p21 response in the cells (Wyllie et al. 1996).

### 2.4.4 G2/M Arrest

A model for the mechanism of the G2/M checkpoint has recently been suggested on the grounds of studies in yeast, mouse and human cells (Weinert 1997, Furnari et al. 1997, Peng et al. 1997, Sanchez et al. 1997). G2 arrest is caused by inhibitory phosphorylation of Cdc2, a prominent member of the Cdk family. These recent studies indicate that Cdc2 phosphorylation results from upstream inactivation of Cdc25, the prime activator of Cdc2. The model proposes that, on detection of DNA damage, activated ATM phosphorylates the protein kinase Chk1, which in turn phosphorylates Cdc25 at its serine residue at position 216. This results in promoted binding of Cdc25 to

its sequestering protein, thus preventing dephosphorylation and activation of Cdc2 by Cdc25. Mitosis is not permissible in the presence of inactive phosphorylated Cdc2 and so the cell arrests at the boundary between G2 and M phases.

Since human cells bearing a non-phosphorylatable Cdc2 are only partially defective in G2 arrest (Jin et al. 1996), other mechanisms of control over this checkpoint probably exist. In particular, conflicting reports abound as to whether p53 exerts any regulation at the G2/M checkpoint.

G2/M arrest certainly occurs independently of p53 and p21, since irradiation of p53-null or p21-null cells successfully induces this checkpoint (Kastan et al. 1991, Waldman et al. 1996), presumably by the ATM-Chk1-Cdc25-Cdc2 pathway. Given that activation of ATM by DNA damage, however, activates p53 as well as Chk1, the question of whether p53 can influence the G2/M checkpoint remains open. For example the generation of tetraploidy and octaploidy, which is indicative of defective mitosis, has been observed in mouse fibroblasts deficient in p53, suggesting that p53 does at least have a role in monitoring M phase (Cross et al. 1995).

## 2.5 Apoptosis

It is now generally accepted that growth arrest alone is not sufficient for cancer therapy to be effective, since at some stage arrested cells will re-enter the replicative cycle. Instead, tumour cells must actually be eliminated by a treatment such as irradiation. The cure rate, for example, of murine tumours *in vivo* can be predicted by the apoptotic response of the tumour cells (Stephens et al. 1991). Clearly, apoptosis is central to the issue of radiosensitivity and, therefore, needs to be considered in some detail.

Apoptosis is a genetically encoded energy-dependent cell death programme, defined morphologically by contraction of the cytoplasm, plasma membrane blebbing, chromatin condensation and internucleosomal DNA cleavage (Wyllie et al. 1980). Apoptosis constitutes the common end-point of numerous different signal transduction pathways, whose relative importance depends on the cell type and triggering signal for apoptosis. The apoptotic response to DNA damage, however, appears to be critically dependent on p53 function, since thymocytes from p53 knock-out mice fail to enter apoptosis when irradiated (Lowe et al. 1993). Although there have been some

suggestions that a p53-independent route operates to initiate apoptosis following insults such as irradiation (Strasser et al. 1994), this is yet to be substantiated.

### 2.5.1 Caspases; the final common pathway of apoptosis

Much of our understanding regarding the mechanisms of apoptosis originates from studies on the nematode *Caenorhabditis elegans* (Ellis et al. 1991), in which the genetic programme leading to cell death has been fully elucidated. The gene products operating in *C. elegans* to effect apoptosis are highly conserved and are now facilitating identification of human homologues, including for example the caspase family.

Caspases are the 'executioners' of apoptosis, responsible for the dismantling of the cell machinery. They constitute the final common pathway of apoptosis, on which the various initiating signals converge . The caspases are cysteine proteases with aspartate specificity, and operate in a cascade which incorporates a degree of redundancy (Li et al. 1995). Ten caspases have been identified to date, grouping into three sub-families referred to as the caspase-1 (the interleukin-1ß-converting enzyme (ICE) proteases), caspase –2 and caspase-3 (also known as CPP-32) subfamilies (Nagata 1997). Activated through a proteolytic processing, they cleave specific apoptotic substrates, leading to the characteristic changes. For example, activated caspase-3 digests the inhibitor of caspase-activated DNase, ICAD, resulting in the freedom of the endonuclease, CAD, to cleave chromatin (Sakahira et al. 1998, Enari et al. 1998). This it does at internucleosomal sites, yielding the DNA fragments of regular size that constitute the pathognomic apoptotic 'DNA ladder'.

### 2.5.2 Activation of the caspases

A key molecule in effecting activation of the caspases is cytochrome c. The release of cytochrome c from the mitochondria and its relocation in the cytosol is followed by caspase activation and committment of the cell to apoptosis (Reed 1997). bax, a pro-apoptotic member of the bcl-2 family, has been shown to induce cytochrome c release from the mitochondria (Rosse et al. 1998). Conversely, escape of cytochrome c from the mitochondria is prevented by overexpression of the pro-survival members of the bcl-2 family, resulting in suppression of apoptosis (Yang et al. 1997, Kluck et al. 1997).

A number of bcl-2 family members, which reside predominantly in the outer mitochondrial membrane, have been identified in humans (Krajewski et al. 1993). While bcl-2 and bcl-$X_L$ oppose apoptosis, bax, bcl-$X_S$, bik, bak and bad promote apoptosis (Boise et al. 1993). The various members of the bcl-2 family are capable of dimerising with one another (Oltvai et al. 1993). The affinity between pro- and anti-apoptotic members is greater, however, than that between those of the same group (Sattler et al. 1997). Since dimerisation between functionally diametric members neutralises their effects on apoptosis, the ratio between inhibitors and promoters of apoptosis in a cell modulates its sensitivity to apoptosis . For example, embryonal death occurs in mice deficient in bcl-$X_L$ as a result of a shift in the balance towards apoptosis (Motoyama et al. 1995). Conversely, the apoptotic response to chemotherapeutic agents is severely impaired in bax-deficient fibroblasts (McCurrach et al. 1997) and is reduced by 50% *in vivo* in bax-deficient mice (Yin et al. 1997).

Thus, altering the balance between pro- and anti- apoptotic members of the bcl-2 family controls committment to apoptosis. This is achieved by determining the sub-cellular localisation of cytochrome c, although the mechanism by which it occurs is not known. Since the bcl-2 family of proteins can form membrane channels *in vitro* , they may participate in regulating mitochondrial permeability to cytochrome c (Reed 1997, Minn et al. 1997).

### 2.5.3 Regulation of the bcl-2 family

Returning to the issue of DNA damage, how does p53 initiate apoptosis? Although this question is hotly debated, the control exerted by p53 over the transcription of the bcl-2 family probably forms at least part of the link between DNA damage and activation of the caspases. Sequence-specific binding sites for p53 have been identified in the bax promoter (Miyashita and Reed 1995), while transcription of both bcl-2 and bcl-$X_L$ is repressed by active p53. Thus, activation of p53 by DNA damage leads to increased expression of pro-apoptotic proteins and concurrent reduction in anti-apoptotic proteins, resulting in a dramatic shift of their ratio in the favour of apoptosis.

A number of studies have indicated, however, that additional mechanisms contribute to the orchestration of apoptosis by p53. Even when the transcriptional activation of p53-inducible genes is blocked, p53-dependent apoptosis can still occur

(Haupt et al. 1995, Caelles et al. 1994). In this scenario apoptosis requires the presence of very high levels of p53 protein. This suggests that p53-mediated apoptosis relies not only on specific transcriptional activation but also biochemical properties of p53 protein itself.

The nature of this alternative mechanism(s) is unclear. The carboxy terminus of the p53 protein appears to interact with certain components of the DNA repair machinery, including DNA helicases (Oberosler et al. 1993), and p53 is even speculated to bind directly at sites of DNA damage. A coupling between certain types of DNA damage and p53 perhaps generates conformational or biochemical changes in the p53 protein, which provide a means of switching the cell into the apoptotic route.

A further level of control over the bcl-2 family is provided by phosphorylation. The ability of bad to bind and inhibit bcl-$X_L$ is lost when bad is phosphorylated, thus allowing bcl-$X_L$ freedom to promote cell survival (Zha et al. 1996). Whether p53 influences phosphorylation of the bcl-2 family is not known.

### 2.5.4 TRAIL and p53-mediated apoptosis

The **T**NF **r**elated **a**poptosis-**i**nducing **l**igand TRAIL is a member of the tumour necrosis factor (TNF) family of death-inducing ligands, whose binding to specific cell surface receptors, including DR5 and DR4, rapidly induces apoptosis (Sheridan et al. 1997, Pan et al. 1997). Intriguingly, tumour cells are far more susceptible to the death signal from TRAIL than normal cells, a fact explained recently by the identification of a 'decoy' receptor for TRAIL (Sheridan et al. 1997). Expression of the 'decoy' receptor, named TRID (**T**RAIL **r**eceptor without an **i**ntracellular **d**omain), is apparently restricted to normal tissues, since it has not been detected in a single tumour cell line so far investigated (Pan et al. 1997). Although the TRID protein contains the external TRAIL-binding region and a stretch of anchoring amino acids, it lacks the intracellular domain essential for initiating the signal leading to caspase activation.

Until recently this apoptotic pathway was regarded as being independent of the response to DNA damage, but another experimental approach has now demonstrated a link to p53. Screening of p53-dependent DNA damage-inducible genes has revealed the gene *KILLER*, named to reflect its role in apoptosis, which turns out to be identical to *DR5* (Wu et al. 1997). DNA damage induces KILLER/DR5 in a p53-dependent manner, a response which was abrogated in tumour cells bearing mutant p53. It is still unclear

whether this effect is direct or mediated by the transactivation activity of p53.

Thus, p53 appears to be able to influence the TRAIL pathway to apoptosis through its effects on KILLER/DR5. This, in addition to a speculative simultaneous inhibition by p53 of TRID, provides an attractive hypothesis for how wt p53 might try to maximise apoptosis in tumour cells. It remains to be answered whether p53-mediated apoptosis is functionally dependent on induction of KILLER or whether it operates in parallel with other p53-mediated apoptotic controls, such as the bcl-2 family.

## 2.6 The need for a deeper scientific understanding

The overview provided here hopefully shows that, while much has been learnt in recent years, the molecular mechanisms by which tumour cells evade apoptosis are unclear. GBM is one of the most malignant tumours to affect humans and, therefore, merits intensive study. The proven beneficial effect offered to GBM patients by adjuvant radiotherapy is encouraging but limited. The therapeutic challenge is to find ways of improving the radiosensitivity of GBMs, but only by extending our scientific understanding of the molecular mechanisms leading to radioresistance in GBMs, can we hope to fulfil this aim.

# 3. AIM OF THE PROJECT

A clearer understanding of the cellular mechanisms involved in the response to ionising radiation is pivotal to the development of new therapeutic strategies for GBM. In order to gain further insight into dynamic functional aspects of cell cycle regulation and the control of apoptosis, this project aims to investigate the molecular changes induced by ionising radiation in molecularly characterised primary cell cultures from both *de novo* and secondary GBMs.

While p53 mutation accounts for the genetic instability and radioresistance seen in secondary GBM, current understanding of the genetic alterations occurring in *de novo* GBM does not account for their equally pronounced genetic instability and radioresistance. In the absence of p53 mutation, these features are indicative of defective functioning of the p53 pathway. Functional comparison of the radiation responses in secondary GBMs and *de novo* GBMs *in vitro* aims to reveal defects in the regulatory pathways controlling the cell cycle and apoptosis in *de novo* GBMs, and thus identify putative radioresistance mechanisms.

This project will investigate changes in expression of p53, its upstream regulator ATM, downstream effectors of cell cycle arrest, p21 and gadd45, apoptosis regulators, including the bcl-2 family and the TRAIL pathway and finally, the inhibitor of p53, mdm2, occuring after irradiation of asynchronous primary cultures.

Since understanding of this pathway remains rudimentary, insights into general functional aspects of the cellular response to genotoxic stress may be gained from this model system.

# 4. MATERIALS AND METHODS

## 4.1 Materials

### 4.1.1 Chemicals and equipment

All chemicals originated from Boehringer Ingelheim Bioproducts, Ingelheim, Germany, unless otherwise stated. Sterile plastic ware for cell culture was obtained from Falcon, New Jersey, NJ, USA. All medium (DMEM and RPMI), FCS, trypsin, glutamine, penicillin and streptomycin originated from Gibco-BRL, Karlsruhe, Germany.
*Taq* DNA polymerase and RNeasy RNA isolation kits were supplied by Qiagen, Hilden, Germany. Takara Ex *Taq* DNA polymerase, used for the nested polymerase chain reaction (PCR) amplification of mdm2 splice variants was obtained from Takara Biomedicals, Shiga, Japan. Random hexanucleotide primers, Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase, 2‘-deoxynucleoside 5‘-triphosphate (dNTPs) set, DNA and protein size markers came from Boehringer, Mannheim, Germany. Agarose originated from Roth, Karlsruhe, Germany.
All reagents for Bradford protein assays were obtained from Biorad, München, Germany. X-ray film X-AR was supplied by Eastman Kodak, Rochester,USA.
Polyvinylidene difluoride (PVDF) Immobilon membranes for Western blots were supplied by Millipore, Bedford, MA, USA.
Mutation detection enhancement (MDE) gel concentrate for use in SSCP analysis was supplied by J.T. Baker Inc., Phillipsburg, NJ, USA.
The cytotoxicity test „Cell Titer 96$^{TM}$ Non-radioactive Cell Proliferation Assay“ was obtained from Promega, Madison, WI, USA.
Direct DNA sequencing was performed by Seq Lab, Göttingen, Germany.

The following pieces of equipment were used;
Cells were cultured in a Stericult 200 incubator. PCR reactions were performed in a PTC-100 thermocycler from MJ Research Inc., Watertown, Mass., USA.
Gel electrophoresis chambers from Biorad, Hercules, CA,USA and power units from Pharmacia LKB, Freiburg, Germany were used.

### 4.1.2 Cell lines and primary cell cultures

The human glioblastoma cell lines LN 229, LN 18 and LN Z308 were kind gifts from Dr. E. van Meir, Lausanne, Switzerland (Van Meir et al. 1994). They have well defined p53 status and were used in this project as controls. LN 229 bears wt p53. LN 18 carries an heterozygous dominant negative point mutation in codon 238 of exon 7, whereby TGT is replaced by TCT, resulting in a serine instead of a cysteine residue. LN Z308 bears a rearrangement in p53, such that a small amount of a truncated p53 RNA transcript but no p53 protein is expressed.

Primary cell cultures were established from a total of twenty one human glial tumours and from normal fibroblasts by K. Münkel and Dr. B. Eifert, Department of Neurosurgery, University of Heidelberg, Germany. The selected tumours included one grade II astrocytoma, one grade II oligoastrocytoma, one grade III astrocytoma, one grade III oligoastrocytoma, six secondary GBMs and eleven *de novo* GBMs. The mean patient age was 51.7 years (range 28-75 years) and 62% of the patient group were male. The primary cultures of fibroblasts, being non-neoplastic cells which form an adherent monolayer *in vitro*, provided a normal control in irradiation experiments.

### 4.1.3 Buffers and solutions

The following standard buffers were used :

| | |
|---|---|
| TE (pH 8.0) : | 10mM tris (pH 8.0), 1mM EDTA (pH 8.0) |
| TAE (pH 8.0) : | 40mM tris-acetate, 2mM EDTA (pH 8.5) |
| TBE : | 89mM tris, 89mM boric acid, 0.2mM EDTA |
| TNE : | 50mM tris-Cl, 140mM NaCl, 5mM EDTA |
| PBS (pH 7.4): | 137mM NaCl, 2.7mM Kcl, 4.3mM $Na_2HPO_4$, 1.4mM $KH_2PO_4$ |
| loading buffer : | 95% formamide, 10mM NaOH, 0.05% bromphenol blue, 0.05% xylene cyanol |
| triton X-100 lysis buffer : | 50mM tris-Cl, 5mM EDTA, 1% triton X-100, 150mM NaCl, 1mM PMSF, 80ng/ml apoprotein, 50ng/ml leupeptin, 4ng/ml pepstatin |

### 4.1.4 Polymerase chain reaction primers

The oligonucleotides listed below were used in PCR at the specified annealing temperatures. All primers were synthesised by MWG-Biotech, Ebersberg, Germany.

| Primer name | Locus | Product length (basepair) | Annealing temp. (°C) | Forward primer sequence<br>Reverse primer sequence |
|---|---|---|---|---|
| hATM | ATM | 218 | 53 | AGAGCCAAAGAGGAAATAGG<br>AAGCCAGAGGGAACAAAG |
| hp53 | p53 | 83 | 48 | GCTTTGAGGTGCGTGTT<br>GTGAGGCTCCCCTTTCT |
| hp21 | p21 | 285 | 52 | GCAGTGTGTCGGGTGAAG<br>CTCAGCAAGCAACGAAGTG |
| hgd45 | GADD45 | 162 | 50 | CAAGTGACAGCCCGATTAT<br>TTGTTACCCTGACCTGAGTG |
| hbax | BAX | 132 | 49 | GATGCGTCCACCAAGAAGC<br>TGCCACTCGGAAAAAGACC |
| hbcl-2 | BCL-2 | 174 | 53 | CACCAAGAAAGCAGGAAAC<br>CAGGATAGCAGCACAGGA |
| hbcl-x | BCL-XL | 245 | 58 | CCCCAGGGACAGCATA<br>CCCATAGAGTTCCACAAAAGT |
| hTRAIL | TRAIL | 232 | 52 | TGAGGAATGGTGAACTGGT<br>CCCCCTTGATAGATGGAA |
| hKILLER | KILLER | 218 | 52 | GCACCACGACCAGAAAC<br>AAACACAGCCACAATCAAGA |
| hTRID | TRID | 151 | 54 | TGTGCTTCTGATTGTGTTTG<br>GCGTTTCTGTCTGTGGG |
| hMDM2 | MDM2 | 143 | 55 | GAGGGCTTTGATGTTCCTGA<br>GCTACTAGAAGTTGATGGC |
| hDRD | dopamine receptor | 113 | 55 | CCACTGAATCTGTCCTGGTATG<br>GTGTGGCATAGTAGTTGTAGTGG |
| hmdm2ex | MDM2 (external) | 1573 | 55 | CTGGGGAGTCTTGAGGGACC<br>CAGGTTGTCTAAATTCCTAG |
| hmdm2in | MDM2 (internal) | various | 55 | CGCGAAAACCCCGGGCAGGCAAATGTGCA<br>CTCTTATAGACAGGTCAACTAG |
| hPTEN | PTEN | 352 | 52 | AAGACCATAACCCACCACA<br>CCACTGAACATTGGTATAGTTT |
| hßglob | ß globin | 500 | 59 | GGTTGGCCAATCTACTCCCAGG<br>GCTCACTCAGTGTGGCAAAG |
| D7S8 | EGFR | 120 | 58 | GGAATGCAACTTCCCAAAATGTGCC<br>ACAGCCATGCCCGCATTGGCTCTAA |
| D7S12 | ß actin | 180 | 61 | CTTGATGAGGTAGTCAGTCAGGTCC<br>TATCCAGGCTGTGCTATCCCTGTAC |
| p3/4 | p53 exon 5 | 183 | 60 | CTTTCAACTCTGTCTCCTTCCTCTTCCTACAGTACT<br>GTCGTCTCTCCAGCCCCAGCTGCTCACCATCGCTAT |
| p13/14 | p53 exon 6 | 112 | 60 | TTGCCCAGGGTCCCCAGGCCTCTGATTCCTCACTGA<br>ACTGCTCACCCGGAGGGCCACTGACAACCACCCTTA |
| p5/6 | p53 exon 7 | 109 | 60 | CCTCATCTTGGGCCTGTGTTATCTCCTAGGTTGGCT<br>CCAGTGTGCAGGGTGGCAAGTGGCTCCTGACCTGGA |
| p7/8 | p53 exon 8 | 136 | 60 | CTCTTGCTTCTCTTTTCCTATCCTGAGTAGTGGTAA<br>CCTCCACCGCTTCTTGTCCTGCTTGCTTACCTCGCT |

### 4.1.5 Antibodies

A polyclonal rabbit anti-p53 antibody and an horse radish peroxidase (HRP)-conjugated anti-rabbit antibody, supplied by Innovative Diagnostik Systeme, Hamburg, Germany, were used in Western blotting.

## 4.2 Methods

### 4.2.1 Cell culture

Cell lines LN 229, LN 18 and LN Z308 were cultured as monolayers in DMEM with 10% FCS, 2 mM L-glutamine, penicillin (100U/ml) and streptomycin (100µg/ml). All primary cell cultures were maintained as monolayers in RPMI , supplemented as described above. Cells were grown at 37°C in 5% $CO_2$ . The passaging time, defined below, for each of the primary cultures and the cell lines was estimated as an indication of rate of cell growth. Where the cultures were beyond their first passage, the passaging time was defined by the number of days in culture between seeding the cells to 30% confluence and their attainment of 80% confluence. At this point in time the primary cultures were sub-cultivated.

### 4.2.2 Radiation treatment

Irradiation of cells was kindly performed in the Department of Radiotherapy, University of Marburg (Director: Prof. Dr. med. R Engenhart-Cabillic). Each of the cultures established from patient tumour material was subjected to irradiation within the first four passages of cell culture. Standard cell conditions were maintained by performing a medium change 24h before treatment and irradiating at a set time to avoid the effects of diurnal variation. Proliferating cells in 6cm petri dishes containing 3ml medium were γ irradiated using a cobalt $^{60}Co$ source (1.3 MeV) at a calibrated radiation dose of 1.470 Gy/min. Dosimetry was fully calibrated before commencing the experimental protocol. A dose response curve was performed on fibroblasts, LN 229 and LN 18 at measurement points from 1 to 12 Gy. On the grounds of these results, 10 Gy were chosen as the experimental dose in this study.

From non-radiated controls and radiated cells at time 2h and 8h after irradiation, RNA and protein were extracted (see below). At 24h after irradiation, irradiated and non-radiated control cells were fixed in 70% methanol for flow cytometry analysis of the cell cycle status and were subjected to a cytotoxicity test.

### 4.2.3 Extraction techniques

#### 4.2.3.1 DNA extraction

DNA extraction was performed according to standard protocol (Sambrook et al. 1989). Briefly, after washing confluent cells twice in PBS, 3ml TNE were applied to the cells. After a 10 min incubation at room temperature, a cell scraper was used to mechanically dislodge the cells. Incubation with Proteinase K (100µg/ml) and 0.5% SDS at 56°C overnight led to nuclear lysis. Finally DNA was purified in a phenol-chloroform-isoamylalcohol solution (25:24:1) and precipitated in ice-cold ethanol. DNA was dissolved in TE and stored at 4°C.

#### 4.2.3.2 RNA extraction

RNA was extracted from cells using a RNeasy kit, according to the supplier's protocol. Cell material was lysed under highly denaturing conditions to inactivate RNases and diluted in a high salt buffer system. The sample was applied to a spin column containing a silica gel-based membrane, which bound total RNA during the subsequent washes. RNA was eluted in RNase-free water.

#### 4.2.3.3 Protein extraction

According to standard protocol (Sambrook et al. 1989), after two washes in cold PBS the proliferating cells were incubated for 10 min on ice in triton X-100 lysis buffer. Cell debris was removed by centrifugation (10000 rpm, 10 min, 4°C) and the supernatent was stored at -80°C.

### 4.2.4 Single stranded conformation polymorphism analysis

100ng of genomic DNA was used for PCR in a 50µl reaction volume containing 0.3µM primer, 10mM Tris-Cl, 75mM KCl, 1.5mM $MgCl_2$, 200µM each dNTP, 0.025µCi $^{33}$PdCTP and 1U *Taq* DNA polymerase with primers for exons 5 to 8 of the p53 gene, as listed. The PCR programme ran at 94°C for 5 min, 60°C for 3 min and 72°C for 3 min for 1 cycle, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. 8µl of the amplification product was added to 20µl of loading buffer and heated at 94°C for 5 min. The denatured DNA was then separated on a 0.5 x Mutation Detection Enhancement (MDE) gel at 4°C, 2 Watts overnight. Gels were dried and exposed to X-ray film for 24 h.

### 4.2.5 Western blot analysis

Protein concentration was measured according to the Bradford protocol (Sambrook et al. 1989). 10µg of protein was loaded onto a 10% polyacrylamide/SDS gel and electrophoresed at 110V constant voltage. Proteins were transferred to a PVDF Immobilon membrane at 200mA for 90 min. The membrane was blocked for 1 hour by 5% skimmed milk powder in TTBS and incubated overnight at 4°C with rabbit polyclonal anti-p53 antibody diluted 1:1000 in TTBS. The membrane was incubated for 1 hour at room temperature with an HRP-conjugated anti-rabbit IgG antibody diluted 1:2000, before incubation for 60 sec with Lumiglo Reagent (20 x concentrate). The membrane was then wrapped in clingfilm and exposed to an X-OMAT MA-film. Expression of p53 protein was analysed according to a semi-quantitative scale, whereby zero protein detection was scored as ‘0’, modest protein detection as ‘+’, marked protein expression as ‘++’ and very pronounced protein expression as ‘+++’.

### 4.2.6 Polymerase chain reactions

PCR was performed using 200ng DNA in a 50µl reaction volume containing 0.3µM primer, 10mM Tris-Cl, 75mM KCl, 1.5mM $MgCl_2$, 200µM each dNTP and 1U *Taq* DNA polymerase. The reaction mixture was heated at 95°C for 5 min to denature the DNA and then subjected to 35 cycles of PCR (94°C for 1 min, specific annealing temperature for 1 min, 72°C for 2 min) followed by a final extension at 72°C for 5 min.

Protocols differing from this standard protocol are described below.

#### 4.2.6.1 Differential polymerase chain reaction for *MDM2* amplification

200ng of genomic DNA was amplified in a differential PCR reaction of 25µl volume, containing 10mM Tris-HCl, 75mM Kcl, 1.5mM $MgCl_2$, 200µM dNTPs, 0.5µM primers for both *MDM2* and the reference dopamine receptor gene, *DRD2*, together with 1U *Taq* DNA polymerase, as previously described (Biernat et al. 1997). DNA denaturation at 95°C for 5 min was followed by 25 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 2 min) and a final extension at 72°C for 5 min. Equivalent or stronger expression of *MDM2* with respect to *DRD2* was regarded as positive indication of *MDM2* amplification.

#### 4.2.6.1 Reverse transcription polymerase chain reaction (RT-PCR)

First strand synthesis was carried out using 250ng RNA in a final volume of 20µl containing 50mM Tris-HCl, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 250µM each dNTP, 0.5µl of hexanucleotide mix and 100U M-MLV reverse transcriptase at 37°C for 2 h. 2.5µl of the first strand reaction was subsequently used for each PCR using primers and conditions as listed in Section 4.1.4. 0.3µM final primer concentration was used in a reaction mix as described above. The number of cycles was limited to 28 to ensure a linear amplification of the PCR products.

#### 4.2.6.2 RT-PCR for mdm2 splice variants

A nested PCR method was used in order to detect specifically mdm2 splice variants, based on a published protocol (Sigalas et al. 1996). 5µl of first strand, generated as described above, was used in a 50µl PCR reaction mix containing 1 x Takara Ex reaction buffer, 1.5mM $MgCl_2$, 0.6µM hmdm2ex primer pair, 200µM each dNTP and 1U Takara Ex *Taq* DNA polymerase. The PCR was carried out for 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2.5 min, followed by an extension at 72°C for 5 min. The second round of the nested PCR was performed under identical conditions with 0.6µM hmdm2in primers and 5µl of the first round PCR reaction mixture. The products of the second PCR were analysed by electrophoresis on a 1%

agarose gel and visualised by ethidium bromide (EtBr) staining and ultraviolet (UV) translumination.

### 4.2.7 Electrophoresis

#### 4.2.7.1 Agarose gel electrophoresis

Samples in loading buffer were applied to polymerised 0.5 - 1 % agarose in 1 x TAE in a horizontal gel chamber. Electrophoresis was performed with maximal 10V/cm and 1 x TAE running buffer. Thereafter the agarose gel was stained with 0.01% EtBr for 5 min and excess was washed off in distilled water for 30 min, before visualising and documenting the nucleic acid fragments with a UV light source. Fragment size was determined by comparison to a standard marker ladder.

#### 4.2.7.2 PAGE and silver staining

10µl of PCR product was added to 2µl of loading buffer and analysed by non-denaturing PAGE (12.5% polyacrylamide) at 125V for 1 h. Gels were silver stained by fixing in 10% ethanol (5 min), oxidising in 1% nitric acid (3 min), incubating in 12mM silver nitrate (20 min) and reducing in a solution containing 0.28M sodium carbonate and 0.019% formalin until bands appear. The reaction was stopped with 10% glacial acetic acid (2 min). The gel was then placed in distilled water (2 min), before drying on Whatman paper. Fragment size was determined by comparison to a standard marker ladder.

### 4.2.8 Flow cytometry

At 24h after treatment, asynchronous irradiated cells and non-radiated control cells were fixed in ice-cold 70% methanol. Flow cytometric measurements were kindly performed in the Institute of Pathology, University of Regensburg, Germany (Director: Prof. Dr. med. F. Hofstädter), where the alcohol fixed cells were incubated with 5µg RNase at 37°C for 1h and then stained with 0.05 mg/ml propidium iodide. For flow cytometric analysis, fluoresence was excited using an air-cooled argon ion laser (emission wavelength 488 nm, output power 40 mW). Data evaluation started with

elimination of doublets and debris on a dot plot of the propidium iodide signals pulse area versus pulse width. Univarate cell cycle analysis was performed with the Phoenix flow systems MULTIPLUS software. Measurements for the cell lines were performed in triplicate and for primary cell cultures in duplicate, where possible given the limited quantity of material, to ensure reproducibility. The results were calculated as means of the replicates. Where two stem cell lines were present within the cell sample, the mean of both sets of percentage values was taken. The data were analysed in the context of the collected results by the author.

### 4.2.9 Cytotoxicity test

At 24h after treatment irradiated cells and non-radiated control cells were trypsinised and resuspended at a cell concentration of 1 x $10^5$ /ml. 100µl of this cell suspension was dispensed into each replicate of 8 wells in a 96 well plate. 15µl of a tetrazolium-containing dye solution were added to each well and incubated at 37°C in a humidified 5% $CO_2$ atmosphere. After 4h 100µl of a Solubilisation/Stop solution was added per well, to solubilise the formazan product converted from tetrazolium by living cells. After a 1h incubation period at 37°C in a humidified 5% $CO_2$ atmosphere and careful mixing, the absorbance was read at 570nm wavelength using an ELISA plate reader. The mean percentage reduction in absorbance between the non-radiated and the irradiated samples, each of which had been in culture for identical time periods, was taken to represent the percentage of cell death within the irradiated samples.

### 4.2.10 Statistical analysis

Statistical analysis of frequency distributions was performed using the Chi-squared test. In view of the small fields of frequency, the Fisher's Exact variant of the Chi-squared test was used according to a standard statistical programme.

# 5. RESULTS

## 5.1 Clinical Parameters

Primary cell cultures were established from a total of 21 glial tumours. As shown in Table 5.1, the selected tumours included one grade II astrocytoma, one grade II oligoastrocytoma, one grade III astrocytoma, one grade III oligoastrocytoma, 6 secondary GBMs and 11 *de novo* GBMs, providing representation of the spectrum of malignant glial tumours. In two cases the tumour had been subjected to radiation therapy before being established in culture (NCH 135 and NCH 128). In all other cultures the tumour cells were naive to the effect of ionising irradiation.

Overall, the mean patient age was 51.7 years, with a range from 28 to 75 years. The secondary GBM and *de novo* GBM patient groups had mean ages of 48.3 years and 54.2 years respectively. 62% of the entire patient group were male. An even distribution of gender was observed in the secondary GBMs and grade II and III glial tumours. A male preponderance (9:2) was seen in the *de novo* GBM group. Over a partially retrospective observation period of 84 months, the length of survival from the time of initial diagnosis for patients with grade II and III tumours ranged from 13 to 84 months and all four patients survived beyond the end of the observation period. The mean survival time for patients with secondary GBMs from the time of their diagnosis with a grade II or III tumour was 31.8 months (n=6), whereby the statistics for survival time after progression to GBM were unfortunately not available. 3 of these 6 patients survived beyond the end of the observation period. The mean survival from the time of diagnosis of *de novo* GBMs was 10.9 months (n=11) and 2 of the 11 patients survived beyond the end of the observation period.

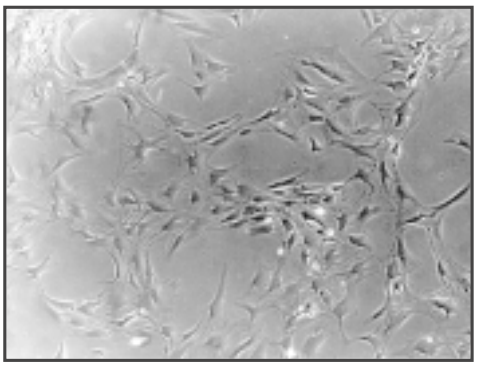
## 5.2 Cell morphology and growth characteristics

The cell morphology in the primary cultures was highly variable, as illustrated in Figures 5.2.1 and 5.2.2. The typical features of relatively large, markedly pleomorphic glial cells with prominent cell body extensions were demonstrable in culture. The glial tumour primary cultures rarely grew to more than 80% cell confluence. No consistent morphological characteristics distinguishing between either the different grades of glial tumour, nor between secondary and *de novo* GBMs could be determined.

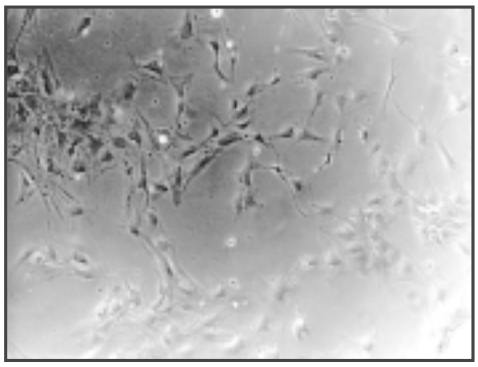
**Table 5.1** shows the clinical data for the patients from which primary cell cultures of glial tumours were established. In this and following tables and figures, astrocytoma and oligoastrocytoma are abbreviated to A and OA, respectively, and tumour WHO grade is shown by Roman numerals. Survival beyond the end of the observation period is indicated by the superfix 'L' after the stated length of survival.

| Tumour | Diagnosis | Patient age (years) | Patient sex | Previous radiotherapy | Survival time (months) |
|---|---|---|---|---|---|
| G118 | OA II | 61 | F | - | 31$^{L}$ |
| G156 | A II | 50 | F | - | 15$^{L}$ |
| G114 | A III | 56 | F | - | 13$^{L}$ |
| NCH 135 | OA III | 32 | M | yes | 84$^{L}$ |
| G101 | 2° GBM | 62 | F | - | 22 |
| G109 | 2° GBM | 57 | M | - | 5 |
| G150 | 2° GBM | 63 | F | - | 12$^{L}$ |
| NCH 59 | 2° GBM | 46 | F | - | 74 |
| NCH 105 | 2° GBM | 28 | M | - | 19$^{L}$ |
| NCH 128 | 2° GBM | 34 | M | yes | 59$^{L}$ |
| G100 | *de novo* GBM | 46 | M | - | 13 |
| G112 | *de novo* GBM | 56 | M | - | 12 |
| G113 | *de novo* GBM | 50 | F | - | 1 |
| G116 | *de novo* GBM | 42 | F | - | 9 |
| G117 | *de novo* GBM | 49 | M | - | 23$^{L}$ |
| G120 | *de novo* GBM | 64 | M | - | 17 |
| G122 | *de novo* GBM | 49 | M | - | 10 |
| G139 | *de novo* GBM | 63 | M | - | 6 |
| G145 | *de novo* GBM | 58 | M | - | 9 |
| G147 | *de novo* GBM | 75 | M | - | 6 |
| G148 | *de novo* GBM | 44 | M | - | 14$^{L}$ |

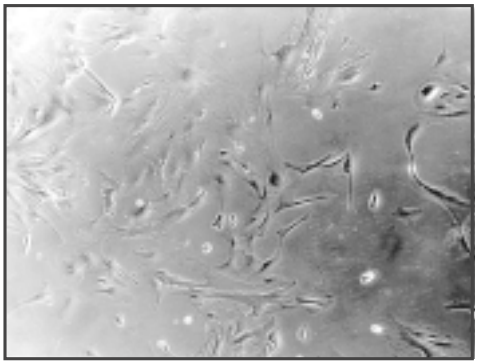
**Figure 5.2.1** Morphology of primary cell cultures established from grade II and grade III astrocytomas and oligoastrocytomas



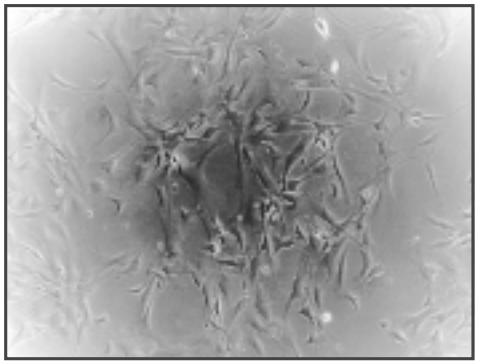
G156
(A II)



G118
(OA II)



G114
(A III)



NCH 135
(OA III)

**Figure 5.2.2** Comparison of the morphology of primary cell cultures from secondary versus *de novo* glioblastomas

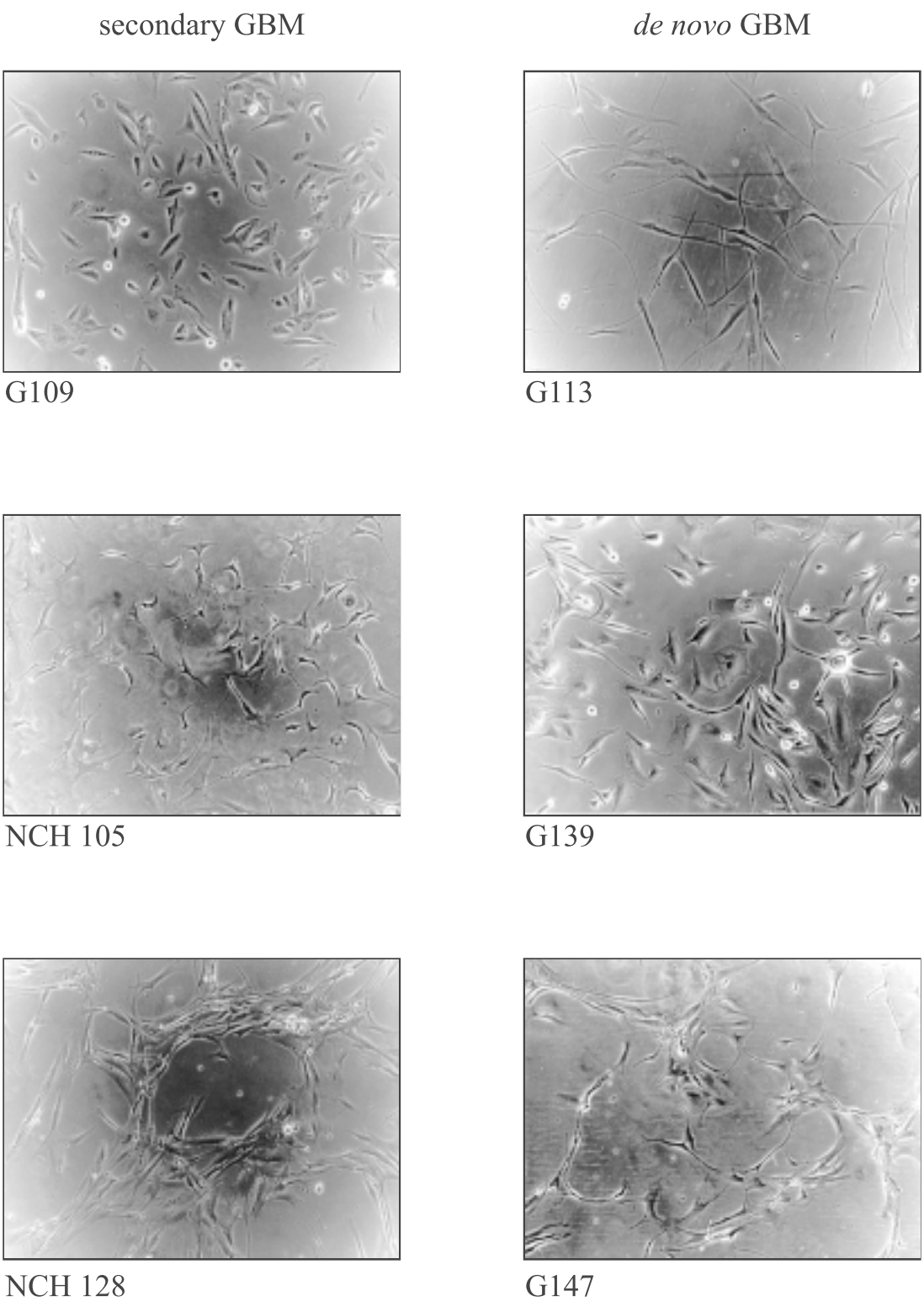
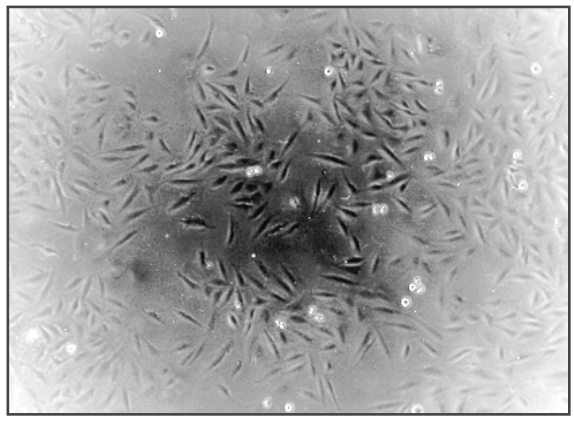
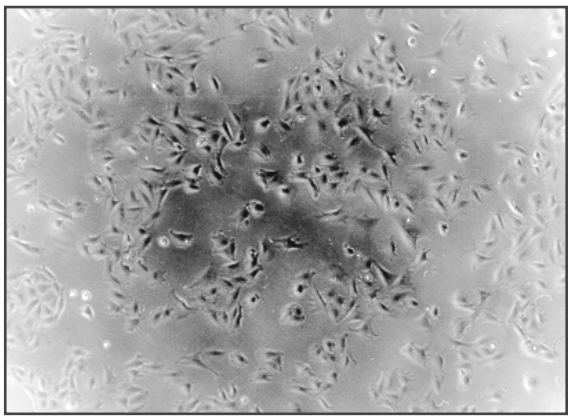


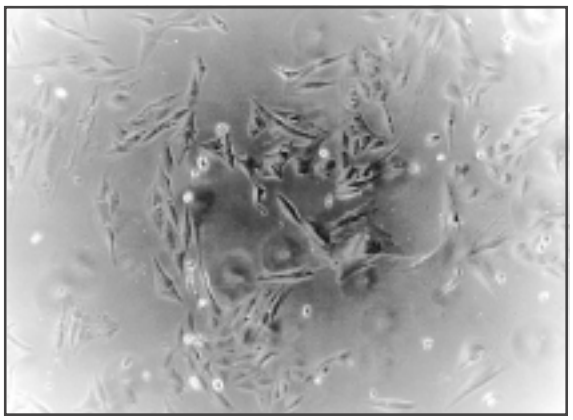
**Figure 5.2.3** Morphology of glioblastoma cell lines LN 229 (wt p53), LN 18 (mutant p53) and LN Z308 (absent p53)



LN 229



LN 18



LN Z308

**Table 5.2** shows the passaging time (days in culture required for proliferation from 30% to 80% cell confluence for cells beyond the first passage) *in vitro* of the primary glial cultures and GBM cell lines. The passage in which cells were exposed to irradiation during the course of the experiments is also shown.

| Tumour culture | Diagnosis | Passaging time (days) | Cell passage irradiated |
|---|---|---|---|
| G118 | OA II | 4 | 4 |
| G156 | A II | 2 | 2 |
| G114 | A III | 5 | 4 |
| NCH 135 | OA III | 1 | 4 |
| G101 | 2° GBM | 10 | 4 |
| G109 | 2° GBM | 3 | 2 |
| G150 | 2° GBM | 2 | 3 |
| NCH 59 | 2° GBM | 1 | 4 |
| NCH 105 | 2° GBM | 3 | 4 |
| NCH 128 | 2° GBM | 1 | 4 |
| G100 | *de novo* GBM | 3 | 4 |
| G112 | *de novo* GBM | 7 | 3 |
| G113 | *de novo* GBM | 2 | 2 |
| G116 | *de novo* GBM | 2 | 3 |
| G117 | *de novo* GBM | 2 | 3 |
| G120 | *de novo* GBM | 3 | 4 |
| G122 | *de novo* GBM | 7 | 2 |
| G139 | *de novo* GBM | 5 | 4 |
| G145 | *de novo* GBM | 2 | 3 |
| G147 | *de novo* GBM | 3 | 2 |
| G148 | *de novo* GBM | 4 | 2 |
| LN 229 | GBM cell line, wt p53 | 2 | 271 |
| LN 18 | GBM cell line, mutant p53 | 1 | 545 |
| LN Z308 | GBM cell line, absent p53 | 4 | 82 |

The morphology of the GBM cell lines LN 229, LN 18 and LN Z308 differed significantly from that of the primary cell cultures, as illustrated in Figure 5.2.3. The cells were smaller, more uniform in size and shape, with less pronounced cell body extensions, and grew to full confluence. The three GBM cell lines were of value as a form of control in the following experiments due to their well defined p53 status.

Table 5.2 shows the passaging time for each of the primary cultures and the cell lines, defined by the number of days between seeding to 30% confluence and attainment of 80% confluence. Passaging times ranged from a minimum of one day to a maximum of ten days. No significant trend in the *in vitro* passaging time was observed in relation to increasing grade of tumour. Also shown in the table are the passages in which each tumour culture underwent irradiation during the experiments.

## 5.3 Molecular characterisation

### 5.3.1 p53 mutational SSCP band shifts

SSCP analysis of exons 5 to 8 of the p53 gene revealed band shifts in 8 out of 21 primary cell cultures of glial tumours, including 6 out of 17 GBMs. All but one of the mutational band shifts were detected in exon 8, which is regarded as the mutation 'hot spot' area of p53. The exception to this finding was G101, in which a band shift was observed in exon 6. Figure 5.3.1 provides an example of an SSCP gel for exon 8, in which band shifts in both directions relative to the wt p53 pattern are illustrated. Table 5.3 summarises the molecular characterisation of the glial tumour primary cultures.

An SSCP band shift indicative of p53 mutation was displayed in all GBMs which had progressed from a grade II or III glial tumour, and in the grade II and III astrocytomas, G156 and G114. No band shift was observed in exons 5 to 8 in either of the mixed oligoastrocytic tumours, G118 and NCH 135, nor in any of the 11 cases of *de novo* GBM.

**Figure 5.3.1** SSCP analysis for exon 8 of p53 showing band shifts indicative of mutations in the positive control (PK, mutation in codon 273) and primary cultures NCH 128, G150, NCH 59, G114 and G109 (from left to right indicated by arrows)

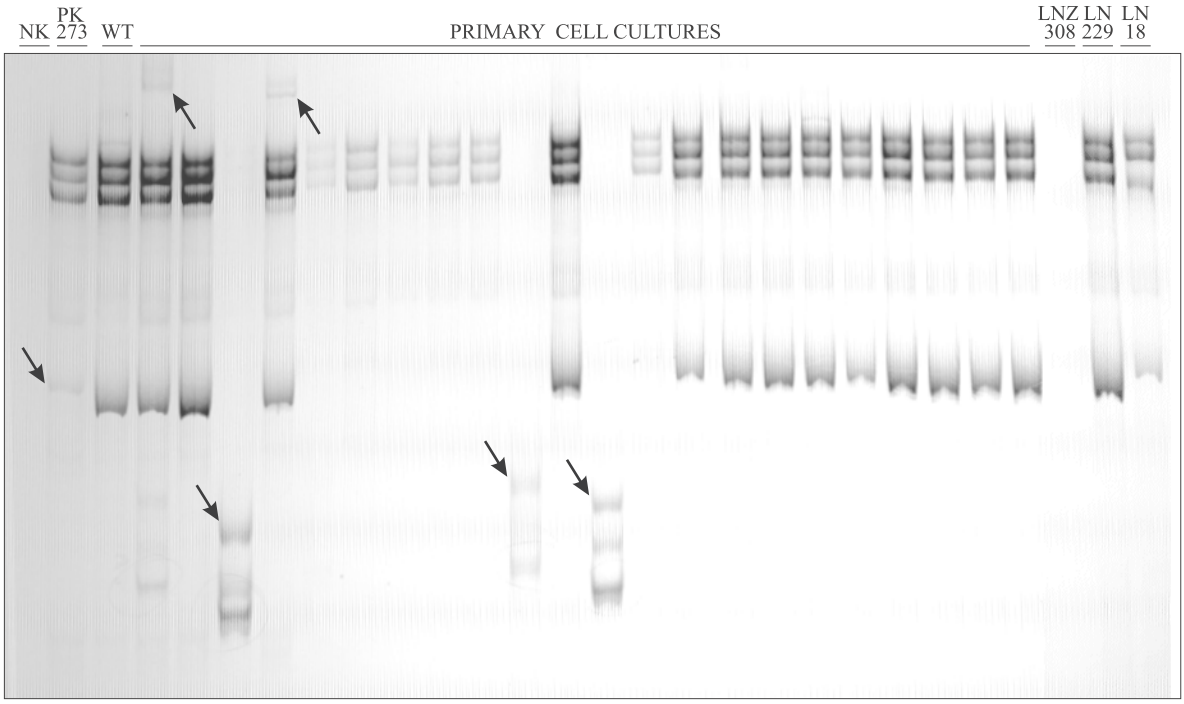


**Table 5.3** shows the results of molecular characterisation. SSCP analysis for p53 showed either a wt pattern, represented by 'wt', or a band shift in the noted exon, suggestive of a mutation. The level of p53 protein expression is represented by '0'; zero, '+'; modest, '++'; marked and '+++'; very pronounced protein expression. Presence of *MDM2* amplification or mdm2 splice variants is indicated by '+' in their respective columns, the number of crosses indicating the number of different sized splices detected in the latter. 'N.D.' indicates sample not tested.

| Tumour | Diagnosis | p53 SSCP band shifts | p53 protein expression | *MDM2* amplification | mdm2 splice variant |
|---|---|---|---|---|---|
| G118 | OA II | wt | + | - | + |
| G156 | A II | ex.8 | 0 | - | - |
| G114 | A III | ex.8 | ++ | - | + |
| NCH 135 | OA III | wt | ++ | - | N.D. |
| G101 | 2° GBM | ex.6 | + | - | - |
| G109 | 2° GBM | ex.8 | + | + | - |
| G150 | 2° GBM | ex.8 | + | - | + |
| NCH 59 | 2° GBM | ex.8 | 0 | - | - |
| NCH 105 | 2° GBM | ex.8 | 0 | - | N.D. |
| NCH 128 | 2° GBM | ex.8 | 0 | - | - |
| G100 | *de novo* GBM | wt | + | - | + |
| G112 | *de novo* GBM | wt | + | - | + |
| G113 | *de novo* GBM | wt | + | - | + |
| G116 | *de novo* GBM | wt | ++ | + | + |
| G117 | *de novo* GBM | wt | 0 | + | - |
| G120 | *de novo* GBM | wt | 0 | - | - |
| G122 | *de novo* GBM | wt | 0 | - | - |
| G139 | *de novo* GBM | wt | +++ | - | + |
| G145 | *de novo* GBM | wt | ++ | - | + |
| G147 | *de novo* GBM | wt | 0 | - | - |
| G148 | *de novo* GBM | wt | ++ | - | - |
| LN 229 | GBM cell line | wt | +++ | + | ++ |
| LN 18 | GBM cell line | ex.7 | +++ | - | + |
| LN Z308 | GBM cell line | absent | 0 | + | - |

### 5.3.2 p53 protein expression

Under non-radiated conditions, p53 protein was detected in 13 of the 21 glial tumour primary cultures. Very pronounced basal p53 protein expression was detected in the GBM cell lines LN 229 and LN 18. No p53 protein was detectable in LN Z308. In 4 of the 8 primary cultures in which p53 showed mutational band shifts on SSCP analysis, p53 protein was detectable by Western blot analysis. In 9 of the 13 cultures where a wt p53 SSCP pattern was present, p53 protein was detectable. 5 of these 9 cases showed either marked or very pronounced p53 protein expression, assessed according to the semi-quantitative scale described previously. Figure 5.3.2 shows that p53 protein expression was particularly pronounced in the *de novo* GBM cultures G116 and G139, where it was quantitatively equivalent to the expression of wt p53 protein in the GBM cell line LN 229. This level of p53 protein accumulation was greater than that observed in any of the primary cultures established from secondary GBMs. The results are summarised in Table 5.3.

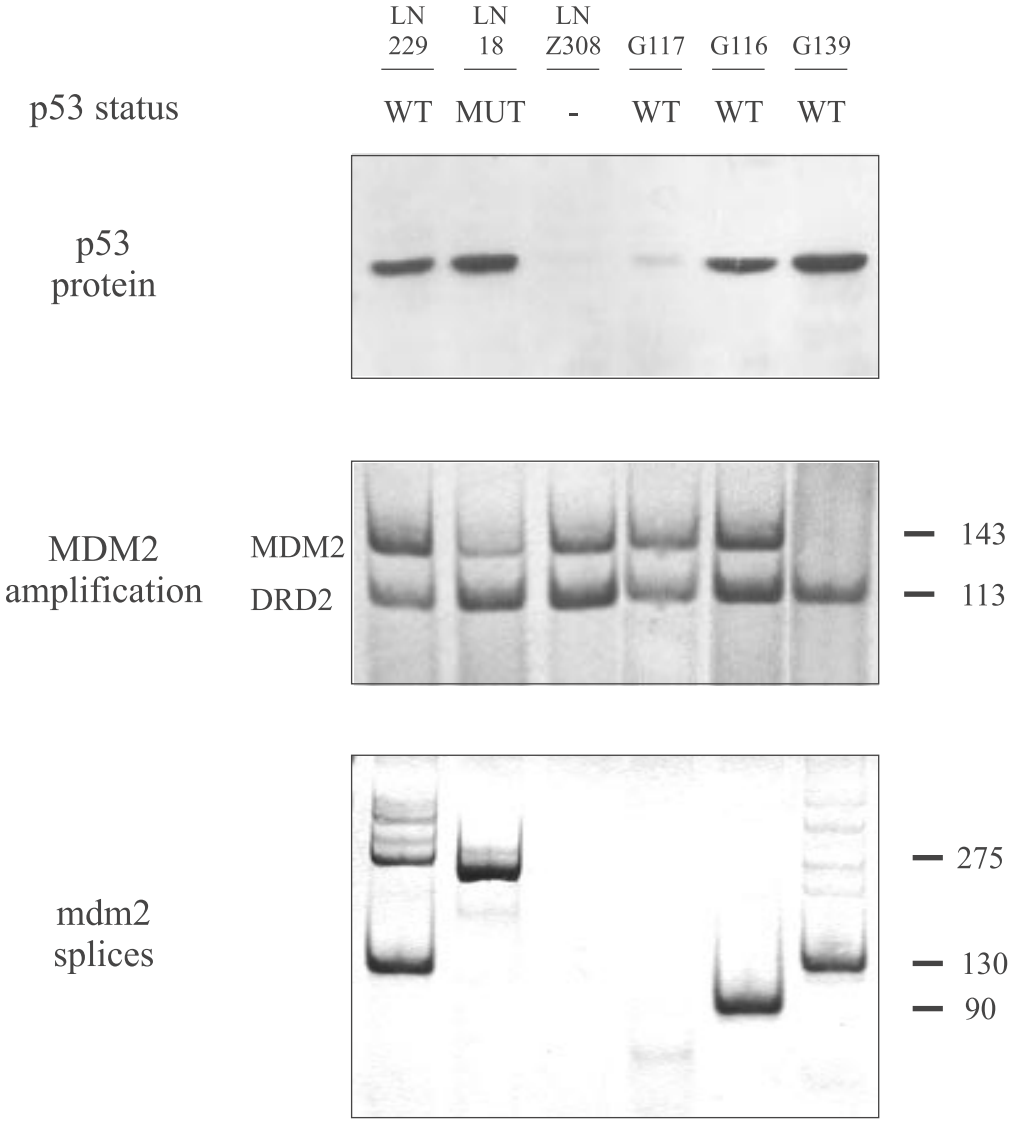
### 5.3.3 *MDM2* gene amplification

*MDM2* amplification was detected in two of the three GBM cell lines; LN 229, which bears wt p53, and LN Z308, in which no p53 protein is expressed, as illustrated in Figure 5.3.2. *MDM2* amplification was not present in the mutant p53 cell line LN 18. Investigation of the glial tumour primary cultures showed that *MDM2* was amplified in 3 of the 21 cultures. Two of the cultures in which *MDM2* amplification was detected, G117 and G116, were derived from *de novo* GBMs, but the third, G109, originated from a secondary GBM.

### 5.3.4 mdm2 splice variants

In order to investigate whether, according to the literature for bladder and ovarian carcinomas (Sigalas et al. 1996), mdm2 is alternatively spliced in human glial tumours *in vitro*, 19 of the primary glial tumour cultures and the three GBM cell lines were screened. The results are shown in Table 5.3. Spliced mdm2 transcripts were detected in 9 out of 19 primary cultures and in the GBM cell lines LN 229 and LN 18, as

**Figure 5.3.2** Expression of p53 protein (upper panel), amplification of the MDM2 gene in comparison to the reference dopamine receptor gene DRD2 (second panel) and expression of mdm2 splice variants (bottom panel) in LN 229, LN 18, LN Z308 and *de novo* GBMs with wt p53



illustrated in Figure 5.3.2. A single variant transcript was expressed in all cases, with the exception of LN 229, which demonstrated two splices. Of those primary cultures with mutational SSCP band shifts for p53, spliced mdm2 transcripts were detected in 2 out of 7 cases, whereas 7 out of 12 primary cultures bearing a wt p53 SSCP pattern displayed the presence of an mdm2 splice variant.

The spliced transcripts detected ranged in size between 90 and 300 base pairs. Sequencing of the spliced transcripts showed loss of at least part of the p53 binding domain in all of the variants detected, as illustrated in Figure 5.3.5.

A significant correlation was determined between the presence of an mdm2 splice variant and detection of p53 protein in the glial tumour primary cultures (Fisher's Exact Test, $p<0.01$). The correlation between the presence of a spliced mdm2 variant and wt p53 protein detection attained the same level of significance ( Fisher's Exact Test, $p<0.01$).

## 5.4 The dose response to ionising irradiation

In order to determine the most appropriate dose of ionising irradiation for use in the following experiments, a dose response curve for normal fibroblasts and the GBM cell lines LN 229 and LN 18, which bear wt and mutant p53 respectively, was performed. The biological effect of the irradiation was measured in terms of the percentage of cells present in G1 phase of the cell cycle at 24 h after irradiation as compared to non-radiated control cells at the same time point, as shown in Figure 5.4..

Normal fibroblasts showed an effective response to irradiation, with an arrest of cells at the G1 checkpoint observed from a dose of 1 Gy, maximal at 2 Gy. A marginal decline in the plateau of the response curve was observed from this point, indicative of an increasing number of apoptotic or necrotic cells at increasing doses of irradiation. In the GBM cell line LN 229, no significant response to irradiation was observed until a dose of 6 Gy was reached. At and above this dose of 6 Gy, the LN 229 cells showed a modest arrest in G1 with the effect plateauing at a maximum between 7 and 12 Gy. The GBM cell line LN 18 demonstrated a slight increase in the percentage of cells in G1 at 2 Gy irradiation, but not at any other irradiation dose, raising doubts as to the significance of the response seen at 2 Gy. A marked decline in the percentage of cells in G1 phase was observed at doses higher than 6 Gy in LN 18.

**Figure 5.3.4** shows schematically the spliced transcripts of mdm2 identified in GBM cell lines and primary cell cultures, as compared to the full length mdm2 RNA. The p53 binding domain, which is essential for the mdm2 function of p53 protein degradation, is shown to be disrupted in the splice variants.

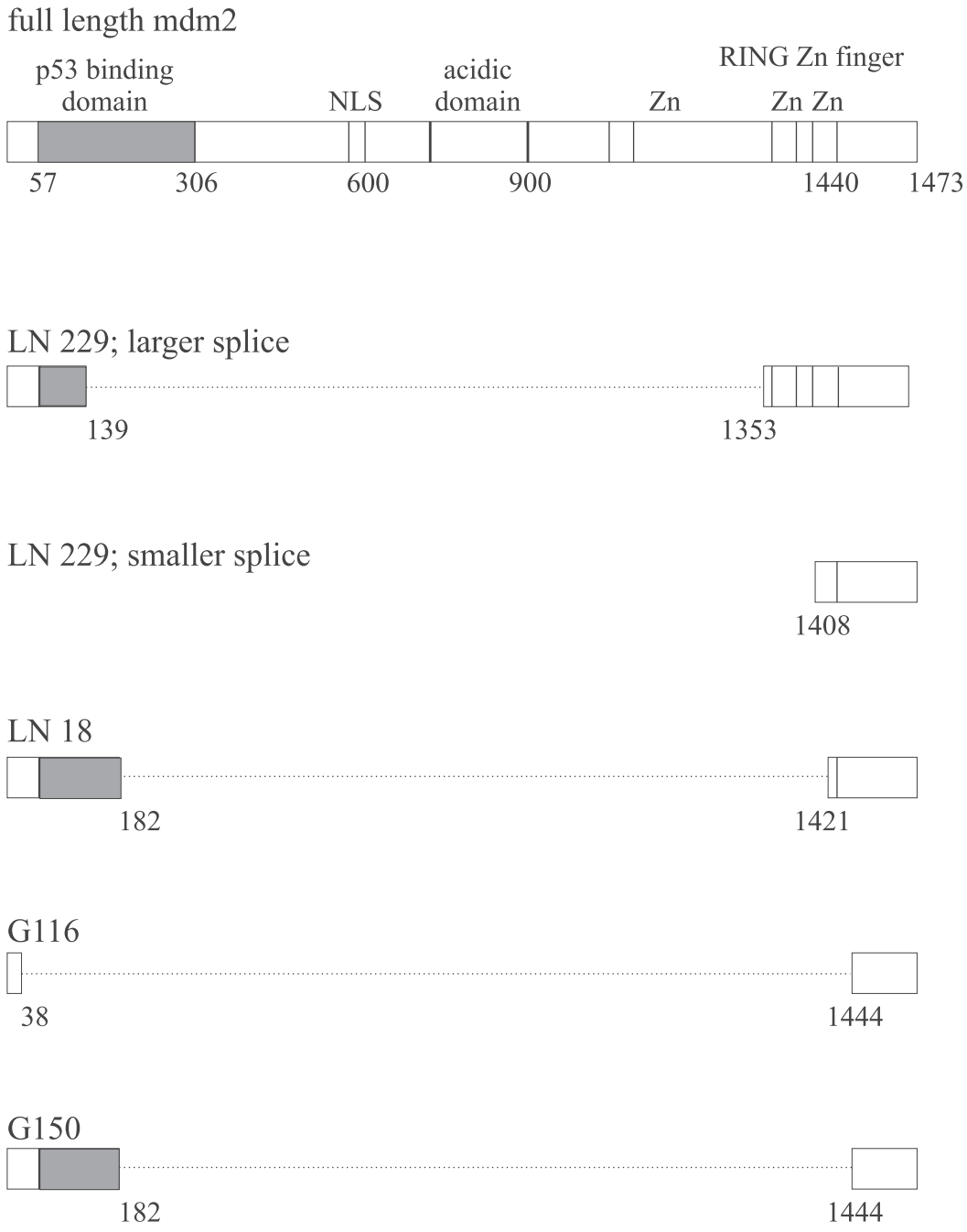
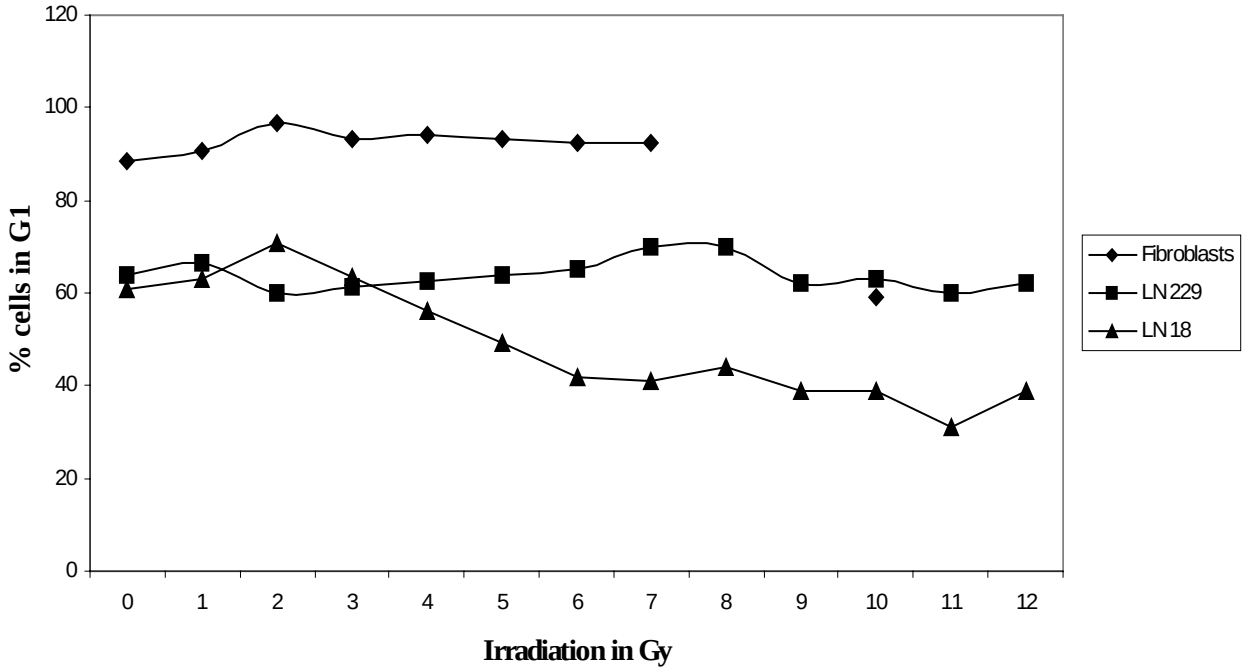


**Figure 5.4** shows the percentage of cells within the G1 phase of the cell cycle at 24 h after increasing doses of ionising irradiation in normal fibroblasts and the GBM cell lines LN 229 (wt p53) and LN 18 (mutant p53). Non-radiated control cells are represented by the dose 0 Gy. Since the response of fibroblasts was not measured above 7 Gy except for a measurement at 10 Gy, a single point for their response at 10 Gy is shown in the diagram.




On the grounds of the results for the wt p53 GBM cell line, LN 229, it was reasoned that in order to study the effects of ionising radiation on glial tumours *in vitro*, it was necessary to use doses greater than 6 Gy. Since the effects on G1 arrest between doses of 7 Gy and 12 Gy were equivalent in LN 229, a dose of 10 Gy was chosen for subsequent experiments on the cell lines and primary cultures.

Since the effects of irradiation on the G1 checkpoint in fibroblasts were detectable at lower doses, and significant cell necrosis was probably induced at the high radiation dose of 10 Gy, fibroblasts were irradiated at 7 Gy to provide a normal control for the subsequent experiments.

## 5.5 The response of glial tumour cell cultures to irradiation

### 5.5.1 Cell Morphology

Irradiation of the glial primary cell cultures and GBM cell lines with 10 Gy did not result in any morphological changes visible by light microscopy. In all cases the cells remained adherent and, as can be seen in the example in Figure 5.5.1, the cells continued to proliferate. Light microscopy revealed that a proportion of normal human fibroblasts became non-adherent after irradiation and that no significant cell proliferation occurred during the 48 h after irradiation.

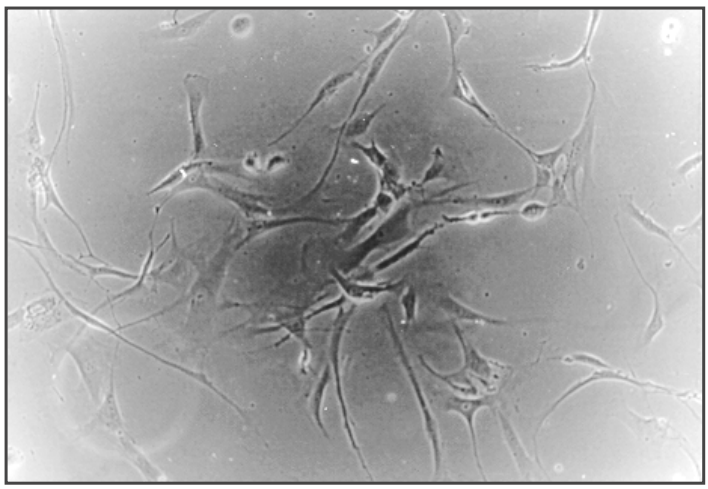
### 5.5.2 The effect of irradiation on cell cycle checkpoints

All primary cell cultures and cell lines were analysed by flow cytometry 24 h after irradiation with 10 Gy and compared to a non-radiated control of a replicate culture at the same cell confluence. The results are summarised in Table 5.5.2.

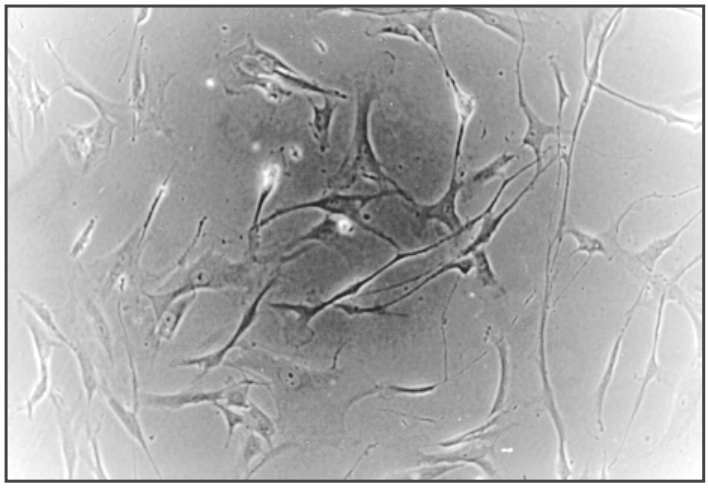
Arrest at the G1 checkpoint after irradiation occurred in only 3 of 19 glial tumour primary cultures. G1 arrest failed in 14 out of 15 GBMs *in vitro*. Failure of the G1 checkpoint in GBMs occurred in the presence of both wt and mutant p53, spanning both secondary and *de novo* groups of GBM. The sole exception to this was the secondary GBM culture NCH 128, which showed a G1 arrest equivalent to that seen in normal fibroblasts. A minimal G1 arrest was observed in G156, established from a grade II astrocytoma and a moderate G1 arrest occurred in the grade III oligoastrocytoma NCH 135. In cultures established from the remaining two cases of grade II and III glial tumours, G1 arrest did not occur following irradiation. Normal fibroblasts showed an arrest at the G1 cell cycle checkpoint of up to 93% of cells after irradiation with 7 Gy (see Section 5.4).

G2/M arrest was observed in 15 out of 19 glial tumour primary cultures and in all three GBM cell lines. Arrest of fibroblasts at the G2/M checkpoint was not observed. The effect of irradiation on the percentage of cells in S phase was variable and inconsistent. No significant arrest at S phase was observed in irradiated normal fibroblasts.

**Figure 5.5.1** Morphology of primary cell culture G116, established from a *de novo* glioblastoma, before and 24h after irradiation with 10 Gy



before
10 Gy



24h after
10 Gy

**Table 5.5.2** shows the differences between the percentages of cells in each phase of the cell cycle after irradiation with respect to non-radiated control cells. A positive number indicates an increase in the percentage of cells after irradiation, i.e. arrest, and a negative number indicates a reduction in the percentage of cells in the respective phase. Samples that were not measured are represented in the table as N.D. (not done).

| Culture | Diagnosis | p53 SSCP band shifts | Change in % cells in G1 | Change in % cells in S | Change in % cells in G2/M |
|---|---|---|---|---|---|
| G118 | OA II | wt | -5.1 | +7.1 | -2.0 |
| G156 | A II | mutant | +2.1 | -4.6 | +2.5 |
| G114 | A III | mutant | -2.6 | -8.0 | +10.6 |
| NCH 135 | OA III | wt | +4.7 | -5.1 | +0.4 |
| G101 | 2° GBM | mutant | -21.5 | -19.1 | +40.6 |
| G109 | 2° GBM | mutant | -7.5 | -3.3 | +10.8 |
| G150 | 2° GBM | mutant | -33.2 | -2.6 | +35.8 |
| NCH 59 | 2° GBM | mutant | | N.D. | |
| NCH 105 | 2° GBM | mutant | -4.5 | -17.0 | +21.4 |
| NCH 128 | 2° GBM | mutant | +7.9 | -9.7 | +1.8 |
| G100 | *de novo* GBM | wt | -1.2 | +1.6 | -0.4 |
| G112 | *de novo* GBM | wt | -9.6 | +12.7 | -3.1 |
| G113 | *de novo* GBM | wt | -56.8 | -0.4 | +57.2 |
| G116 | *de novo* GBM | wt | -35.6 | +6.0 | +29.6 |
| G117 | *de novo* GBM | wt | -19.3 | -29.9 | +49.2 |
| G120 | *de novo* GBM | wt | | N.D. | |
| G122 | *de novo* GBM | wt | -18.8 | +14.3 | +4.5 |
| G139 | *de novo* GBM | wt | -24.4 | -2.1 | +26.5 |
| G145 | *de novo* GBM | wt | -3.8 | -12.0 | +15.8 |
| G147 | *de novo* GBM | wt | -39.7 | +40.5 | -0.7 |
| G148 | *de novo* GBM | wt | -1.8 | -17.2 | +19.0 |
| LN 229 | GBM cell line | wt | -1.2 | -18.6 | +19.8 |
| LN 18 | GBM cell line | mutant | -22.1 | -21.5 | +43.6 |
| LN Z308 | GBM cell line | absent | -6.4 | -24.9 | +31.3 |
| fibroblasts | normal | wt | +8.3 | -7.9 | -0.3 |

### 5.5.3 The effect of irradiation on p53 and its regulators

#### 5.5.3.1 mRNA expression of p53 and ATM

Figure 5.5.3.1 shows a representative example of p53 mRNA expression in both secondary and *de novo* GBMs. As with all further data presented, expression of the house-keeping gene ß actin provided a control for each sample. ß actin displayed constant and quantitatively equivalent expression throughout the series of primary cultures and cell lines, confirming the quantitatively reproducible synthesis of complementary DNA from RNA during RT-PCR. Specificity in each reaction was confirmed by the appearance of a single PCR amplification product of expected size, and negative controls for all primers excluded contamination.

p53 mRNA was variably detected before irradiation in control fibroblast primary cultures and all glial tumour primary cultures and GBM cell lines, with the exception of the p53-null cell line LN Z308, in which p53 RNA transcripts were absent. Marked expression of p53 mRNA was observed in six of the glial tumour primary cultures, but analysis by Fisher's Exact Test demonstrated no significant correlation between basal p53 mRNA and protein expression levels.

The mRNA expression of p53 either remained stable or was reduced at 2 h after irradiation. At 8 h after irradiation moderate expression of p53 mRNA was a consistent finding throughout the primary cultures, despite variations in their baseline levels of expression. mRNA expression of the upstream regulator of p53, ATM, was detected in all primary cultures and cell lines, including LN Z308 and fibroblasts. The level of baseline expression was variable and showed no consistent response to irradiation, as illustrated in Figure 5.5.3.1.

#### 5.5.3.2 mRNA expression of mdm2

Constitutive mdm2 expression was observed throughout the series of glial primary cultures and GBM cell lines, illustrated in Figure 5.5.3.2. High levels of basal mdm2 expression occurred even in the absence of *MDM2* amplification. A similarly strong basal mdm2 expression was detected before irradiation in control fibroblasts.

In response to irradiation, mdm2 expression in the fibroblast and glial tumour primary cultures remained stable at high expression levels. In all three GBM cell lines,

**Figure 5.5.3.1** mRNA expression levels of ß actin, ATM and p53 at 2 h and 8 h after irradiation with 10 Gy as compared to non-radiated controls (’0’) in OA°III (first column), secondary GBM (second and third columns) and *de novo* GBM (final three columns)

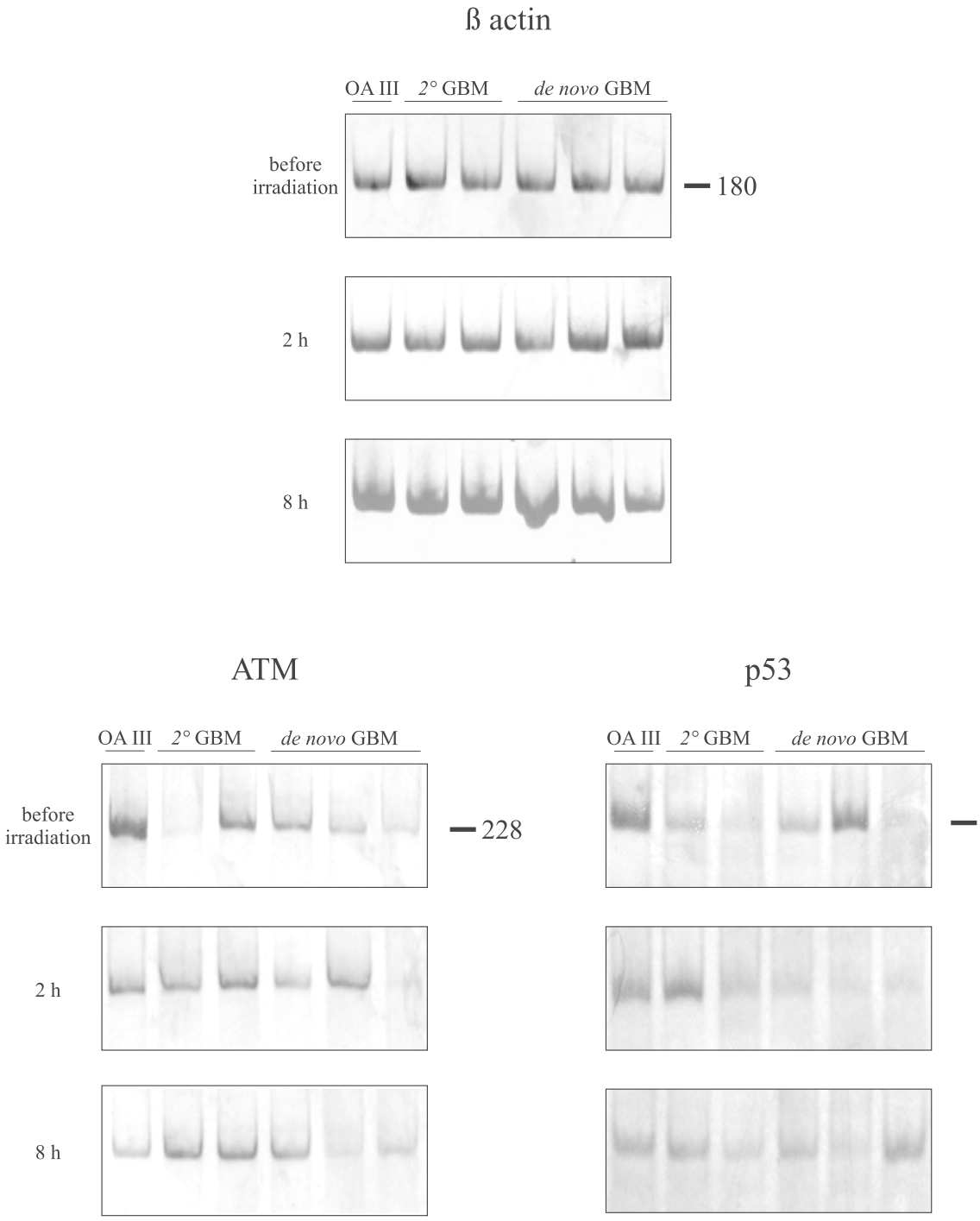
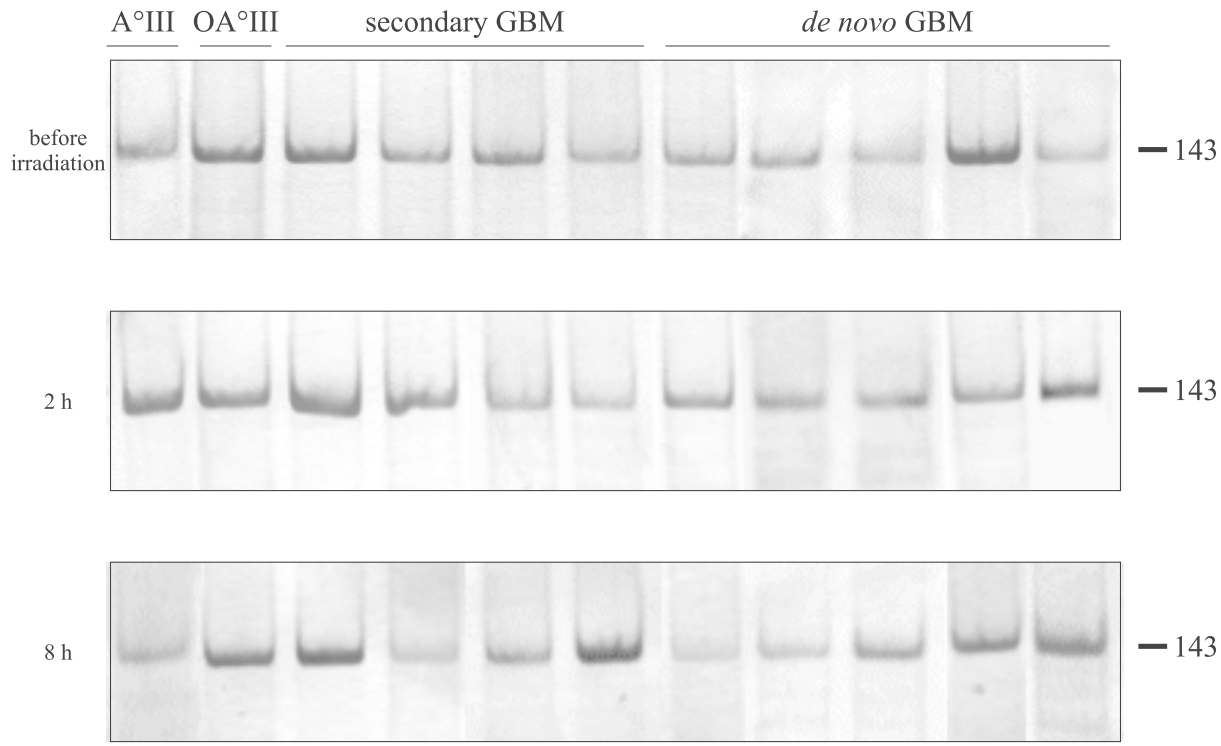


**Figure 5.5.3.2** mRNA expression levels of mdm2 at 2 h and 8 h after irradiation with 10 Gy as compared to non-radiated controls (’0’) in grade III astrocytoma (first column), grade III oligoastrocytoma (second column), secondary GBM (next four columns) and *de novo* GBM (final five columns)



strong basal expression of mdm2 mRNA was not affected significantly by irradiation.

#### 5.5.3.3 p53 protein expression after irradiation

Expression of p53 protein was not detectable in control fibroblasts, either before or after irradiation. In glial tumours *in vitro*, the effect of irradiation on the level of p53 protein expression was variable, depending on whether increased p53 protein expression was present before irradiation (see Section 5.3.2). In those primary glial cultures with no detectable basal p53 protein expression, the level of p53 protein either remained undetectable (3 out of 8 cases) or showed a modest increment (5 out of 8 cases) after irradiation.

A pronounced reduction in p53 protein was observed after irradiation in 11 of the 13 glial tumour primary cultures which exhibited increased basal p53 protein expression, as shown in Table 5.5.3.3. In the 2 remaining cases, wt p53 was modestly ('+') expressed before irradiation and demonstrated a brief increase in detectable levels after irradiation. The 11 cases where reduction in p53 protein expression was observed after irradiation spanned both secondary and *de novo* GBM cultures.

The GBM cell lines LN 18 and LN 229, in which p53 protein was very strongly basally expressed ('+++'), showed a moderate reduction in the level of detectable p53 protein after irradiation (see Figure 5.5.5.1).

**Table 5.5.3.3** shows the basal expression levels of p21 mRNA and p53 protein together with the direction of change in expression levels after irradiation with 10 Gy. Undetectable expression is represented by '0' and basal overexpression by '+'.

| Culture | Diagnosis | basal p21 expression | p53 protein expression | p21 expression after irradiation | p53 protein level after irradiation |
|---|---|---|---|---|---|
| G118 | OA II | 0 | + | no change | brief increase |
| G156 | A II | 0 | 0 | no change | no change |
| G114 | A III | + | + | no change | reduction |
| NCH 135 | OA III | 0 | + | brief increase | reduction |
| G101 | 2° GBM | + | + | reduction | reduction |
| G109 | 2° GBM | + | + | no change | reduction |
| G150 | 2° GBM | 0 | + | no change | reduction |
| NCH 59 | 2° GBM | + | 0 | reduction | sustained increase |
| NCH 105 | 2° GBM | + | 0 | reduction | sustained increase |
| NCH 128 | 2° GBM | + | 0 | reduction | brief increase |
| G100 | *de novo* GBM | + | + | reduction | reduction |
| G112 | *de novo* GBM | + | + | reduction | reduction |
| G113 | *de novo* GBM | 0 | + | brief increase | brief increase |
| G116 | *de novo* GBM | + | + | no change | reduction |
| G117 | *de novo* GBM | + | 0 | reduction | no change |
| G120 | *de novo* GBM | 0 | 0 | brief increase | late increase |
| G122 | *de novo* GBM | 0 | 0 | brief increase | no change |
| G139 | *de novo* GBM | + | + | reduction | reduction |
| G145 | *de novo* GBM | + | + | brief increase | reduction |
| G147 | *de novo* GBM | 0 | 0 | sustained increase | brief increase |
| G148 | *de novo* GBM | 0 | + | sustained increase | reduction |
| LN 229 | GBM cell line | + | + | reduction | reduction |
| LN 18 | GBM cell line | + | + | reduction | reduction |
| LN Z308 | GBM cell line | + | 0 | no change | no change |
| fibroblasts | normal | 0 | 0 | brief increase | brief increase |

### 5.5.4 The effect of irradiation on expression of p21 and gadd45

In control fibroblasts the p53-inducible genes *WAF1,* which encodes p21, and *GADD45* were transcriptionally activated at 2 h after irradiation from minimal basal mRNA levels, as shown in Figure 5.5.4.1. mRNA levels of gadd45 remained elevated at 8 h after irradiation, while those of p21 had returned to pre-irradiation levels.

RT-PCR revealed that, compared to the basal levels of p21 observed in control fibroblasts, p21 was overexpressed before irradiation in 5 out of 6 secondary GBMs and 6 out of 11 *de novo* GBMs. Basal overexpression of p21 mRNA was observed in only one of the four grade II and III glial tumours. The results are summarised in Table 5.5.3.3. Eight of the 12 glial tumour primary cultures with increased basal p21 expression also exhibited elevated basal expression of gadd45 mRNA and increased basal p53 protein expression. A statistically significant correlation between overexpression of p21 mRNA and p53 protein was not present (Fisher's Exact Test).

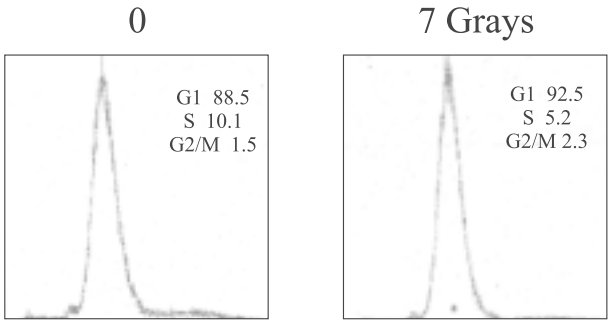
All three of the GBM cell lines displayed high basal levels of p21 mRNA, which were mirrored by elevated gadd45 expression in all but LN 18 (see Figure 5.5.5.1). After irradiation, the basally upregulated levels of p21 and gadd45 were diminished in LN 229 and unaltered in LN Z308. In LN 18 the basally upregulated level of p21 mRNA was reduced after irradiation, whereas gadd45 was induced from a minimal basal level of expression.

Figure 5.5.4.2 and Table 5.5.3.3 show the effect of irradiation with 10 Gy on p21 mRNA expression in the glial tumour primary cultures. Induction of p21 mRNA at 2 h after irradiation occurred in all 5 *de novo* GBMs in which p21 was minimally expressed before irradiation. In 3 of these 5 cases the level of p21 expression had returned to baseline levels by 8 h after irradiation. In 8 out of the 11 *de novo* and secondary GBMs exhibiting basal p21overexpression, irradiation caused a reduction in the p21 expression.

Statistical analysis showed no significant correlation between the reduction in p21 mRNA and p53 protein levels occurring after irradiation.

The pattern of response of gadd45 mRNA levels to irradiation was similar to that seen for p21. In all 7 *de novo* GBMs where basal gadd45 expression was minimal, irradiation led to induction of gadd45 mRNA. The 4 *de novo* GBMs expressing abnormally high levels of gadd45 mRNA before irradiation responded without

**Figure 5.5.4.1** The effect of irradiation with 7 Gy on cell cycle control in fibroblast primary cell cultures at 2 h and 8 h as compared to non-radiated control ('0'). Flow cytometry analysis in irradiated and non-radiated cells at 24 h shown.



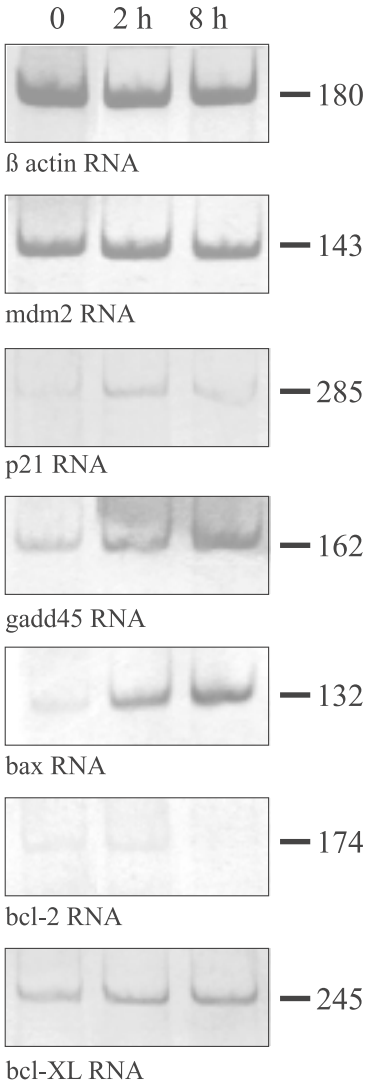
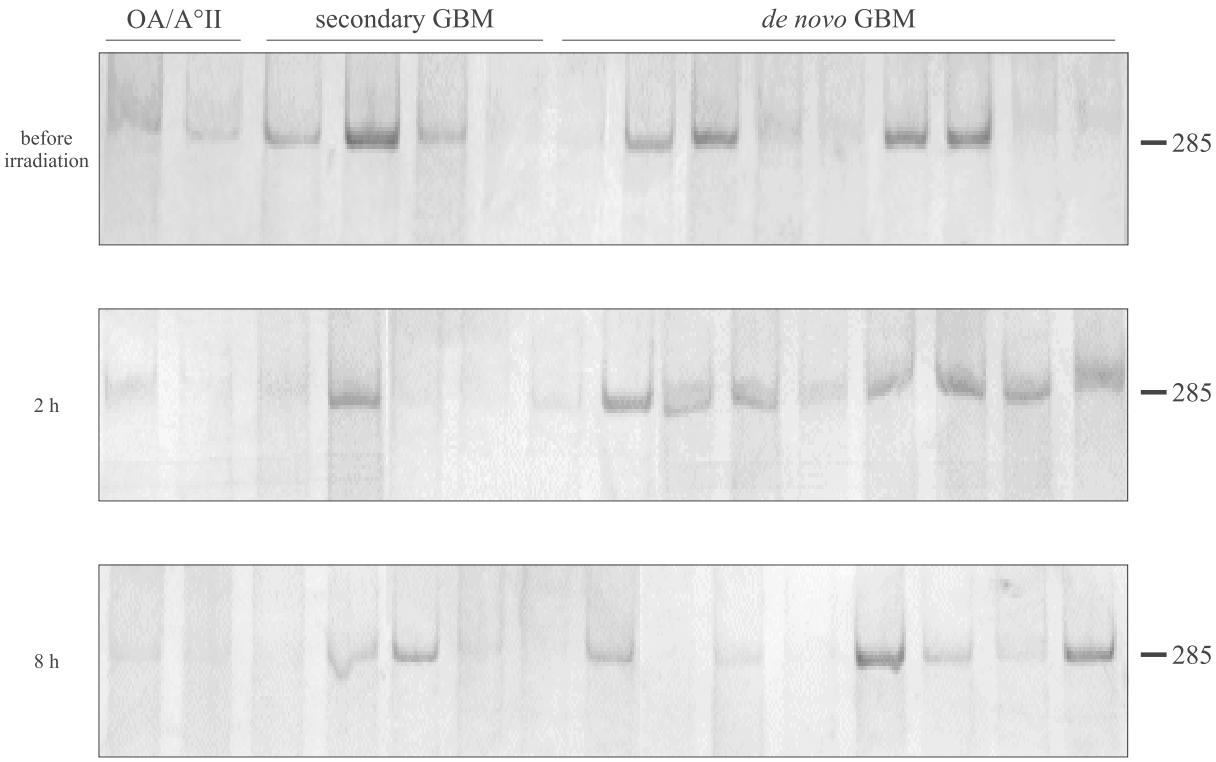



**Figure 5.5.4.2** mRNA expression levels of p21 at 2 h and 8 h after irradiation with 10 Gy as compared to non-radiated controls (’0’) in grade II tumours (first two columns), secondary GBM (next four columns) and *de novo* GBM (final nine columns)



exception to irradiation by a reduction in gadd45. The gadd45 response to irradiation in secondary GBMs and grade II and III glial tumours was not consistent.

### 5.5.5 Relationship between p21 and gadd45 induction and G1 arrest

Figures 5.5.5.1 to 5.5.5.5 illustrate the range of responses observed in p53, p21, gadd45 and in the cell cycle itself after irradiation of the glial tumour cultures.

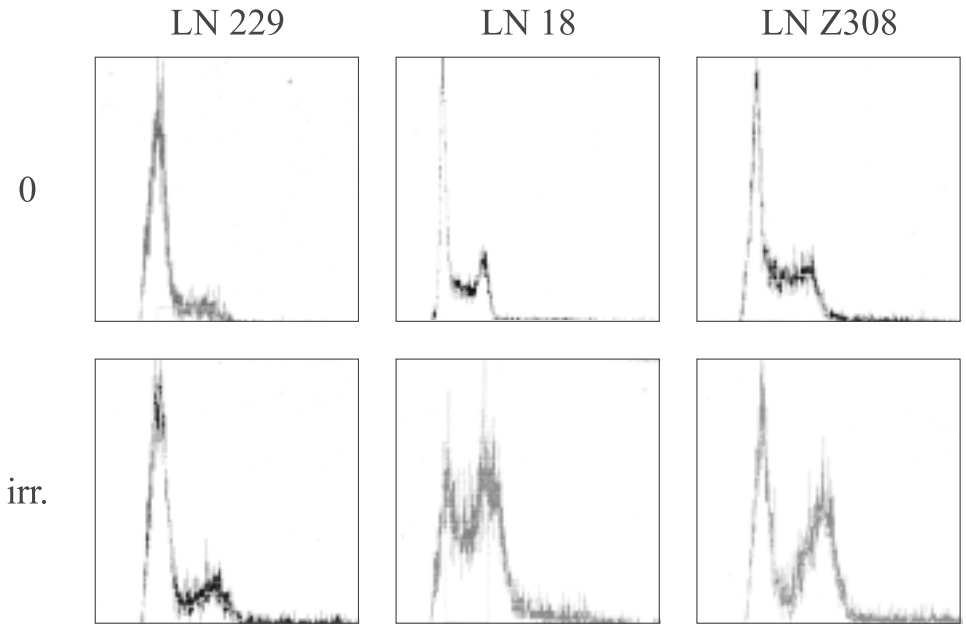
Irradiation of the wt p53 GBM cell line, LN 229, did not lead to induction of either p21 or gadd45 and failed to induce G1 arrest (Figure 5.5.5.1). Basal levels of p21 and gadd45 mRNA and p53 protein were all high in LN 229. The response of the mutant p53 cell line LN 18 was similar to that seen in LN 229. G1 arrest also failed in the p53-null GBM cell line LN Z308, where basally upregulated p21 and gadd45 mRNA levels were not altered by irradiation. A substantial arrest of cells at the G2/M checkpoint occurred in all three GBM cell lines.

Figure 5.5.5.2 demonstrates the changes occurring in the cell cycle and its regulators in primary cell cultures derived from two grade III glial tumours and two secondary GBMs. NCH 135, established from a grade III oligoastrocytoma with wt p53 SSCP pattern, showed an effective arrest of cells in G1, paralleled by significant induction of both p21 and gadd45 mRNA at 2 h after irradiation and reduction in the basally detectable p53 protein. In the remaining three tumour cultures shown, all of which demonstrated mutational p53 SSCP band shifts, neither p21 activation nor G1 arrest was achieved after irradiation, but G2/M arrest did occur. The sole exception to this pattern of response in the secondary GBM primary cultures was NCH 128, where a definite G1 arrest occurred.

Figure 5.5.5.3 provides examples of 2 of the 6 *de novo* GBMs exhibiting basal overexpression of p21. In both these examples, p21 mRNA, gadd45 mRNA and p53 protein were all basally overexpressed. Irradiation resulted in reduction of p53 protein and gadd45 mRNA, and reduced or unaltered p21 mRNA levels. Correspondingly, G1 arrest failed but substantial G2/M arrest was effected by irradiation.

Figures 5.5.5.4 and 5.5.5.5 illustrate *de novo* GBM cultures in which G1 arrest failed despite successful transcriptional activation of p21 after irradiation. Minimal basal p21 expression associated with the absence of wt p53 protein stabilisation in three of these cultures.

**Figure 5.5.5.1** Flow cytometry analysis and expression of cell cycle regulators in irradiated (10 Gy) and non-radiated glioblastoma cell lines. In this and following figures, non-radiated controls are represented by ‘0’ and irradiated samples by ‘irr.’.




| % cells | 0 | irr. | 0 | irr. | 0 | irr. |
|---|---|---|---|---|---|---|
| G1 | 64.0 | 62.8 | 61.2 | 39.1 | 45.9 | 39.5 |
| S | 26.2 | 7.6 | 28.2 | 6.7 | 43.8 | 18.9 |
| G2/M | 9.8 | 29.6 | 10.6 | 54.2 | 10.3 | 41.6 |

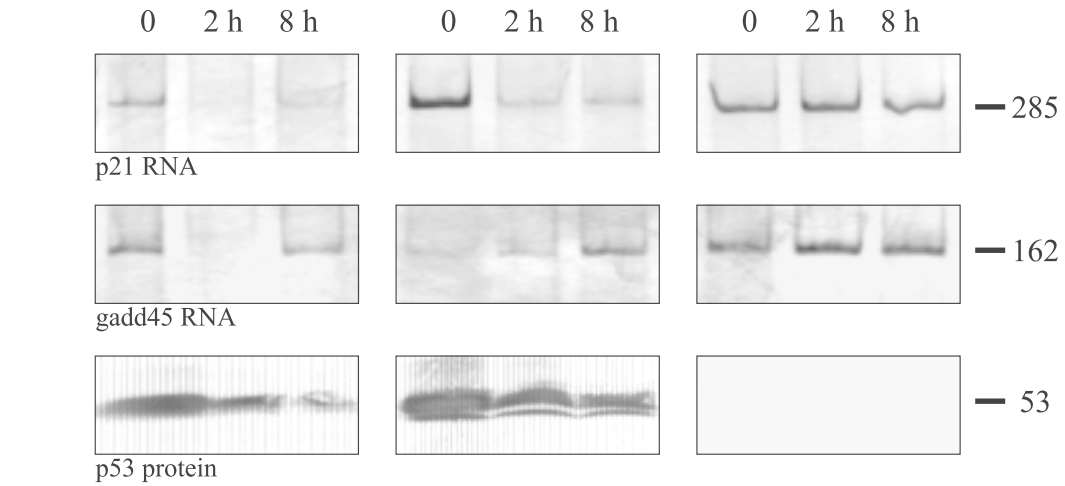
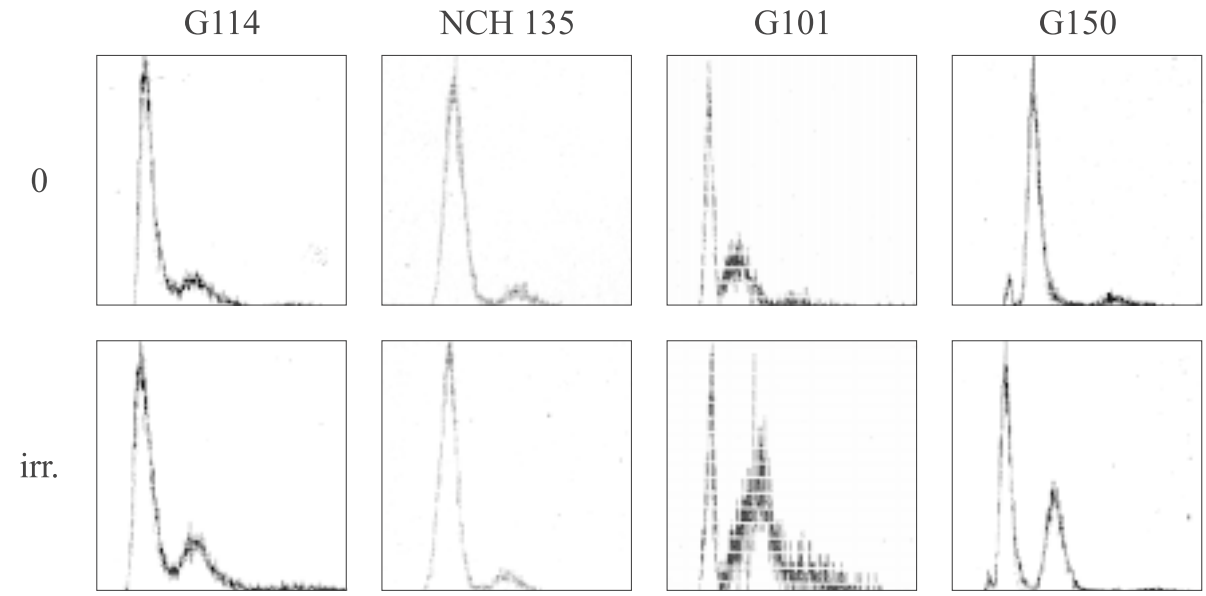


**Figure 5.5.5.2** Flow cytometry analysis and expression of cell cycle regulators in irradiated (10 Gy) and non-radiated primary cell cultures from an A III (G114), A III (NCH 135), and secondary GBMs (G101 and G150).
('0' = non-radiated control, 'irr.' = irradiated sample)




| % cells | 0 | irr. | 0 | irr. | 0 | irr. | 0 | irr. |
|---|---|---|---|---|---|---|---|---|
| G1 | 62.3 | 59.7 | 83.5 | 88.2 | 54.9 | 33.4 | 91.0 | 57.8 |
| S | 33.6 | 25.6 | 6.7 | 1.6 | 42.8 | 23.7 | 5.4 | 2.8 |
| G2/M | 4.1 | 14.7 | 9.8 | 10.2 | 2.3 | 42.9 | 3.6 | 39.4 |

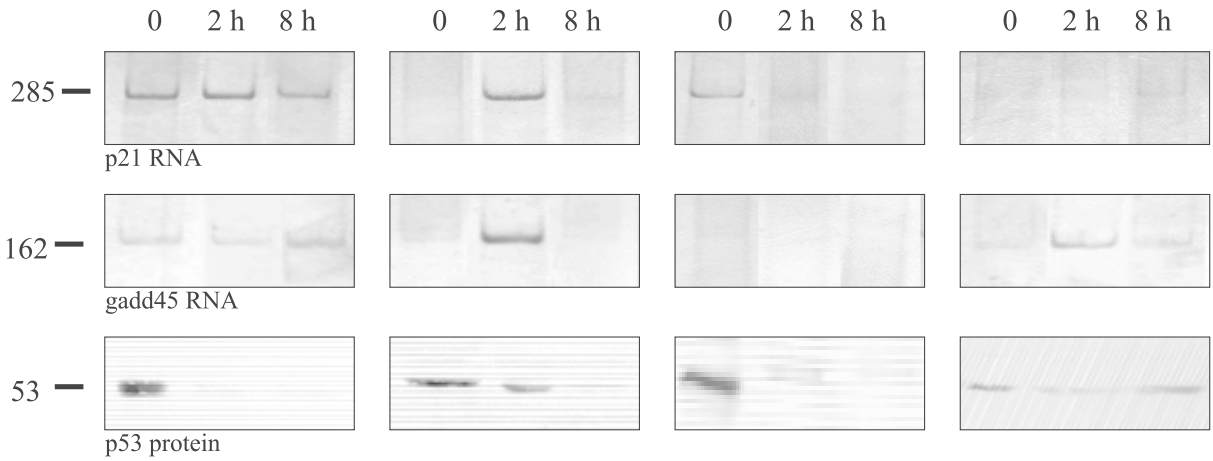
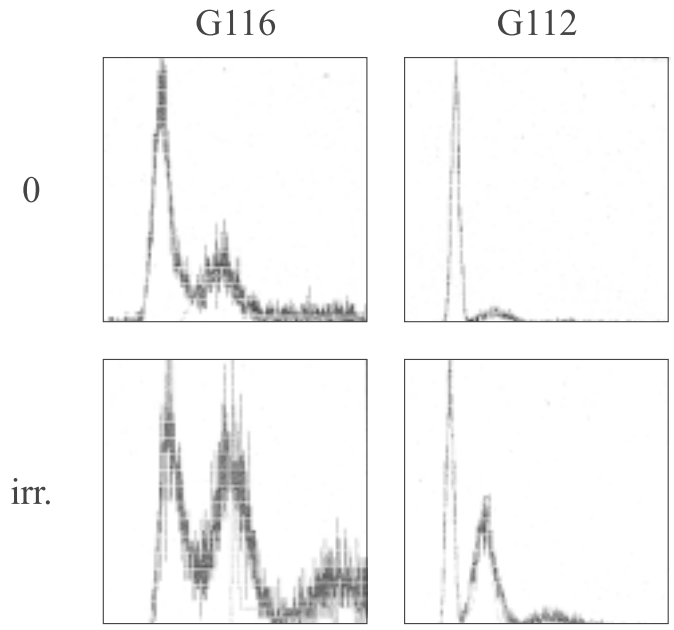


**Figure 5.5.5.3** Flow cytometry analysis and expression of cell cycle regulators in irradiated (10 Gy) and non-radiated primary cell cultures derived from *de novo* GBMs, in which basal wt p53 protein is overexpressed.
('0' = non-radiated control, 'irr.' = irradiated sample)




| % cells | 0 | irr. | 0 | irr. |
|---|---|---|---|---|
| G1 | 64.8 | 29.2 | 66.2 | 47.6 |
| S | 28.8 | 34.8 | 31.8 | 10.3 |
| G2/M | 2.4 | 36.0 | 2.1 | 42.1 |

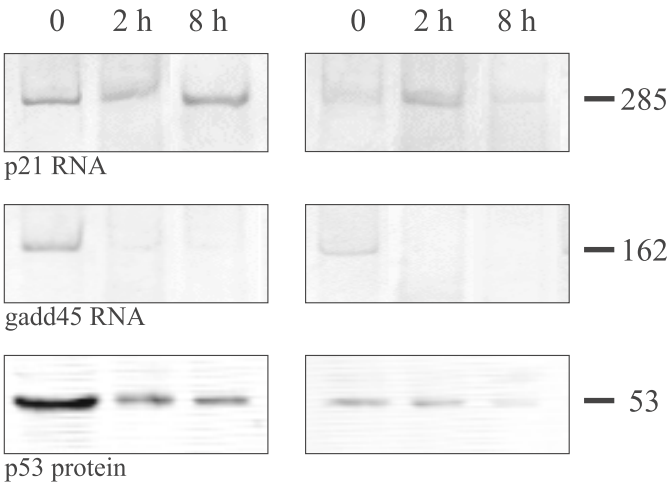
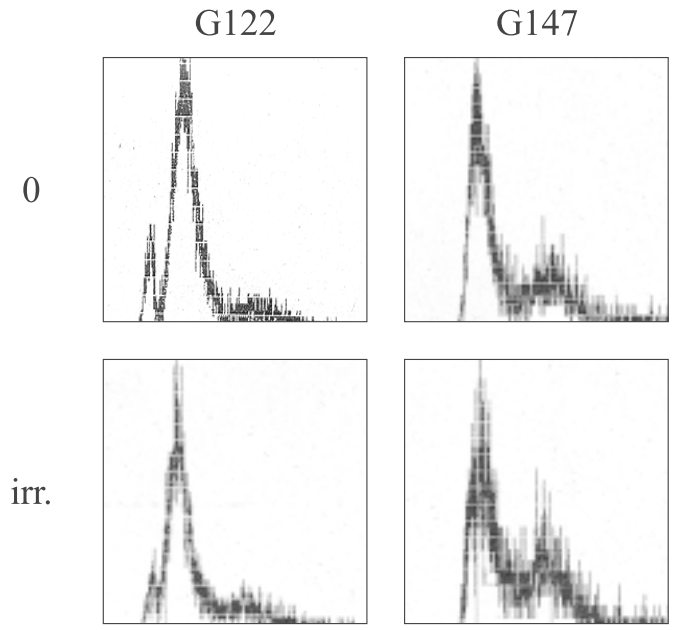


**Figure 5.5.5.4** Flow cytometry analysis and expression of cell cycle regulators in irradiated (10 Gy) and non-radiated primary cell cultures derived from *de novo* GBMs, in which basal wt p53 protein is not detectable.
('0' = non-radiated control, 'irr.' = irradiated sample)




| % cells | 0 | irr. | 0 | irr. |
|---|---|---|---|---|
| G1 | 84.5 | 65.7 | 57.6 | 17.9 |
| S | 13.7 | 28.0 | 33.1 | 73.6 |
| G2/M | 1.8 | 6.3 | 9.3 | 8.6 |

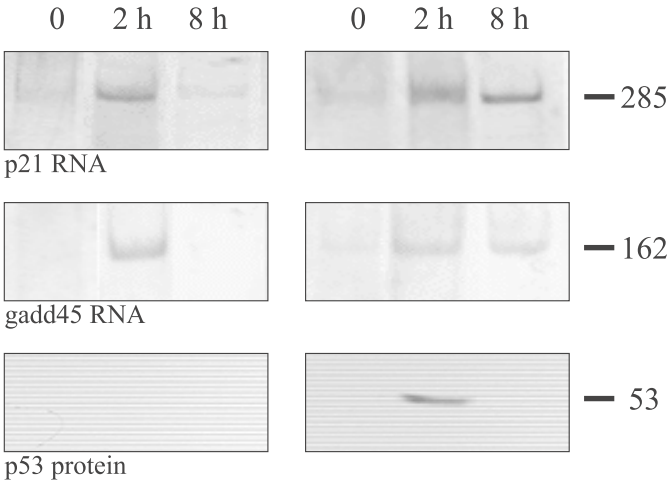
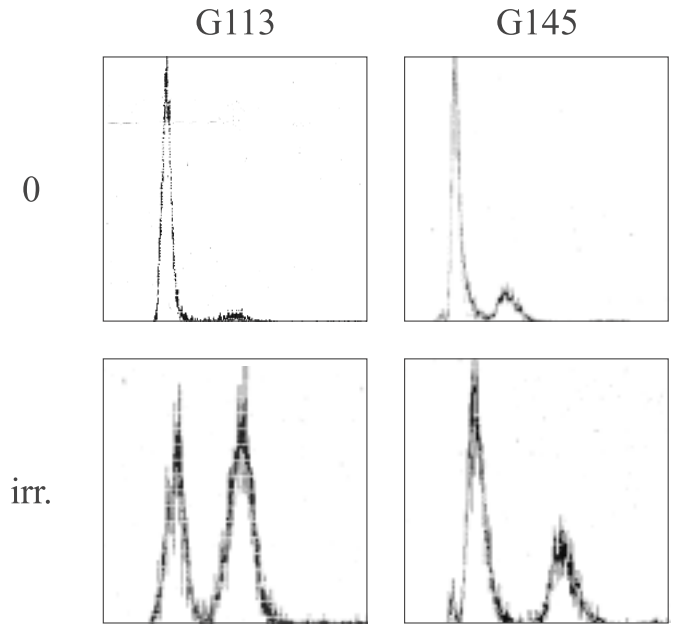
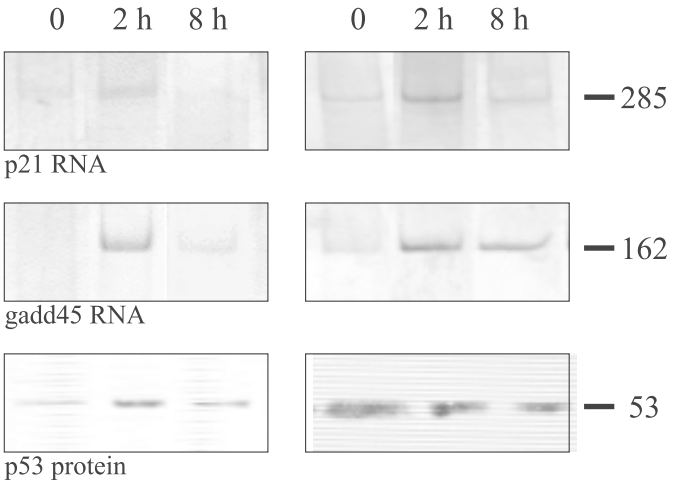


**Figure 5.5.5.5** Flow cytometry analysis and expression of cell cycle regulators in irradiated (10 Gy) and non-radiated primary cell cultures derived from *de novo* GBMs, in which G1 arrest failed despite induction of p21 and gadd45.
('0' = non-radiated control, 'irr.' = irradiated sample)




| % cells | 0 | irr. | 0 | irr. |
|---|---|---|---|---|
| G1 | 93.3 | 36.5 | 70.9 | 67.1 |
| S | 3.5 | 3.1 | 14.7 | 2.7 |
| G2/M | 3.1 | 60.2 | 14.4 | 30.2 |



### 5.5.6 Cell viability after irradiation

An indication of the cytotoxic effect suffered by irradiated cells was obtained from a test of the viability of irradiated cells compared to non-radiated control cells, at the same time point. A 15.5% reduction of cell viability (standard deviation (SD) 0.4%) in irradiated fibroblasts at a total of 28 h after irradiation was observed. Irradiation of the GBM cell lines LN 229 and LN Z308 led to 1.0% and 10.2% reduction in cell viability respectively (SDs 0.4% and 0.4% respectively). The cell line LN 18 demonstrated a cellular proliferation of 1.5% (SD 0.3%) after irradiation rather than a cytotoxic effect. The primary culture NCH 135, which did achieve a G1 arrest after irradiation, showed only 7.1% reduction in cell viability (SD 0.1%) after irradiation. Other primary glial cultures showed proliferation after irradiation or a lesser degree of cytotoxicity as compared to the culture, NCH 135.

### 5.5.7 The effect of irradiation on regulators of apoptosis

#### 5.5.7.1 The bcl-2 family

In irradiated control fibroblasts, mRNA levels of the pro-apoptotic regulator bax increased significantly at 2 h after irradiation from minimal basal levels and showed continuing transcriptional activation at 8 h after irradiation (Figure 5.5.4.1). Expression of bcl-2, which was basally weak, declined to an undetectable level at 8 h after irradiation. The long splice variant of bcl-X showed no significant change after irradiation in its moderate levels of expression in fibroblasts.

In all 21 glial tumours *in vitro*, strong mRNA expression of bax was detected before irradiation. After irradiation, the level of mRNA expression of bax was not significantly altered, as illustrated in Figure 5.5.7.1. The anti-apoptotic regulators bcl-2 and bcl-$X_L$ were invariably expressed before irradiation in the glial tumour primary cultures, bcl-2 more weakly than the relatively strongly expressed bcl-$X_L$. No significant change was detected in the expression of either bcl-2 or bcl-$X_L$ after irradiation of the primary cultures.

**Fig 5.5.7.1** mRNA expression of bax, bcl-2 and bcl-XL in non-radiated controls (represented by '0') and irradiated (10Gy) primary cultures derived from grade II tumours (first two columns), secondary GBM (next two columns) and *de novo* GBM (final seven columns).

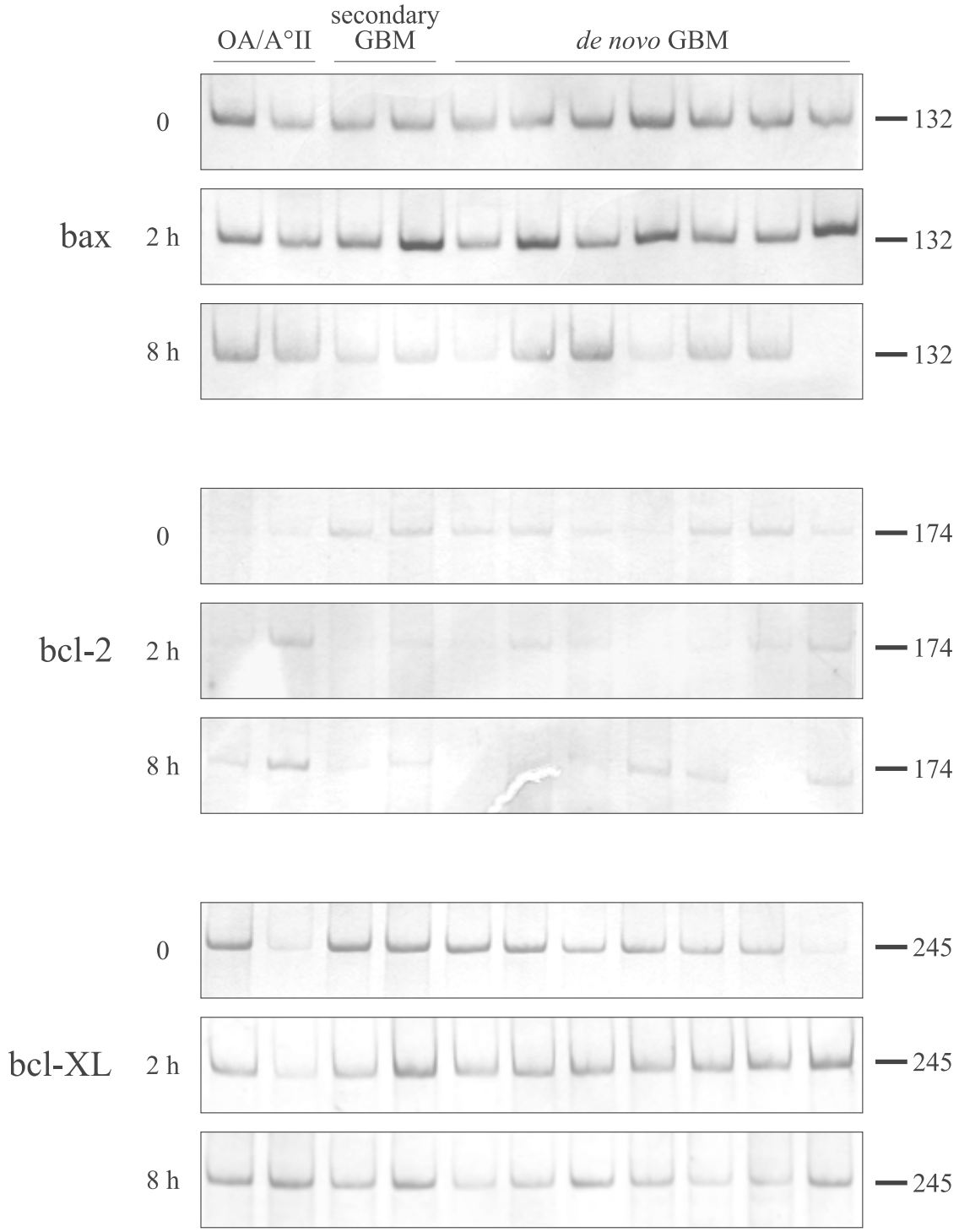
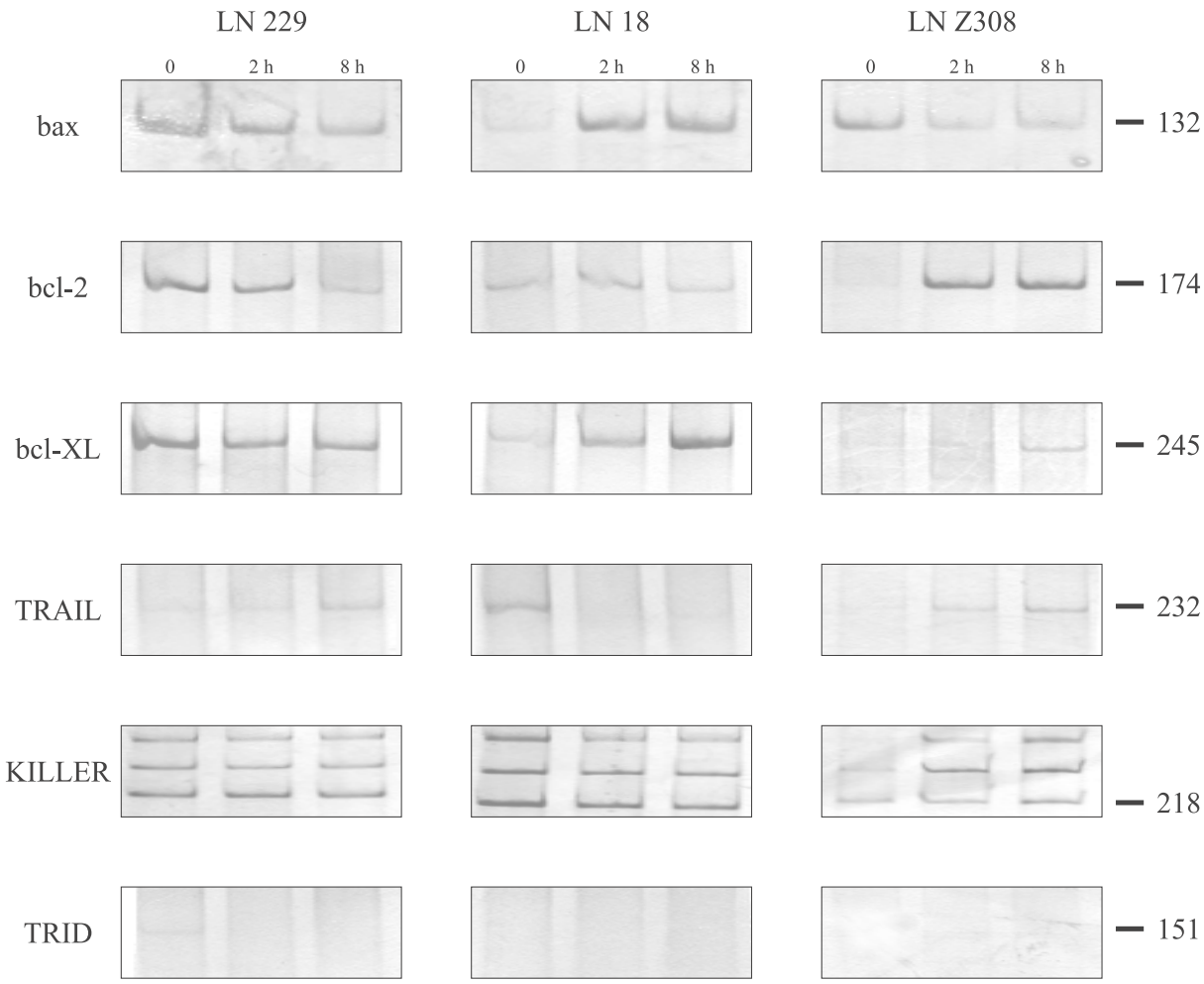


**Figure 5.5.7.2** mRNA expression levels of apoptotic regulators at 2 h and 8 h after irradiation with 10 Gy compared to non-radiated controls (0) in the GBM cell lines LN 229 (wt p53), LN 18 (mutant p53) and LN Z308 (absent p53).

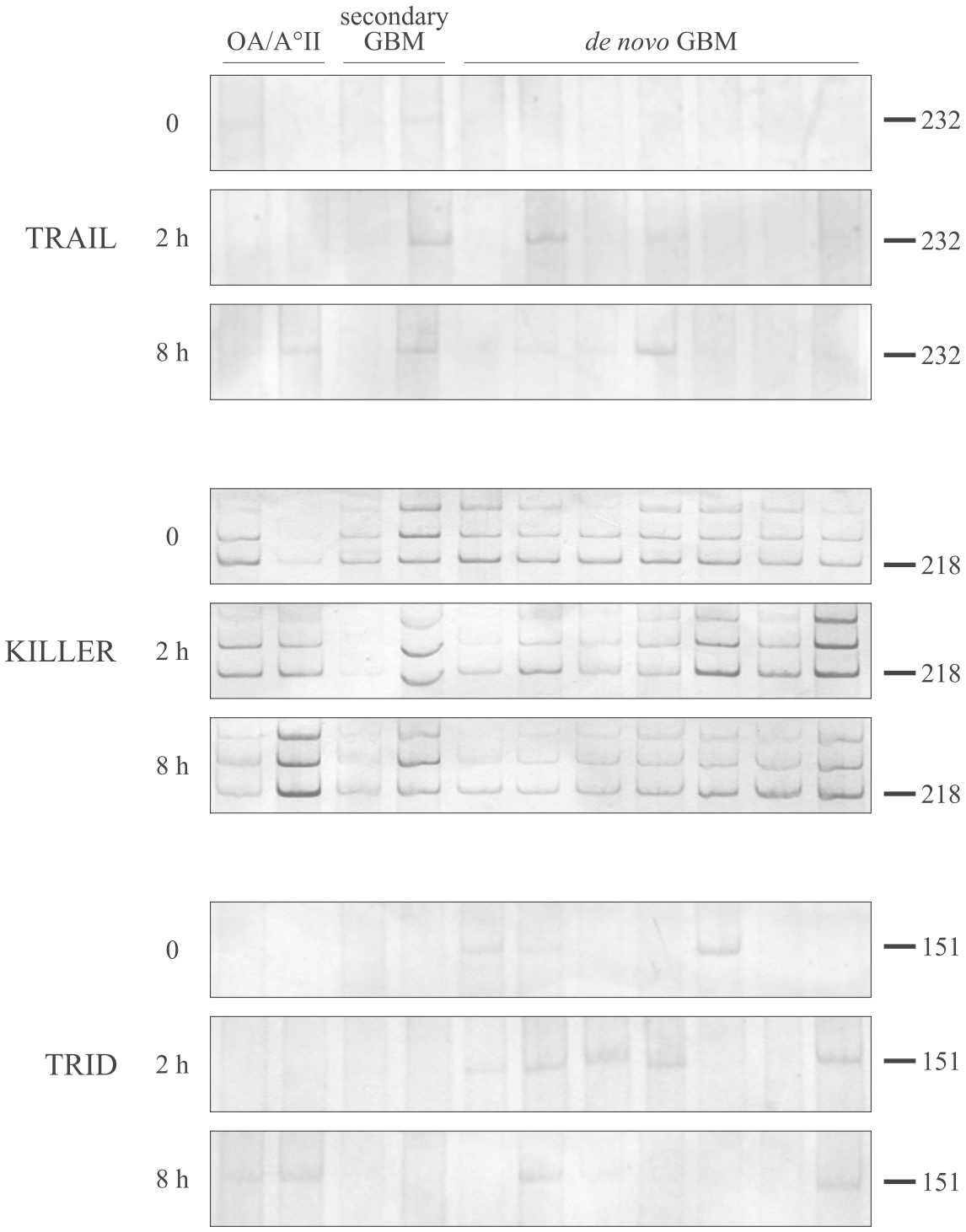


The expression of the bcl-2 family members in the GBM cell lines showed some variation, as illustrated in Figure 5.5.7.2. In LN 229, which bears wt p53, strong expression of bax, bcl-2 and bcl-$X_L$ was present before irradiation and remained stable following irradiation. In contrast, in LN 18, which bears mutant p53, expression of all three regulators was relatively weak before irradiation, but at 2 h and 8 h after irradiation equivalent induction of both bax and bcl-$X_L$ was observed. The expression of bcl-2 was unaltered. In the p53-null GBM cell line LN Z308, bax was strongly expressed before irradiation but was down-regulated after irradiation, bcl-2 was markedly upregulated from 2 h after irradiation and, while bcl-$X_L$ was barely detectable before irradiation, it showed upregulation at 8 h after irradiation.

### 5.5.7.2 The TRAIL pathway

The KILLER/DR5 receptor was present in all glial primary cultures both before and after irradiation. There was no change in expression of KILLER after irradiation throughout the series. As can be seen in Figure 5.5.7.3, two further larger PCR fragments were detected in addition to the predicted KILLER/DR5 fragment, and likewise showed no response to irradiation. The ligand TRAIL was minimally expressed in 3 out of 21 primary cultures before irradiation, and was not detectable in the remainder. After irradiation weak induction was detected in 9 out of 21 cases, particularly in the secondary GBM cultures (4 out of 6 cases). Weak expression of the decoy receptor TRID was detected in 7 out of 21 glial tumour primary cultures before irradiation. Induction of the decoy receptor TRID to modest levels occurred in 12 out of 21 cases. TRAIL was also weakly induced in the cell lines LN 229 and more markedly in LN Z308, in the presence of the KILLER/DR5 receptor but in the absence of the decoy receptor TRID, which showed no response to irradiation (Figure 5.5.7.2).

**Figure 5.5.7.3** mRNA expression of TRAIL, KILLER and TRID in non-radiated controls (represented by '0') and irradiated (10Gy) primary cultures derived from grade II tumours (first two columns), secondary GBM (next two columns) and *de novo* GBM (final seven columns)



# 6. DISCUSSION

Current understanding of the cellular response to genotoxic stress evoked by irradiation is rudimentary. Although key molecular players, such as p53 and its downstream target genes, have been identified, their intricate regulation and interactions have not yet been fully elucidated. For radioresistant tumours such as GBMs, a deeper understanding of the mechanisms involved in the response to ionising radiation is crucial in order to permit the development of improved therapeutic strategies.

GBMs can be divided into at least two subgroups. Secondary GBMs bear a mutation in the tumour suppressor gene p53, while *de novo* GBMs are characterised by amplification of the *EGFR* gene and deletion of chromosome 10 (Kleihues and Cavanee 1997). The mechanisms leading to radioresistance and genetic instability in secondary GBMs can be directly related to their loss of p53 function, consequent to mutation. The equally pronounced genetic instability and radioresistance seen in *de novo* GBMs, however, cannot be accounted for by their characteristic genetic alterations, which bear no reported signalling connection to apoptotic pathways. Instead, the radioresistance of *de novo* GBMs indicates that, inspite of the presence of wt p53, the p53 pathway is functionally defective in these GBMs.

The hypothesis that the presence of a wt p53 gene is not synonymous with normal p53 function in *de novo* GBMs forms the crux of the study presented here. The aim was to identify candidate sites of defective regulation of the cell cycle and apoptosis in *de novo* GBMs. In essence, this project questions what mechanisms lead to radioresistance in *de novo* GBMs.

The molecular changes induced *in vitro* by ionising radiation in primary cell cultures from both secondary and *de novo* GBMs, as well as three GBM cell lines, have been investigated. Molecular characterisation of the GBM primary cultures has demonstrated frequencies of p53 SSCP band shifts, increased basal p53 protein expression and *MDM2* amplification similar to those reported in the literature for GBM tumour material (Kouzarides 1995, Biernat et al. 1997). The mutational SSCP band shifts for the p53 gene coincided with those tumours within the secondary GBM group, or grade II or III astrocytic tumours. Since no mutation in p53 outside exon 5 to 8 has been reported for glial tumours (Kleihues and Cavanee 1997), the finding of wt SSCP

band patterns for exons 5 to 8 in those cultures established from *de novo* GBMs is most likely to represent intact p53 gene status.

Novel short spliced transcripts of mdm2, the negative regulator of p53, have been identified in both the GBM primary cultures and cell lines. This finding has been supported by a recent publication, reporting the presence of mdm2 splice variants, larger however than those identified here, in GBM tumour material (Matsumoto et al. 1998).

Investigation of the effects of ionising irradiation on this series of GBMs *in vitro* has revealed that significant apoptosis does not occur, G1 arrest fails in more than 90% but the G2/M checkpoint is successfully instigated in up to 80% of cases. p21 overexpression has been identified in both secondary and *de novo* GBM cultures. In those cultures with basally overexpressed p21, irradiation failed to induce p21, actually repressing it in approximately 70%, and failure in G1 arrest ensued. Despite adequate induction of p21 in up to half of *de novo* GBMs after irradiation, G1 arrest was not achieved, indicative of a defect in the p53 pathway downstream from p21 in these cases.

The following section will discuss the results in detail, with particular respect to the functioning of the p53 response and radioresistance mechanisms in *de novo* GBMs.

## 6.1 Apoptosis

The aim of irradiation treatment of tumour cells is the induction of apoptosis. This study has shown that significant apoptosis does not occur after irradiation of GBM cell lines or glial tumour primary cultures, including those established from *de novo* GBMs. This finding corresponds to the clinical finding of an equally poor response to irradiation in secondary and *de novo* GBMs (Baxendine-Jones et al. 1997). Thus, the question of what mechanisms lead to radioresistance in *de novo* GBMs is valid in this series of *in vitro* cultures. Investigation of the mRNA expression levels of the most important members of the bcl-2 family identified no explanation for abrogation of the apoptotic pathway, but did bring to light several interesting questions which are discussed below.

### 6.1.1 The bcl-2 family

In GBM primary cultures *in vitro* of both secondary and *de novo* subgroups, the pro-apoptotic regulator bax was strongly expressed both before and after irradiation, without evidence of significant induction after irradiation. Neither was a significant change detected in the expression of bcl-2 or bcl-$X_L$ after irradiation in either secondary or *de novo* GBMs. Before irradiation, bcl-$X_L$ was strongly expressed throughout the glial tumour primary cultures but expression of bcl-2 was relatively weak.

The results presented here support the hypothesis that the p53 response itself is dysfunctional in *de novo* GBMs. The absence of either significant induction of bax or repression of bcl-2 and bcl-$X_L$ after irradiation in *de novo* GBM cultures, where the p53 gene is intact, shows that the response of p53 itself to irradiation does not function normally in *de novo* GBMs *in vitro.* Since active p53 directly activates the bax promoter (Bogler et al. 1995), a defect in *de novo* GBMs appears to lie either at the level of p53 itself or upstream, for example at ATM. Consistent with current opinion, this argument makes the assumption that bax is transcriptionally regulated by p53 alone. The p53-null GBM cell line LN Z308, however, expresses bax at relatively high basal levels, indicating that bax expression is not under the sole control of p53. Furthermore, bcl-$X_L$ and in particular bcl-2 show marked induction after irradiation in LN Z308. These results indicate that, in addition to control by p53, the bcl-2 family is regulated by p53-independent mechanisms which are capable of transcriptionally activating pro-life signals in irradiated tumour cells. There is no report in the literature to date, however, of a such a bcl-2 family regulator. The identification of this putative control mechanism is clearly of paramount importance to resolving the problem of radioresistance in GBMs. Like p53, such a regulator would need to be capable of binding to the bcl-2 family promoters, but with the opposite effect of inducing anti-apoptotic and repressing pro-apoptotic members. In the absence of potential candidates, the approach of differential display might provide the most effective means of identifying this putative pro-life regulator of the bcl-2 family.

As noted above, the expression of bcl-2 was relatively weak in GBM primary cultures *in vitro,* but bcl-$X_L$ was expressed at levels comparable to those of bax and was not repressed after irradiation. This finding raises the possibility that the pro-life signal provided by bcl-$X_L$ in GBM primary cell cultures is strong enough to prevent apoptosis.

It has been shown, however, that bcl-$X_L$ preferentially heterodimerises with the pro-apoptotic regulator bad rather than bax (Zha et al. 1996). In the light of the strong bcl-$X_L$ expression detected here in GBMs *in vitro*, it would be interesting to investigate the expression of bad in these cultures; were bad to be relatively weakly expressed in the presence of a strong bcl-$X_L$ signal, this might suffice to retain cytochrome c within the mitochondria and thus prevent caspase activation.

Investigation of bad in these glial tumour cell cultures is also of possible relevance to determining the significance of *EGFR* amplification, which is the characteristic genetic alteration in *de novo* GBMs. A link has been recently established between growth factor receptors and the pro-apoptotic regulator bad (Datta et al. 1997). Activation by insulin-like growth factor 1 (IGF-1) of its receptor has been shown to result in phosphorylation of the serine-threonine kinase c-AKT, via phosphotidyl-inositide-3'-OH kinase activation. c-AKT activation culminates in the phosphorylation of bad. Since only unphosphorylated bad is able to heterodimerise with and thereby neutralise the effect of the anti-apoptotic factors bcl-2 and bcl-$X_L$, phosphorylation of bad results in a strengthened survival signal. Thus, growth factors such as IGF-1 are capable of suppressing apoptosis in favour of proliferation. Theoretically the tyrosine kinase EGF receptor may also be capable of leading to phosphorylation of c-AKT, although there is no report to this effect in the literature to date. If such a signal transduction from the EGF receptor were to operate, an amplification and constitutive activation of the EGF receptor, as seen in *de novo* GBMs, could result in suppression of apoptosis and consequent radioresistance. Thus, although this study has concentrated on the transcriptional level of control of the p53 pathway, post-translational regulation mechanisms may be of even greater importance.

A final question concerns the status of bax. In colon cancers of the microsatellite mutator phenotype, in which p53 is usually wt, frameshift mutations in the *BAX* gene are a frequent finding (Rampino et al. 1997). To date, there has been no report of whether *BAX* mutations occur in GBM. Clearly, a loss of function mutation in *BAX* could lead to radioresistance and so this is an important question which needs to be answered, particularly for *de novo* GBMs.

### 6.1.2 The TRAIL pathway

In view of recent reports that the KILLER/DR5 receptor for the TRAIL ligand is p53-inducible (Wu et al. 1997), the TRAIL pathway was also investigated in the series of glial tumour primary cultures and GBM cell lines. Since the TRAIL ligand was not basally expressed and only weakly induced after irradiation in less than half of the glial primary cultures, the TRAIL pathway does not appear to carry the potential to induce apoptosis in these cells. While KILLER/DR5 was basally expressed in all investigated cultures, no significant change in its expression could be demonstrated after irradiation, indicating that p53 is not capable of effective induction of KILLER/DR5 in glial tumour cells *in vitro*. This lends further support to the hypothesis that p53-dependent induction of its specific direct target genes is defective in GBMs, not only where p53 is mutated but also in *de novo* GBMs in the presence of a wt p53 gene. Interestingly, the PCR for KILLER/DR5 consistently identified two additional larger fragments, which are suggestive of alternative splicing of the receptor, but require confirmation by sequencing. The expression pattern of the two larger fragments, however, did not differ from that of the predicted fragment.

The decoy receptor TRID was weakly expressed after irradiation in approximately half of the GBMs *in vitro*. This contrasts to the published report that TRID is expressed only in non-neoplastic cells, with the function of protecting normal cells from irradiation-induced apoptosis (Pan et al. 1997). While the absence of TRID expression in tumour cells is believed to render the tumours radiosensitive, perhaps the finding here of TRID expression in GBMs contributes to their radioresistance. In view of the minimal TRAIL expression before and after irradiation, however, the significance of TRID induction in GBMs needs to be regarded critically.

## 6.2 Radiosensitivity and the G1 checkpoint

Although the aim of irradiation treatment of tumour cells is the induction of apoptosis, an intimate relationship exists between both G1 and G2/M arrest and the triggering of apoptosis. Clearly p53 has great influence on all of these events, but it is still not clear how and from which cell cycle state p53 initiates apoptosis . The

following section will discuss the relationship between the G1 checkpoint and radiosensitivity in the light of the results in the glial tumour primary cultures and GBM cell lines.

### 6.2.1 G1 arrest and apoptosis

Arrest at the G1 checkpoint failed to occur after irradiation in 16 out of 19 glial tumour primary cultures and in all three GBM cell lines. G1 arrest failed in 4 out of 5 secondary GBM cultures, consistent with their mutant p53 status. The sole exception was a secondary GBM which had undergone radiotherapy *in vivo*. The significance of prior irradiation on the cell cycle control is not known, but notably, G1 arrest was achieved in each of the two previously irradiated glial tumour cultures in this series (NCH 135 established from a grade III oligoastrocytoma and the secondary GBM, NCH 128). The patients in both of these cases enjoyed a lengthy survival of 84 and 59 months respectively, suggestive of a relative radiosensitivity.

In none of the primary cultures established from *de novo* GBMs was a G1 arrest successfully invoked after irradiation. The failure of G1 arrest observed in *de novo* GBMs *in vitro* supports the hypothesis that, despite an intact p53 gene, the p53 pathway is functionally defective in *de novo* GBMs.

The failure of G1 arrest in *de novo* GBM cells *in vitro* contrasts sharply with the response of other tumour types which bear wt p53. A study examining the response of a wide range of non-glial tumour cell lines showed that, in the presence of wt p53, irradiation did induce an arrest in G1 phase (O'Connor et al. 1997). In the cell line LN 229 and all the *de novo* GBM primary cultures studied here, however, inspite of their wt p53 status, G1 arrest failed after irradiation.

The finding of a failed G1 arrest in more than 90% of GBM primary cultures may be of particular relevance to the radioresistant nature of these cells. A significant correlation between the level of irradiation-induced G1 arrest and degree of radiosensitivity has been demonstrated in a series of tumour cell lines of varying radiosensitivities (McIlwrath et al. 1994). Failure to arrest in G1 correlated with radioresistance and, furthermore, this reflected a loss of normal p53 function. This is in agreement with the concept that normal p53 function is required for sensitivity of tumour cells to DNA-damaging insults such as irradiation (Fan et al. 1994).

The traditional model of p53 activity proposes that activation of p53 by DNA damage causes G1 arrest, permitting repair before DNA synthesis in S phase, unless the damage is beyond repair, in which case p53 eliminates the cell by apoptosis. In the irradiated GBM cells *in vitro* investigated here, both apoptosis and G1 arrest fail. Does the failure in G1 arrest result in failure of apoptosis in these GBM cells?

Several lines of evidence suggest that G1 arrest is not necessary for p53-mediated apoptosis to occur. For example, haematopoietic cells undergo p53-mediated apoptosis despite failing to arrest in the G1 phase of the cell cycle (Yonish-Rouach et al. 1993). Likewise, p21-null fibroblasts undergo p53-dependent apoptosis but cannot arrest in G1 (Brown et al. 1997). Such findings provide strong evidence that G1 arrest is not a prerequisite for p53-dependent apoptosis, thus invalidating the traditional model of p53 activity mentioned above. Perhaps the failure of both apoptosis and G1 arrest in radioresistant tumour cells merely reflects parallel consequences of disrupted p53 function?

A further twist to the story comes from the finding that transfection of excess functional Rb can rescue cells from p53-mediated apoptosis (Haupt et al. 1995). This suggests that Rb may play a role in inhibiting p53-mediated apoptosis. When Rb is rendered non-functional by mutation, not only is G1 arrest abrogated, but apoptosis is also enhanced (Morgenbesser et al. 1994). Similarly, cells overexpressing E2F, which is negatively regulated by Rb, are prone to p53-mediated apoptosis (Wu and Levine 1994).

Such findings indicate that the inability of a cell to arrest adequately in G1 favours p53-mediated apoptosis. Thus, the invalidated concept that G1 arrest is necessary for apoptosis may simply have been the wrong way round and, rather, *failure* of G1 arrest initiates p53-mediated apoptosis. This would add a further level of sophistication to p53's protection of the genome, by countering mutations in its downstream cell cycle regulators. But if this is the case, why is apoptosis not initiated in the *in vitro* GBMs studied here, since their G1 checkpoint has certainly failed?

### 6.2.2 The significance of p21 overexpression

The intricate relationship between control of the G1 checkpoint and susceptibility to irradiation-induced apoptosis described above has particular bearing to the overexpression of p21, which has been detected in this series of glial primary cultures and cell lines.

Basal overexpression of p21 is a striking feature of the series studied here, affecting more than 80% of secondary GBMs and 55% of *de novo* GBMs. This finding agrees with a previous report of elevated p21 expression in human GBM tumour tissue compared to normal brain tissue (Jung et al. 1995). Since p21 mutations are not found in GBMs (Koopmann et al. 1995), overexpressed p21 presumably maintains its activity, leading to an increased level of Cdk inhibition. This assumption is supported by the finding that ectopic overexpression of p21 in a human astrocytoma cell line, U-373MG, led to a marked decrease in Cdk2 activity (Chen et al. 1996). Theoretically, increased Cdk inhibition would in turn result in an increased level of binding of E2F1 by hypophosphorylated Rb protein. Since overexpression of E2F1 is known to enhance p53-dependent apoptosis (Wu and Levine 1994), the reduction in levels of available E2F1, consequent to p21 overexpression, may be postulated to impede p53-dependent apoptosis.

Such a mechanism, linking p21 overexpression to blocked apoptosis, would provide a model capable of explaining the radioresistance seen in GBMs which overexpress p21, a group including more than half of the *de novo* GBMs. Such a mechanism may also explain the astounding absence of p21 mutations, observed not only in GBMs but in all human tumours (Koopmann et al. 1995); in the absence of p21-dependent inhibition of Cdk, excessive levels of unbound E2F1 would be permitted to accumulate, rendering the cell prone to apoptosis. In other words, the presence of a p21 mutation would prove fatal to the cell. In support of this theory, an enhancement of apoptosis has been shown in p21-null fibroblasts (Brown et al. 1997). Furthermore, p21 has been shown to protect from apoptosis in differentiating myocytes (Wang and Walsh 1996) and neuroblastoma cells (Poluha et al. 1996). Clearly, futher work, including verification of the E2F1 status in GBM cells where p21 is overexpressed, is necessary in order to substantiate this hypothesis.

p21 certainly warrants further investigation as a possible prognostic factor. If p21 overexpression does indeed correlate with radioresistance, then it would be of particular benefit to be able to identify those patients in which p21 levels are normal, since radiotherapy is likely to be particularly beneficial in these cases. If the overexpression of p21 is confirmed to block apoptosis, this would provide a number of new therapeutic avenues to explore. For example, can introduction of exogenous E2F1 into GBM cells enhance apoptosis induced by irradiation? Likewise, p21 antisense treatment of GBM tumour cells might be able to restore the balance in favour of apoptosis.

What mechanisms lead to p21 overexpression? p21 overexpression might be speculated to be a consequence of the increased p53 protein expression frequently observed in GBMs. In this series of *in vitro* glial tumours, however, no significant correlation between p21 and p53 overexpression was detectable. Given the relatively small size of this study, investigation of p21 overexpression and p53 protein stabilisation in a larger series would perhaps cast a different light on this postulate.

The observation of marked basal expression of p21 in the p53-null GBM cell line raises the possibility that p53-independent mechanisms of p21 induction also exist in GBMs. Although the activation of p21 after DNA damage has been shown to be a p53-dependent process (el-Deiry et al. 1994), basal p21 expression has been shown in p53-null SaOS-2 cells to be induced independently of p53 following oxidative stress (Russo et al. 1995). p53-independent mechanisms of p21 activation, perhaps consequent to oxidative stress, merit further investigation in GBMs.

### 6.2.3 The response of p21 to irradiation

An obvious outstanding question is why do the irradiated GBM cells not manage to arrest in G1 phase of the cell cycle? On considering this issue, it is clear that the p21 response to irradiation in GBM cells *in vitro* falls into two categories. In those cultures where minimal basal p21 expression was manifest, induction of p21 mRNA was invariably achieved after irradiation but G1 arrest failed nonetheless. Irradiation of cultures with basal p21 overexpression failed to achieve p21 induction in more than 90%, and no G1 checkpoint halt was achieved. The implications arising from these two different responses to irradiation in GBM cells will be considered in the next section.

#### 6.2.3.1 G1 checkpoint failure despite p21 induction

In 45% of the investigated *de novo* GBM cultures, significant induction of p21 occurred after irradiation. Despite p21 induction, however, not one of these cell cultures achieved an arrest at the G1 checkpoint. This implies that a defect downstream from p21 occurs in this sub-group of *de novo* GBMs.

A possible candidate is the *RB* gene which is deleted in approximately 30% of GBMs (Henson et al. 1994). In the absence of Rb protein, the regulatory influence of p21 on Rb is redundant and the transcription factor E2F1 remains fully unbound, free to effect immediate passage into the S phase. Thus, G1 arrest is not possible. Since the percentage of GBMs demonstrating failure of G1 arrest despite p21 induction approximates quantitatively to the fraction suffering *RB* deletion, further investigation is required to establish whether *RB* is indeed deleted in this sub-group of *de novo* GBMs.

But although a deficit of Rb function would certainly explain the abrogation of G1 arrest after irradiation in GBMs with adequate p21 induction, it fails to explain the radioresistance of these tumours. As previously discussed, loss of Rb function should infact enhance the apoptotic response to irradiation (Morgenbesser et al. 1994). Can it be true that *RB* deletion actually carries a favourable prognosis in GBM? Such a correlate has certainly not been noted in the literature. It is more probable that a defect in the apoptotic pathway co-exists with *RB* alteration in GBMs. For example, were hyperphosphorylation of bad, discussed in Section 6.1, transpire to be a feature of GBMs with *EGFR* amplifications, the combined effect of *RB* deletion and *EGFR* amplification would account for both failure of G1 arrest and radioresistance.

Clearly, such a scenario contrasts starkly to that where p21 overexpression is postulated to impede apoptosis in GBMs. The latter mechanism is dependent on the expression of Rb protein, which ‘kidnaps’ E2F1. Therefore, it is important establish whether *RB* deletion and p21 overexpression are mutually exclusive in this series of GBM cultures.

A further relevant candidate capable of disrupting the G1 checkpoint despite adequate p21 induction, is the INK4 family member, p16. Deletion of p16 is reported in approximately two thirds of *de novo* GBMs (Biernat et al. 1997). In parallel to p21, activated p16 inhibits Cdk2 and Cdk4 (see Figure 2.4). p16 is not, however, p53-

dependent and is not thought to play a direct role in the response to irradiation. A crucial outstanding question is whether p16 is essential for G1 arrest. If parallel p16 activation were necessary for a halt at the G1 checkpoint, then p16 deletion could account for failure of G1 arrest, despite adequate activation of p21 after irradiation.

#### 6.2.3.2 Failure of the G1 checkpoint and of p21 induction

The G1 checkpoint failed in the *in vitro* GBMs in which p21 was not activated after irradiation in all but one case. In none of the secondary GBM cultures investigated was p21 induction effected. In approximately half of the *de novo* GBM cultures, induction of p21 after irradiation failed, concurring in each case with basal p21 overexpression..Overall, in GBM cultures with basal p21 overexpression, irradiation failed to increase p21 expression in more than 90% of cases and, of these, irradiation led to a marked reduction in p21 mRNA levels in more than 60 %.

These results help to explain the paradoxical failure of G1 arrest after irradiation in the subgroup of GBMs overexpressing p21, since a reduction in p21 levels occurs after irradiation in more than 60 % of the subgroup. Trancriptional repression of p21 after irradiation presumably results in the release of E2F1, forcing the cells to forego the G1 checkpoint, despite the abundant basal expression of p21.

Failure of p21 activation in the secondary GBMs studied *in vitro* is readily explicable on the basis of their p53 mutational status, as indicated by SSCP analysis. The finding that p21 induction after irradiation fails in approximately half of the investigated *de novo* GBM cultures, despite the presence of wt p53, however, provides convincing evidence of a profound defect at or upstream from the level of p53 in this subgroup of *de novo* GBMs. Although a parallel reduction of p53 protein was observed in 6 out of the 10 glial cultures in which p21 levels fell after irradiation, no statistically significant correlation was revealed on analysis by the Fisher's Exact Test. Given the relatively small numbers of tumour cultures investigated, a larger study to clarify this issue is warranted.

## 6.3 p53 protein

The results discussed so far have shown that p53 function is defective not only in secondary GBMs, where it is mutated, but also in *de novo* GBMs which bear wt p53. The failed activation of p53's downstream target genes *WAF1*, encoding p21, and *BAX*, observed in a significant proportion of irradiated *de novo* GBM cultures, provides convincing evidence to this effect. Since the defect in p53 function does not lie within the p53 gene in *de novo* GBMs, perhaps the p53 protein itself is defective. The next section will consider the results from the investigated *in vitro* glial tumours with regard to the p53 protein.

### 6.3.1 p53 protein stabilisation

wt p53 is not normally detected by Western blot analysis because the half-life of wt p53 protein is very short, while mutant p53 protein, which has a longer half-life, is frequently detectable. p53 protein in its wt form, however, is detected by Western blot analysis in approximately 60% of *de novo* GBM tumour material (Anker et al. 1993, Rubio et al. 1993). The finding of detectable wt p53 protein is indicative of an abnormally prolonged half-life and consequent stabilisation of wt p53 in these *de novo* GBMs.

In this study, investigation of p53 expression by Western blotting revealed a pronounced accumulation of p53 protein in the GBM cell lines LN 229 and LN 18, and absent p53 protein in LN Z308. Detection of p53 protein in the mutant p53 cell line LN 18 was to be expected. The pronounced expression of p53 in LN 229, however, represents a stabilisation of wt p53 protein. Investigation of the GBM primary cultures showed increased expression of p53 protein in half of the tumours with mutational p53 SSCP band shifts. p53 protein was moderately to very strongly detectable in up to 70% of the cultures in which a wt p53 SSCP pattern had been demonstrated. These results indicate that wt p53 protein stabilisation occurs in approximately 70% of the investigated *de novo* GBM cultures.

What possible mechanisms might lead to stabilisation of wt p53 protein in *de novo* GBMs and what significance does stabilised p53 protein have for the GBM cell? In considering this question, the identification of splice variants of mdm2, the negative regulator of p53, may have some bearing.

### 6.3.2 The significance of mdm2 splice variants

Our results show that short mdm2 splice variants are expressed in approximately half of this series of human glial tumours *in vitro*. In particular, mdm2 spliced transcripts were found in approximately 60% of the glial tumour primary cultures bearing wt p53. Sequencing of the splice variants demonstrated a splicing which disrupted the p53 binding domain of the mdm2 protein. Furthermore, a significant correlation between the presence of mdm2 splice variants and increased expression of wt p53 protein was determined (Fisher's Exact Test, $p<0.01$).

Although the phenomenon of wt p53 protein stabilisation has long been noted in *de novo* GBMs, the mechanism by which the half-life of wt p53 is prolonged is still unclear. It has been shown recently that mdm2 directly instigates p53 protein degradation when their multimeric complexes are bound via the p53 binding domain in the amino terminal of mdm2 (Haupt et al. 1997). Thus, the quantitative molecular ratio between p53 and mdm2 is of importance in determining the half-life of p53. In 8-10% of glioblastomas with wt p53, *MDM2* is amplified and mdm2 protein is overexpressed (Biernat et al. 1997). Given the consequent shift in the molecular ratio, one would expect that *MDM2* amplification would exclude accumulation of wt p53 protein. In this study, however, it was shown that *MDM2* amplification coincided with wt p53 protein stabilisation in the GBM primary culture G116 and cell line LN 229. Both these cultures also express alternatively spliced mdm2 transcripts, deficient in their p53 binding domain. Is the paradox of wt p53 protein stabilisation coincident with amplified *MDM2* perhaps explained by the finding that mdm2 is alternatively spliced in these *in vitro* GBMs?

On consideration of the results presented here, the constellation of p53 status, *MDM2* status and presence of spliced mdm2 transcripts is able to provide a feasible explanation for the finding of stabilised p53 protein, particularly that of wt form. In those GBMs bearing wt p53 and amplified *MDM2* (LN 229, G116 and G117), one would expect increased degradation of p53 protein in view of the amplification in *MDM2*. Hence, one would not expect to find detectable p53 in the Western blot. This is indeed the case for G117. G116 and LN 229, however, display marked p53 protein expression, which coincides with the presence of mdm2 splice variants. Since the mdm2 splice variants lack their p53 binding domain and are thus prevented from instigating

p53 protein degradation, the degradative effect of overexpressed mdm2 on p53 is theoretically counteracted by the generation of these splices. G117 displays no spliced mdm2 variants and thus does not escape p53 protein degradation.

Likewise, in *de novo* GBMs without *MDM2* amplification, pronounced stabilisation of wt p53 protein was observed where short mdm2 splice variants were also expressed, with only one exception (G148). In the absence of excessive p53 protein degradation by overexpressed mdm2, a spliced variant of mdm2 would be predicted to achieve a greater stabilising influence. Accordingly, the observed quantitative differences in the extent of wt p53 protein accumulation appear to relate to the presence or absence of *MDM2* amplification. For example, particularly pronounced wt p53 expression was detected in G139, where a spliced mdm2 transcript was present in the absence of *MDM2* amplification. In contrast, G116, which does bear *MDM2* amplification, displayed a lesser degree of wt p53 protein accumulation.

Spliced mdm2 transcripts did not occur exclusively in the presence of an intact p53 gene. LN18, in which p53 is mutated, displayed a spliced mdm2 transcript and marked stabilisation of p53 protein. However, mutant p53 protein was also stabilised in the absence of mdm2 splice variants, for example in G101 and G109, presumably as a result of the inherent stability of the mutant protein.

Since p53 itself induces mdm2, one might postulate that p53 influences the splicing mechanism. This is supported by the observation that mdm2 splices are not generated when p53 is absent (LN Z308). Furthermore, when at least one wt p53 allele is present (LN 18) alternative splicing of mdm2 appears to be possible and splice variants of mdm2 are particularly frequent (up to 60% of cases) where the p53 gene remains intact.

If, as this suggests, p53 does bear some control over the ability to alternatively splice mdm2, one is bound to ask whether mdm2 splice variants confer an advantage to the cell. An accumulation of wt p53 protein in a tumour cell might increase its chances of either effecting DNA repair or initiating apoptosis and thus combating the tumorigenic process. Evidence that accumulated wt p53 protein does have a biological effect was provided by adenovirus-mediated transfer of wt p53 into glioma cells with consequent increased expression of wt p53 protein (Gomez Manzano et al. 1996). In glioma cells bearing endogenous mutant p53 the effect of wt p53 protein accumulation

was induction of apoptosis, whereas those glioma cells with intact p53 exhibited marked inhibition of cellular proliferation. Perhaps in GBM cells wt p53 attempts to upregulate itself, particularly when threatened by an amplification in *MDM2*, by generating spliced transcripts of mdm2 , deficient in their p53 binding domain, which consequently permit p53 accumulation.

Full length mdm2 is known to be oncogenic, exerting multiple pro-proliferative effects, as previously discussed. Studies in which p53-/ pRb- human osteosarcoma (SaOS-2) cells were transfected with truncated forms of mdm2 identified the minimum essential transforming region of mdm2 to be the p53 binding domain (Dubs Poterszman et al. 1995). Thus, spliced mdm2 transcripts which lack this binding domain could be postulated to lose their transforming potential, supporting the hypothesis that these isoforms have a protective role in the cell.

Conversely, another group has correlated the presence of short mdm2 splice variants in ovarian and bladder carcinoma with malignancy and furthermore has demonstrated that the splices have malignant transformation potential in NIH 3T3 cells (Sigalas et al. 1996). This raises the question of whether the alternative splicing of mdm2, putative leading to p53 protein stabilisation, is detrimental to the cell. Were the sequence of events confirmed to lead from alternative mdm2 splicing to p53 protein accumulation and hence to p21 overexpression, one might speculate that mdm2 splicing constitutes a tumorigenic mechanism.

The hypothesis formulated here that alternative splicing of mdm2 results in wt p53 protein stabilisation is supported by all the reported results except for the *de novo* GBM culture G148, in which marked stabilisation of wt p53 protein was found in the absence of a mdm2 splice variant. This suggests that p53 stability is also dependent on other regulatory mechanisms. Of possible relevance is the recent report that the *INK4* gene product, $p19^{ARF}$, blocks mdm2-dependent p53 protein degradation (Zhang et al. 1998, Pomerantz et al. 1998). Thus, $p19^{ARF}$ acts to increase levels of p53 protein in the cell by inhibiting mdm2 function. The interaction between $p19^{ARF}$ and mdm2 appears to occur directly at the carboxy terminal of the mdm2 protein and promotes mdm2 degradation. Since the $p19^{ARF}$ -encoding gene region, which shares its second and third exons with p16, is commonly deleted in GBM, a loss of $p19^{ARF}$ infact arises in GBMs. This in itself would not explain wt p53 protein accumulation, since deletion of $p19^{ARF}$

would be expected to enhance p53 protein degradation, but does indicate that multiple parallel pathways are probably involved in controlling p53 stability.

### 6.3.3 Degradation of stabilised p53 protein after irradiation

In almost 90 % of glial tumour primary cultures with increased basal p53 protein expression, irradiation led to a marked reduction in the protein level. This suggests that irradiation stimulated degradation of the stabilised p53 protein.

What mechanism might account for the radiation-induced degradation of p53 protein? Since the basal expression of mdm2 can be seen from the results to be appreciable, post-translational modification of the mdm2 protein after irradiation could rapidly activate its ability to degrade p53 protein. Furthermore, $p19^{ARF}$ normally inhibits mdm2 activity (Zhang et al. 1998, Pomerantz et al. 1998). The *INK4* gene locus, which encodes $p19^{ARF}$, is commonly deleted in GBMs (Schlegel et al. 1996). Thus, in the absence of its own negative regulator, mdm2 may be primed to be particularly aggressive in its attack on p53 in such GBMs.

In summary, p53 protein in GBM cultures has been shown to behave abnormally, not only where it is mutated, but also where a wt gene is present, as in *de novo* GBM cultures. Therefore, these findings support the hypothesis that presence of an intact p53 gene in GBMs is not synonymous with normal p53 function. The significance of wt p53 stabilisation is still unclear. It may represent a rescue attempt within the tumour cell, with the aim of promoting cell cycle control and apoptosis, but then why is it rapidly degraded after irradiation? Conversely, stabilisation of wt p53 protein might constitute a tumorigenic mechanism, inducing p21 overexpression and consequently impeding apoptosis. Such speculations merit further investigation.

## 6.4 Radiosensitivity and the G2/M checkpoint

*In vitro* GBMs have been shown in this study to respond to ionising radiation with a G2/M arrest in up to 80% of cases, regardless of their p53 status. This finding demonstrates that entry into the G2/M checkpoint is controlled independently of G1 arrest in GBM cells, and in particular that it is a p53-independent event.

In contrast to G1 arrest, maximal G2 arrest does not correlate with radiosensitivity (McIlwrath et al. 1994) but rather with radioresistance (McKenna et al. 1991). In fact, it has been shown that irradiation-induced apoptosis correlates positively with the brevity of the G2 arrest (Yao et al. 1996 , Powell et al. 1995). Shortening the duration of G2 arrest by treatment with caffeine, which activates Cdc2, results in a sensitisation of p53-deficient tumour cells to irradiation. Thus, the pronounced G2/M arrest observed in the irradiated GBMs *in vitro* may contribute to the radioresistance of the tumours.

p53 has been shown to be responsible for accelerating the exit from G2 arrest (Guillouf et al. 1995), whereas cells lacking wt p53 display a pronounced delay in G2 after exposure to irradiation (Metting and Little 1995). Thus, p53 may actually have the effect of terminating G2 arrest. Perhaps this provides a hint that the G2/M checkpoint is the spring board to p53-mediated apoptosis; G1 arrest provides the opportunity to repair DNA damage. This does not exclude the possibility that damage, particularly if severe, persists into the subsequent phases of the cell cycle. DNA damage would certainly persist if G1 arrest fails altogether. In this scenario the cell arrests at the G2/M checkpoint. If at this point active p53 is present, it terminates the G2 arrest and initiates apoptosis. In the absence of active p53, G2 arrest is maintained until repair is completed, signalled by the disappearance of ATM. This raises the question of whether the pronounced reduction in stabilised p53 protein, seen in many of the GBM primary cultures after irradiation, strands the GBM cell in G2/M arrest, thus contributing to radioresistance.

The model described here would be consistent with the finding that apoptosis can occur in the absence of G1 arrest, and relies on the fact that initiation of G2/M arrest is independent of p53. It would probably rely on the existence of differential p53 activation states, since on one hand p53 has to be inactivated before the cell can exit G1 arrest, but on the other hand, active p53 terminates subsequent G2/M arrest and commits the cell to apoptosis. This is difficult to tie together unless p53 has differing

activation states, dependent on the type of DNA damage or means of activation.

In support of this is the observation that ATM-independent activation of p53 by irradiation does not result in either normal p21 activation or G1 arrest, but does induce bax, resulting in apoptosis (Barlow et al. 1997). This implies that distinct, differentially activated p53 forms exist to provide the switch mechanism between DNA repair and apoptosis. A stumbling block in this argument is the finding that AT cells, deficient in ATM, fail to arrest in both G1 and G2/M, but can still apoptose after irradiation (Jorgensen and Shiloh 1996). This raises the question whether either of the two main cell cycle checkpoints are necessary for committment to apoptosis. Such vital questions remain to be answered.

## 6.5 Limitations to *in vitro* studies of glial tumours

In order to improve our understanding of the dynamic molecular responses operative in human tumours, *in vitro* studies play an important role and yield valuable results. The response of tumour cells *in vitro*, however, cannot be assumed to be representative of the *in vivo* situation.

This project has attempted to minimise the *in vitro* and *in vivo* differences by studying glial tumour primary cell cultures in early passages. The genetic and molecular characteristics of tumour cells are believed to be increasingly altered by repeated cell passaging. The glial tumour primary cultures studied here demonstrated relatively slow growth kinetics and marked cellular polymorphy, suggestive of polyclonality. In contrast, the GBM cell lines investigated in parallel were highly monomorphic, implying that they had undergone monoclonal selection during the course of repeated passaging. Accordingly, the primary cultures were used for all experiments within the first four passages in order to provide an *in vitro* system which at least reflects the *in vivo* tumour biology more closely than can established cell lines.

The *de novo* group of GBMs are characterised genetically by amplification in *EGFR* and absence of p53 mutation. Thus, in order to fully confirm the tumour classification in the investigated series, the *EGFR* status would have provided valuable information. The investigation of GBM primary cultures has a specific limitation, however, since the amplification of *EGFR* is known to be eliminated within the first *in*

*vitro* passage. Because it occurs so rapidly, this appears to be an active process (Bigner et al. 1990), but the mechanism is not understood. Thus, determination of *EGFR* amplification within primary cell cultures of GBMs is not directly possible. Unfortunately neither frozen tumour material nor normal tissue from the patients in this series was available to permit either direct analysis of *EGFR* or LOH studies.

As discussed in the Introduction, amplification of *EGFR* in GBMs co-exists with chromosome 10 aberrations, including chromosome 10 monosomy (Leenstra et al. 1994). *PTEN* has been identified as a candidate tumour suppressor gene on chromosome 10 (Li et al. 1997, Steck et al. 1997) and has been postulated to reflect *EGFR* status in GBMs. Within the scope of a parallel project in the same department, *PTEN* status is currently being investigated in this series of glial tumour primary cultures.

## 6.6 The way ahead

The results from this project have shed some light on possible mechanisms of radioresistance in GBMs. The GBM cells respond to irradiation with failed apoptosis, failed G1 arrest but pronounced G2/M arrest. Since the profile of absent G1 arrest and a long G2/M arrest has been shown, on both counts, to correlate with radioresistance, it is important to establish in these cultures why G1 arrest fails and why G2/M arrest is so pronounced.

In secondary GBMs, the loss of p53 function arising from mutation accounts for the aberrant irradiation response. But it is clear that, even in those GBMs with wt p53, the *de novo* GBMs, the p53 response is dysfunctional. In a little more than half of these cases the defect appears to lie at or above the level of p53, since induction of p21 and bax after irradiation fails. Whether stabilised wt p53 protein has a role to play in this abnormal response requires investigation. In the remaining *de novo* GBMs, G1 arrest fails despite induction of p21, suggestive of a downstream defect, perhaps at the Cdk-Rb level. Whether deficits in Rb function account for these cases merits further study.

The results have also highlighted possible mechanisms by which apoptosis may be impeded. The overexpression of p21 which occurs in over two thirds of GBM cells *in vitro* may be of particular importance, as a result of its inhibitory influence on the

pro-apoptotic transcription factor E2F1. The means by which E2F1 exerts this effect are unknown. In view of the finding that anti-apoptotic members of the bcl-2 family are upregulated independently of p53 in the GBM cell line LN Z308 after irradiation, a search for novel bcl-2 family regulators may be merited. In GBM cells where p21 overexpression is not manifest, urgent questions as to the status of the apoptotic regulator bad, and its possible connection to *EGFR* amplification, arise. This highlights the probable importance of post-translational modification as a means of regulation of the p53 response, in addition to the transcriptional control which has been investigated in this project.

Thus, the way forward has become a little more evident. The application of this information to the benefit of the patient, however, remains the ultimate- and perhaps most difficult- challenge.

# 7. SUMMARY

Malignant human glial tumours bear a grave prognosis. The average survival time of patients diagnosed with the most malignant of the glial tumours, glioblastoma multiforme (GBM), is only one year (Kleihues and Cavanee 1997). Although adjuvant radiotherapy is of proven benefit, its effect is limited. A clearer understanding of the cellular response to ionising radiation, particularly the molecular mechanisms by which tumour cells evade apoptosis, is pivotal to the development of new therapeutic strategies for GBM.

GBMs can be divided into at least two subgroups. Secondary GBMs bear a mutation in the tumour suppressor gene p53, while *de novo* GBMs are characterised by amplification of the *EGFR* gene and deletion of chromosome 10. The mechanisms leading to radioresistance and genetic instability in secondary GBMs can be directly related to their loss of p53 function, consequent to mutation. The equally pronounced genetic instability and radioresistance seen in *de novo* GBMs, however, cannot be accounted for by their characteristic genetic alterations, which bear no reported signalling connection to apoptotic pathways. Instead, the radioresistance of *de novo* GBMs indicates that, inspite of the presence of wt p53, the p53 pathway is defective in these GBMs.

The hypothesis that the presence of a wt p53 gene is not synonymous with normal p53 function in *de novo* GBMs forms the crux of the study presented here. The aim was to identify candidate sites of defective regulation of the cell cycle and apoptosis in *de novo* GBMs by comparing their molecular response to ionising radiation with that of othe glial tumours, particularly the secondary GBMs.

Molecular characterisation of glial tumour primary cultures has demonstrated frequencies of p53 SSCP band shifts, increased basal p53 protein expression and *MDM2* amplification similar to those reported in the literature for GBM tumour material (Kouzarides 1995, Biernat et al. 1997). Novel short spliced transcripts of mdm2, the negative regulator of p53, have been identified in both the GBM primary cultures and cell lines. Investigation of the effects of ionising irradiation on GBMs *in vitro* has revealed that significant apoptosis does not occur, G1 arrest fails in more than 90% of cases but the G2/M checkpoint is successfully instigated in the majority. p21 overexpression has been identified in both secondary and *de novo* GBM cultures. In

those cultures with basally overexpressed p21, irradiation failed to induce p21 and failure in G1 arrest ensued. Despite adequate induction of p21 in up to half of *de novo* GBMs after irradiation, G1 arrest was not achieved.

The results from this project have shed light on possible mechanisms of radioresistance in GBMs. In secondary GBMs, the loss of p53 function arising from mutation accounts for the aberrant irradiation response. But it is clear that, even in those GBMs with wt p53, the *de novo* GBMs, the p53 response is dysfunctional. In a little more than half of these cases the defect appears to lie at or above the level of p53, since induction of p21 and bax after irradiation fails. Whether stabilised wt p53 protein has a role to play in this abnormal response requires investigation in a larger series of tumours. The identification of short alternatively spliced mdm2 transcripts, deficient in their p53 binding site, which correlate significantly with increased basal expression of p53 protein, suggests that mdm2 splice variants may be responsible at least in part for wt p53 protein stabilisation in *de novo* GBMs. In the remaining *de novo* GBMs, G1 arrest fails despite induction of p21, suggestive of a downstream defect, perhaps at the Cdk-Rb level, which needs to be further investigated.

The results have also highlighted possible mechanisms by which apoptosis may be impeded. The overexpression of p21 which occurs in over two thirds of GBM cells *in vitro* may be of particular importance, as a result of its inhibitory influence on the pro-apoptotic transcription factor E2F1. The means by which E2F1 exerts its effect on apoptosis are unknown. In view of the finding that anti-apoptotic members of the bcl-2 family are upregulated independently of p53 in the GBM cell line LN Z308 after irradiation, a search for novel bcl-2 family regulators is merited. In GBM cells where p21 overexpression is not manifest, urgent questions as to the status of the apoptotic regulator bad, and its possible connection to *EGFR* amplification via the c-AKT signalling pathway, arise.

In summary, p53 protein in GBM cultures has been shown to behave abnormally, not only where it is mutated, but also in the presence of wt p53 in *de novo* GBM cultures. Therefore, these findings support the hypothesis that presence of an intact p53 gene in GBMs is not synonymous with normal p53 function. In particular, the finding of aberrant p21 expression in more than half of *de novo* GBMs may be of prognostic value and may possibly provide new therapeutic targets for enhancing the effect of radiotherapy in patients suffering from this distressing condition.

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