The Activity of Arachidonic Acid and Gamma-Linolenic Acid on Human Gliomas

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Declaration

I confirm that the work presented in this thesis was the result of experiments conducted by myself and that the thesis composition is my own.

Studies which were carried out in collaboration with colleagues are fully acknowledged in the text.

To Dad

With all my love

Jillian

With very special thanks and all my love

For Brian

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Index of Abbreviations

AA	arachidonic acid
α-LnA	alpha-linolenic acid
AI	apoptotic index
ANOVA	analysis of variance
BK	bradykinin
BrdU	bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
CDKN2	cyclin-dependant kinase
CNS	central nervous system
CT	computerised tomography
DAG	diacylglycerol
DCF	2',7'-dichlorofluorescein
DGLA	dihomo-gamma-linolenic acid
DHA	docosahexaenoic acid
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dUTP	deoxyuridine triphosphate
EGFR	endothelial-derived growth factor receptor
EPA	eicosapentaenoic acid
FAK	focal adhesion kinase
FCS	foetal calf serum
FCγR	Fc receptor for immunoglobulin G2
FITC	fluorescein isothiocyanate
Fl-1	fluorescence-1 - DCF-associated fluorescence, 515-545nm (green)
Fl-2	fluorescence-2 - PI-associated fluorescence, 564-607nm (red)
FMLP	formyl-methionine-leucine-phenylalanine
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
GLA	gamma-linolenic acid
GSH	reduced glutathione
GS-NO	s-nitroso-glutathione
GTP	guanosine triphosphate
Gy	gray
HEPES	hydroxyethyl piperazine ethanesulphonic acid
HODE	hydroxyoctadecadienoic acid
ICE	interleukin-converting enzyme
IFAP	Intermediate filament-associated protein
IgG	immunoglobulin G
IL-1	interleukin-1
IL-2	interleukin-2
IL-6	interleukin-6

$InsP_3$	inositol trisphosphate
LA	linoleic acid
LnA	linolenic acid
LTB_4	leukotriene B4
LTB_5	leukotriene B5
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
mv	millivolt
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NADPH	nicotine adenine dinucleotide phosphate (reduced)
NGF	nerve growth factor
NMDA	N-methyl D-aspartate
NO	nitric oxide
PIP_3	phosphatidyl-inositol trisphosphate
PAF	platelet activating factor
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PI	propidium iodide
PKC	protein kinase C
PLA_2	phospholipase A_2
PLAP	phospholipase A_2 activating protein
PLC	phospholipase C
PLD	phospholipase D
PLL	poly-L-lysine
PMA	phorbol myristate acetate
PUFA	polyunsaturated fatty acid
pRb	retinoblastoma protein
RNA	ribonucleic acid
roi	reactive oxygen intermediate
ros	reactive oxygen species
SAS	statistical analysis software
SEM	standard error of the mean
SNAP	S-nitroso-N-acetylpenicillamine
SOD	superoxide dismutase
SPER/NO	[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]
TAG	triacylglycerol
TGF	transforming growth factor
TNF	tumour necrosis factor
TBS	tris-buffered saline
TPA	teraphthalic acid
TUNEL	terminal uridine deoxynucleotide nick end labeling
VEGF	vascular endothelial growth factor

VnR	vitronectin receptor
WHO	World Health Organisation

Abstract

Despite recent advances in tumour cell biology, the prognosis for patients suffering from malignant glioma remains poor. Although primary glioma rarely metastasises outside the central nervous system (primary being defined as the mass of tumour cells at the original site of the neoplastic event) median survival of adults is less than 1 year after diagnosis.The efficacy of existing therapeutic interventions is limited by poor penetration of chemotherapeutic drugs across the blood brain barrier, the inherent radioresistance of glioma tissue and the infiltrating nature of the tumour. Further progress is likely to be achieved through analysis of the complex biology of these tumours and the development of novel therapeutic strategies. The purpose of this study was to investigate the therapeutic potential of the n-6 essential fatty acids arachidonic acid and gamma-linolenic acid, which may inhibit tumour proliferation by acting as substrates for the production of potentially cytotoxic reactive oxygen intermediates and stimulating apoptotic cell death, both alone and in conjunction with radiation.

Experiments were undertaken to investigate the effects of exogenous arachidonic acid and gamma-linolenic acid on cellular peroxidation, proliferation, viability and apoptosis. These investigations were carried out on single cell suspensions of morphologically heterogeneous fresh human glioma tissue and associated normal brain, human phagocytes and the rat C6 glioma cell line. It was shown that oxidative activity was impaired in human glioma tissue. Addition of 4-40μM arachidonic acid and gamma-linolenic acid induced a concentration dependant increase in tumour reactive oxygen intermediate production and apoptotic activity. Although the kinetics of reactive oxygen intermediate formation in the presence of arachidonic acid and gamma-linolenic acid followed an exponential function in both normal and tumour cell preparations, tumour cells showed a significantly higher sensitivity to exogenous essential fatty acid stimulus. The kinetics of this stimulation were grade dependent, with high grade tumours responding in a more rapid and sustained manner in comparison with lower grade tumours. The morphological heterogeneity of the human glioma preparations was confirmed with immunohistochemical analysis and flow cytometry using monoclonal and polyclonal anti-Glial Acidic Fibrillary Protein (GFAP). GFAP positive cells responded to exogenous arachidonic acid and gamma-linolenic acid with increased reactive oxygen intermediate production, indicating a high sensitivity of glioma cells to essential fatty acid stimulus. Reactive oxygen intermediate production was also investigated in phagocyte preparations of patients undergoing pulmonary resection for lung cancer. It was found that reactive oxygen intermediate generation was stimulated in patient and control phagocytes by exogenous 1-40μM arachidonic acid and gamma-linolenic acid both pre and post-operatively. Increased reactive oxygen intermediate formation was detected in the cell population identified as leukocytes in preparations of human primary glioma, although this response was less than that of associated tumour. It was also found that surgery was associated with an increase in phagocyte reactive oxygen intermediate at 2 and 7 days post-operatively in lung cancer patients. The interactive effects of arachidonic acid, gamma-linolenic acid and therapeutic radiation were demonstrated in the rat C6 glioma cell line. The rate of reactive

oxygen intermediate production in response to exogenous arachidonic acid and gamma-linolenic acid increased within the first hour, and elevated oxidative activity was detected for up to three hours. However, a different pattern of reactive oxygen intermediate generation was observed in response to radiation alone. Similarly, an early apoptotic response was observed following exogenous arachidonic acid and gamma-linolenic acid stimulation. In comparison, radiation induced stimulation of apoptosis occurred over the 12 hour period of incubation and was maximal between 6 and 8 hours post-irradiation. An enhanced radiation response was observed when the stimulation of apoptosis induced by essential fatty acid stimulus alone was low, suggesting that essential fatty acids and radiation may interact to potentiate reactive oxygen intermediate generation and apoptosis.

In conclusion, this study has provided evidence that glioma tissue has low basal oxidative activity in comparison with associated normal brain, and that addition of exogenous arachidonic acid and gamma-linolenic acid stimulates peroxidative and apoptotic activity in glioma tissue a grade dependant manner. Studies on the cellular heterogeneity of human glioma samples indicate that the stimulation of reactive oxygen intermediate production by exogenous arachidonic acid and gamma-linolenic acid occurs in GFAP positive cells. This indicates high sensitivity of human glioma to exogenous essential fatty acid stimulus. Phagocyte populations from lung cancer and malignant glioma patients also respond with increased reactive oxygen intermediate production to exogenous arachidonic acid and gamma-linolenic acid, although the magnitude of this increase is less than that observed for tumour cells. In addition, there is evidence of potentiation of the oxidative and apoptotic response of the rat C6 cell line to exogenous arachidonic acid and gamma-linolenic acid in the presence of therapeutically relevant doses of radiation. These results are consistent with a clinical role for arachidonic acid and gamma-linolenic acid in the treatment of malignant glioma.

Chapter 1:

The Role of Essential Fatty Acids in Cell Proliferation and Death: Relation to Reactive Oxygen Species Formation and Human Primary Glioma

1.1 Introduction

Gliomas are the most common group of intrinsic brain neoplasms in children and middle aged adults (Prados and Levin, 2000). These tumours are currently classified according to presumed cellular origin and histological features associated with increasing malignancy, generally using the WHO system or the system of Daumas-Duport et al (1988). The WHO classification defines these tumours on the basis of cellularity, nuclear and cellular pleomorphism, mitoses, endothelial proliferation and necrosis, with glioblastoma, the highest grade tumour in this system, generally possessing high degrees of each of these characteristics (Berens and Giese, 1999). Anaplastic astrocytomas do not generally contain regions of endothelial proliferation or necrosis, are less cellular and pleomorphic and contain fewer mitoses. Astrocytomas are characterised by moderate cellularity and minimal pleomorphic change, with only occasional mitoses. The Daumas-Duport system grades tumours of glial origin on the presence or absence of four major criteria: nuclear atypia, mitoses, endothelial proliferation and necrosis. Grade one neoplasms have none of these features, grade two have one, grade three have two and grade four have at least three (Daumas-Duport, 1988).

Although there have been significant technical advances in surgical and radiation treatments for brain tumours in recent years, the impact of these developments on clinical outcome has been disappointing (Berens and Giese, 1999 and Neider et al, 2000). This poor prognosis is due in part to the progression of low grade tumours towards more malignant phenotypes and the poor clinical response of malignant gliomas to existing treatment options (Darling, 1990). Surgery is limited by tumour invasion into surrounding normal brain (Turazzi and Licata, 2000), radiotherapy by

the inherent radioresistance of malignant glioma and poor normal tissue tolerance (Mansur et al, 2000) and chemotherapy by the poor penetration of chemotherapeutic drugs across the blood brain barrier (Brandes and Pasetto, 2000 and Olivi et al, 2000). Improvements in patient survival are therefore likely to arise from novel therapeutic interventions which increase tumour cytotoxicity, either alone or in conjunction with conventional treatment options (Neider et al, 2000, Brandes and Pasetto, 2000 and Darling, 1990).

A number of investigations have indicated that essential fatty acids may have clinical relevance in the treatment of human maligant glioma. Essential fatty acids are highly unsaturated lipids which cannot be synthesised *de novo* and must be acquired from the diet or other external sources (Burr and Burr, 1929 and Burr and Burr, 1930). They are generally classified into two groups - omega-3 (n-3) and omega-6 (n-6), the number in brackets indicating the position of the first double bond from the methyl terminus of the hydrocarbon chain (Burr and Burr, 1929 and Burr and Burr, 1930). Omega-3 and omega-6 essential fatty acids are not metabolically interconvertible, and they often have different structural, physiological and biochemical functions *in vivo* (Kinsella, 1988 and Simopoulos, 1991). The empirical structure of a typical fatty acid molecule is shown in Figure 1.1a and the molecular configuration of the C20 essential fatty acid arachidonic acid is shown in Figure 1.1b. Typical naturally occurring sources of essential fatty acids are shown in Table 1.1, and processes involved in their metabolism are shown in Figure 1.2.

Although there is limited information on the activity of essential fatty acids in human glioma cells, *in vitro* investigations have indicated that essential fatty acids are associated with loss of glioma cell viability in established cell lines, possibly through potentiation of cellular oxidative activity (Das, 1990a, Das, 1990b, Traynelis et al, 1995 and Hrelia et al, 1996). These studies concur with other data which indicate that essential fatty acids inhibit the growth of transformed cells by acting as substrates for the production of potentially cytotoxic reactive oxygen species and lipid peroxides (Cornwell and Morisaki, 1984 and Gonzalez, 1992). Breast (Cheeseman et al, 1984)

and hepatic tumour tissues (Bartoli and Galeotti, 1979) have low levels of peroxidative activity, possibly due to dietary deficiency and/or lack of metabolic conversion of essential fatty acid precursors (Bartoli and Galeotti, 1979, Cornwell and Morisaki, 1984 and Vatten et al, 1993). This may result in loss of cellular oxidative activity and hence dysregulation of molecular pathways involved in cell proliferation and death (Cheng and Levy, 1979, Galeotti et al, 1984 and Galeotti et al, 1986). The processes involved in the initiation of lipid peroxidation are shown in Figure 1.3.

In addition to mediating direct tumour cytotoxicity, essential fatty acids may augment the therapeutic response to conventional cancer therapy. As there is evidence that radiotherapy and chemotherapy may induce tumour cell death, in part at least, through stimulation of lipid peroxidation (Ewing and Jones, 1987, Ma et al, 1991 and Alaoui et al, 1992), provision of exogenous essential fatty acids may provide pro-oxidative substrate upon which radiation can act (Yamanaka et al, 1978, Oberley and Buettner, 1979 and Yamaguchi, 1994).The relevance of this hypothesis to glioma therapy has been demonstrated *in vitro*, where essential fatty acids increased the radiosensitivity of rat astrocytoma cell lines while exhibiting minimal toxicity to normal rat astrocytes (Vartak et al, 1997 and Vartak et al, 1998).

While the mode of glioma cell death resulting from essential fatty acid administration, either alone or in conjunction with radiation, has not yet been fully characterised, preliminary evidence indicates that this action may be due to stimulation of a morphologically stereotyped and genetically regulated process termed apoptosis (Nakatsu et al, 1996 and Zhu et al, 1996) rather than a pathological form of cell death termed necrosis which occurs in response to tissue insults including hypoxia and membrane disruptants (Kerr et al, 1972, Wyllie, 1974, Wyllie et al, 1980 and Bellamy et al, 1995). These preliminary studies therefore suggest that essential fatty acids may be involved in tumour-specific molecular pathways mediating programmed cell death and radiosensitivity.

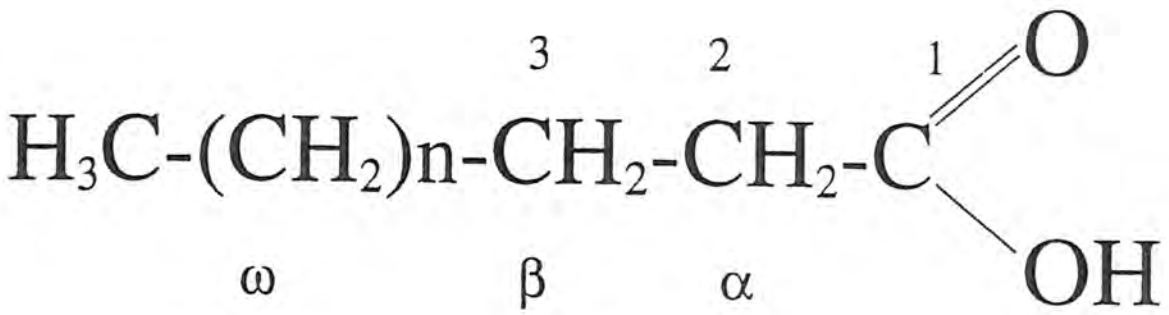


Figure 1.1a. Typical Structure of a fatty acid molecule. Fatty acid carbon atoms are numbered from the carboxyl terminus. Carbon atoms 2 and 3 are referred to as α and β respectively, and the methyl carbon atom at the end of the chain is called ω or δ. The structure of a fatty acid molecule is usually symbolised by the x:y n-z notation where x represents the total number of carbon atoms in the hydrocarbon chain, y, the total number of double bonds and z, the position of the first double bond from the methyl terminus.

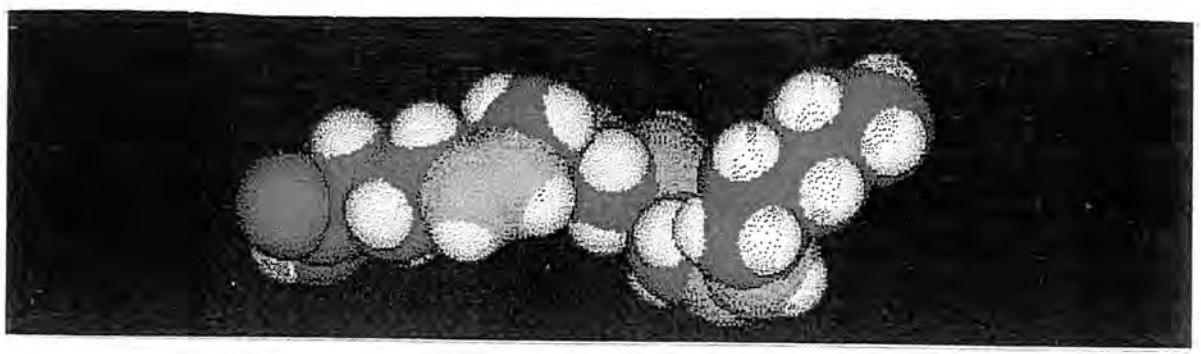
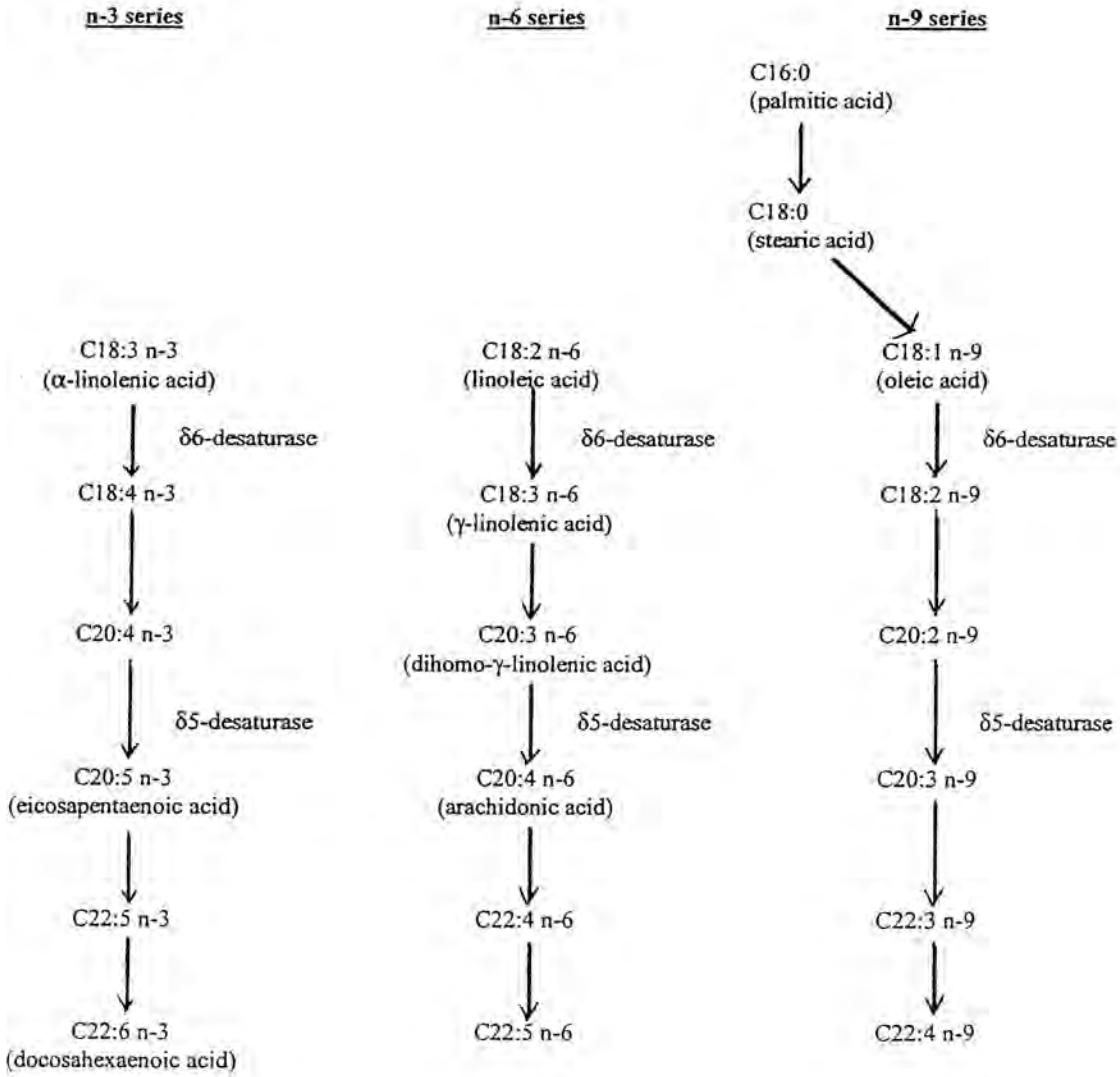


Figure 1.1b. Energy optimised configuration of the C20 essential fatty acid arachidonic acid as determined by the Oxford Molecular Program optimised over 400 cycles. Green: carbon, white: hydrogen, yellow: aliphatic double bond, red: oxygen atoms of carboxyl terminus. Molecular modelling was performed by I. Dawson and A. Leaver (Department of Pharmacology).

Table 1.1. Characteristics and Sources of the Principal Naturally Occurring Fatty Acids

family	name	structure	major source
saturated	palmitic acid	16:0	dairy produce and red meat
	stearic acid	18:0	dairy produce and red meat
unsaturated	oleic acid	18:1 n-9	various vegetable oils including olive oil
	linoleic acid	18:2 n-6	dairy produce, offal, human milk and vegetable seed oils e.g. sunflower, safflower corn and olive oils
	γ-linolenic acid	18:3 n-6	various seeds including evening primrose, borage and blackcurrant
	α-linolenic acid	18:3 n-3	green vegetables, soya and linseed oil
	arachidonic acid	20:4 n-6	dairy produce, meat and various vegetable oils
	eicosapentenoic acid	20:5 n-6	fish, fish oil and shellfish
	docosahexaenoic acid	22:6 n-3	fish, fish oil and shellfish

Figure 1.2. Overview of the metabolic pathways traditionally believed to be involved in the metabolism of the n-3, n-6 and n-9 series essential fatty acids.



*these metabolic conversions proceed slowly in subjects consuming typical western diets. This is because high concentration of 18:2 n-6 fatty acid precursor in cell membrane phospholipids enables successful competition for active sites on desaturase and elongase enzymes. Additional pathways for essential fatty acid metabolism involving the transfer of 24 carbon fatty acids from the endoplasmic reticulum to peroxisomes are discussed in Section 1.4.

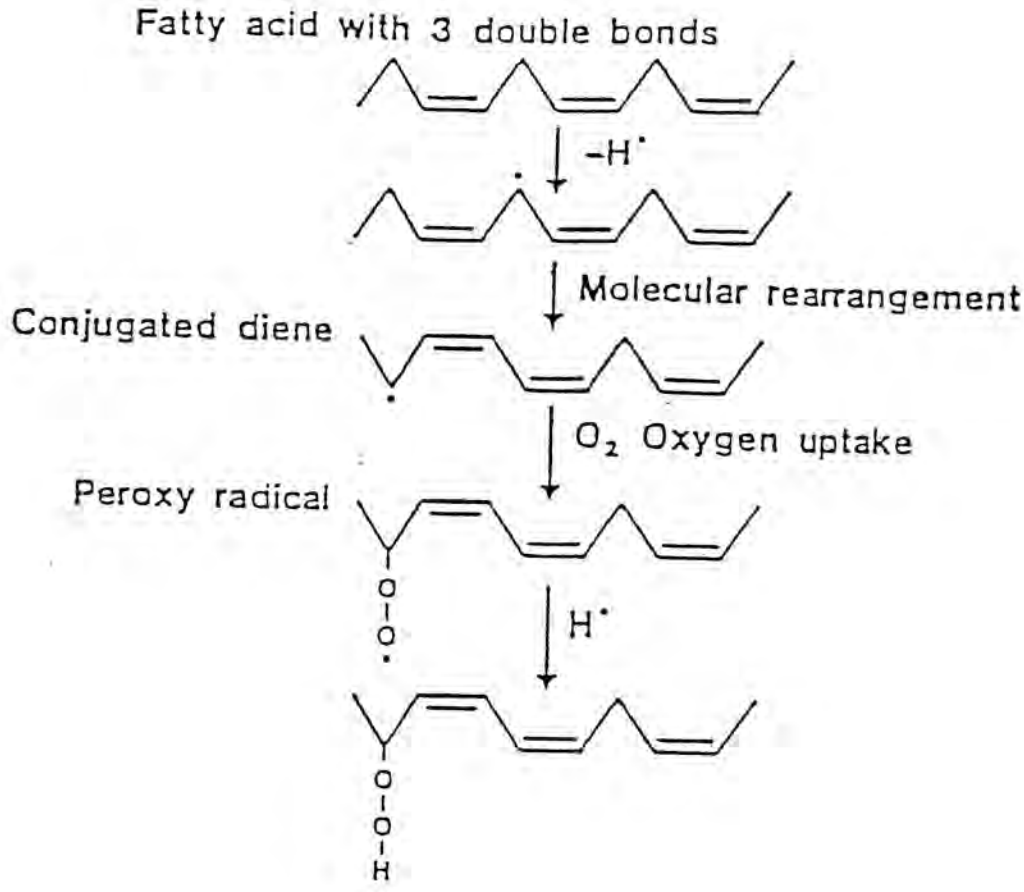


Figure 1.3. Processes involved in the initiation of lipid peroxidation (from Krinsky et al, 1992).

1.2 The Role of Essential Fatty Acids and Oxidative Metabolism in Tumour Proliferation and Death *in vivo* and *in vitro*

The role of essential fatty acids in tumour cytotoxicity was suggested through epidemiological studies which investigated the involvement of dietary lipid in tumour pathogenesis. The relatively low incidence of certain forms of cancer among populations whose diets contained little or no saturated fat, e.g. the Eskimos and Japanese, implied there was an inverse association between polyunsaturated fatty acid consumption and the occurrence of malignant disease (Armstrong and Doll, 1975 and Pritchard et al, 1989). Additionally, epidemiological studies indicated that the probability of migrant Eskimo and Japanese populations developing breast and colon cancer was not significantly different from native western populations. This suggested that environmental rather than genetic factors were important determinants of the pathogenesis of these diseases (Armstrong and Doll, 1975).

Although these epidemiological and dietary studies demonstrated a positive link between saturated fat consumption and cancer of the colon (Committee on Diet and Health, 1989 and Willet et al, 1990), the involvement of saturated dietary lipid in the pathogenesis of breast tumours is less well established (Willet et al, 1992). Little or no association between total fat intake and breast cancer has been observed in most epidemiological studies (Goodwin and Boyd, 1987 and Willet et al, 1992), although a pooled analysis of twelve case controlled investigations suggested a weak but statistically significant positive association between saturated fatty acid intake and the incidence of breast cancer (Howe, 1992).

The limited nature of the evidence supporting the involvement of saturated fatty acids in breast tumour pathogenesis lead to the proposal that the type of fat consumed may be more important than the dietary ratio of unsaturates:saturates, and that essential fatty acids may mediate certain anti-tumour activities (Pritchard et al, 1989 and Horrobin, 1990a). Evidence supporting this hypothesis has been acquired from *in vivo* investigations. Low levels of alpha-linolenic acid in adipose breast tissue are associated with increased risk of breast cancer (Klein et al, 2000), and dietary studies

have demonstrated that administration of the essential fatty acids eicosapentaenoic acid and docosahexaenoic acid to rats was associated with a reduction in the development of breast tumours and metastasis (Pritchard et al, 1989 and Rose et al, 1995). However, additional studies are required to further characterise the role of unsaturated lipids in mammary tumour pathogenesis.

The anti-tumour activities of essential fatty acids *in vivo* have been demonstrated in other malignancies. Omega-3 and omega-6 fatty acids inhibited the proliferation of spontaneously occurring tumours (Tinsley et al, 1981), solid tumour allografts (Hillyard and Abraham, 1979) and tumours induced chemically (Chan et al, 1977 and Nicholson et al, 1990) or by irradiation (Hillyard and Abraham, 1979 and Silverman et al, 1980). Additionally, arachidonic acid, gamma-linolenic acid, docosahexaenoic acid and eicosapentaenoic acid inhibited tumour growth *in vitro* in over one hundred transformed cell lines (Roos and Choppin, 1984, Burns and Spector, 1987, Horrobin, 1990a, Stubbs et al, 1992, DeBranvo et al, 1994, deKock et al, 1994, Finstad et al, 1994, Fujiwara et al, 1986, Meijer et al, 1987, deAntueno et al, 1988 and Cantrill et al, 1997). The majority of these cell types responded to gamma-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid with either a retardation of cell growth or an induction of cell death.

Although the cytotoxic activities of n-3 and n-6 essential fatty acids have been demonstrated in a large number of *in vitro* and *in vivo* investigations, there is inconsistency regarding the relative potencies of arachidonic acid, gamma-linolenic acid, docosahexaenoic acid and eicosapentaenoic acid. This may be due to varying concentrations of intracellular mediators known to influence lipid peroxidation, for example anti-oxidants and bivalent cations. Additionally, the activities of the n-9 fatty acid oleic acid and the n-6 essential fatty acid linoleic acid in tumour tissue have not been well characterised. Most studies indicated that oleic acid had little effect on tumour cytotoxicity (Horrobin, 1990a, Motaung et al, 1999 and Menendez et al, 2001). This suggested fatty acid-specific modulation of pathways involved in the inhibition of cell proliferation and/or the stimulation of cell death. However, a recent

study indicated that oleic acid increased tumour proliferative activity in mouse murine mammary gland adenocarcinoma and salivary gland tumours, and increased the incidence and multiplicity of metasteses. Linoleic acid had little effect in this investigation (Actis et al, 1999 and Munoz et al, 1999), although other studies suggested that linoleic acid increased proliferative activity in breast, colon, prostate and pancreatic tumour cells (Rose et al, 1991, Gonzalez, 1993, Rose et al, 1993, Appel et al, 1994, Welsch et al, 1995 and Godley et al, 1996).

Although the molecular-genetic pathways involved in linoleic acid and oleic acid-mediated stimulation of tumour proliferation have not been well characterised, a recent report indicates that linoleic acid-mediated stimulation of tumour cell growth occurs through potentiation of phospholipase C and protein kinase C activity (Park et al, 2000). Other studies suggest that rather than possessing direct tumourigenic activity, increasing the availability of non-essential fatty acids to tumour cells may augment deficiency of long chain highly unsaturated membrane lipids, and that this may constitute a pro-tumourigenic condition (Monis and Eynard, 1981, Eynard et al, 1997a, Eynard et al, 1997b, Eynard, 1998, Actis et al, 1999 and Munoz et al, 1999). Omega-6 essential fatty acid deficiency may be potentiated through lack of metabolic conversion of linoleic acid, as delta-6 desaturase activity is known to be impaired in transformed tissue (Dunbar and Bailey, 1976).

A number of studies have indicated that essential fatty acid deficiency may increase the risk of tumour development by reducing cellular oxidative activity. Essential fatty acid deficiency resulting from loss of elongation and desaturation products in cultured human keratinocytes attenuated toxicity due to lipid peroxidation (Wey et al, 1993), and supplementation of porcine endothelial cells with polyunsaturated fatty acids increased lipid peroxidation and toxicity (Hart et al, 1991). In comparison, endothelial cells supplemented with saturated and monounsaturated fatty acids had less peroxidative activity and toxicity than the unsupplemented controls (Hart et al, 1991). Inhibition of tumour proliferation in the breast (Pritchard et al, 1989) and glioma cell lines (Das, 1995) by gamma-linolenic acid and in the pancreas by

eicosapentaenoic acid (Zhang and Go, 1996) is associated with accumulation of the products of lipid peroxidation. Tumour cell cytotoxicity is increased by the addition of Fe^{2+} and Cu^{2+}, which stimulate lipid peroxidation (Cantrill et al, 1997 and Sziraki et al, 1999). This effect was inhibited by antioxidants up to five days after essential fatty acid administration (Begin et al, 1988, Cantrill et al, 1997, Vartak et al, 1997 and Vartak et al, 1998).

Gamma-linolenic acid mediated inhibition of cell proliferation in the GHP-212 neuroblastoma cell line was also associated with stimulation of cellular oxidative activity (Hrelia et al, 1996). Although gamma-linolenic acid was metabolised to dihomo-gamma-linolenic acid and arachidonic acid in these cells, the highest cytotoxic effect was observed when gamma-linolenic acid was not converted to its metabolites. This suggests that gamma-linolenic acid toxicity to these tumour cells is not dependent on its metabolites but is due to gamma-linolenic acid itself (Hrelia et al, 1996).

1.3 The Role of Essential Fatty Acids and Oxidative Metabolism in Glioma Proliferation and Death *in vivo* and *in vitro*

(a) The cytotoxic activities of essential fatty acids in malignant glioma

The majority of studies investigating the cytotoxic activities of essential fatty acids in malignant glioma cells have been carried out *in vitro*. Gamma-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid reduced clonogenic survival in rat 36B10 astrocytoma cells (Vartak et al, 1997 and 1998), peroxidised low density lipoprotein inhibited proliferative activity in human glioma cell lines (Kikuchi et al, 1997) and *cis*-unsaturated fatty acids inhibited proliferation and stimulated cell death in a variety of glioma cell lines (Das et al, 1990a). Although information on the cytotoxic activity of essential fatty acids in malignant glioma *in vivo* is limited, there is evidence that polyunsaturated fatty acid (PUFA) administration was associated with the rejection of transformed microglial tumour cells in rats (Frei et al, 1994). Furthermore, an intracerebral infusion study indicated that gamma-linolenic acid is

not cytotoxic to normal rat brain (Das et al, 1995).

Although intercranial infusions of gamma-linolenic acid have shown efficacy in inhibiting glioma proliferation (Frei et al, 1994), little is known about the dose response characteristics of glioma cells to essential fatty acid therapy *in vivo*. However, a recent study used a spheroid model to address this important pharmacological issue (Bell et al, 1999). Spheroids derived from a variety of glioma cell lines were grown in collagen gel and exposed to a range of concentrations of gamma-linolenic acid (0-1mM) for five days. Low concentrations of gamma-linolenic acid (less than 100μM) increased both apoptosis and proliferation, with a net increase in spheroid growth and invasion. In contrast, high concentrations of gamma-linolenic acid (greater than 100μM) reduced spheroid growth. Although the proliferative activity of low dose gamma-linolenic acid may be a hazard in the clinical treatment malignant glioma, its low toxicity against normal cells means higher doses could be used to reduce tumour size.

The cytotoxicity of gamma-linolenic acid *in vivo* has also been investigated in a small clinical trial (Das et al, 1995). Fifteen patients were selected on the basis of histological and radiological evidence of malignant glioma, and following surgical resection the subjects were given 10mg of gamma-linolenic acid orally each day for ten consecutive days. After gamma-linolenic acid therapy was completed, the patients received a CT scan followed by monthly clinical examinations. Administration of gamma-linolenic acid induced glioma regression and improved patient survival, with 80% of the subjects being alive two years after surgery. However, only fifteen patients were assessed in this study, and no control groups were included. Additionally, plasma concentrations of gamma-linolenic acid were not measured, although other studies in rats indicate that essential fatty acids are easily absorbed and readily cross the blood brain barrier when administered at equivalent concentrations (Dhopeshwarkar and Mead, 1973, Spector, 1998, Punchard et al, 2000 and Tso et al, 2002). Further studies are therefore required to characterise the response of malignant glioma to essential fatty acid therapy *in vivo*.

Essential fatty acid-mediated cytotoxicity in glioma cells may be associated with modulation of cellular oxidative activity. Inhibition of tumour proliferation in glioma cell lines was associated with stimulation of lipid peroxidation (Das et al, 1990a and Das et al, 1990b), cis-parinaric acid, an 18C PUFA was cytotoxic to human and rat cell lines via an oxidative pathway (Traynelis et al, 1995) and polyethylene-stabilised glucose oxidase, an enzyme capable of producing reactive oxygen species, induced significant growth delay in subcutaneous rat 9L gliomas (Ben-Yoseph and Ross, 1994). Oxidative stress induced through glutathione depletion has also been associated with C6 glioma cell death (Mawatari et al, 1996). This effect was inhibited by serotonin, which reduced lipid peroxidation (Shinagawa, 1994). However, it has been reported that the lipid peroxidation inhibitors U78517F and U74006F had no significant effect on tumour growth or viability (Megyesi et al, 1990 and Del Maestro et al, 1991).

Analysis of basal cellular oxidation in human brain tumour tissue and glioma cell lines has provided additional evidence that reactive oxygen species are implicated in the control of cell division and death. An *in vitro* study indicated that lipid peroxidation was higher in low grade astrocytoma cell lines in comparison with those derived from more malignant lesions (Louw et al, 1997), and the PUFA content of glioma cells was 50% of that observed in normal astrocytes (Preuss et al, 2000). Additionally, when cellular oxidative activity was investigated *ex vivo* in human glioma and meningioma tissue, basal oxidation was consistently lower in tumour cells in comparison with associated normal brain (Levcheko and Demchuk, 1991). However, no statistically significant differences in basal oxidative activity were detected between gliomas of different grades in this study (Levcheko and Demchuk, 1991).

In C6 glioma cells, arachidonic acid-induced loss of cell viability was associated with cell swelling, which occurred due to H_2O influx resulting from increased sodium permeability (Chan and Fishman, 1982, Staub et al, 1994a and Staub et al, 1994b). This effect was reduced following the administration of SOD, and virtually abolished

by the aminosteroid U-74389F, which is an antagonist of lipid peroxidation (Staub et al, 1994b). It has also been shown that the hydroxyl radical is associated with stimulation of lipid peroxidation and membrane blebbing in C6 glioma cells (Goldberg et al, 1991). As membrane blebbing is a morphological characteristic associated with apoptosis (Kerr et al, 1972 and Wyllie et al, 1980), this observation suggests that lipid peroxidation may induce tumour cell cytotoxicity by modulating molecular pathways which mediate programmed cell death.

Other studies suggest that the activity of anti-oxidant enzymes is a determinant of cell proliferation rather than reactive oxygen species formation. The sensitivity of the glioblastoma cell lines A-172 and U-87 MG to docosahexaenoic acid and eicosapentaenoic acid was not related to intracellular content of the products of lipid peroxidation, but rather cellular anti-oxidant activity (Schonberg et al, 1997). An *in vitro* study also indicated that the malignant progression of low grade astrocytoma was associated with increased expression of intracellular glutathione (Louw et al, 1997). It has also been observed that while gamma-linolenic acid upregulated the antioxidant enzyme CAT in normal astrocytes, it had no significant effect in glioma cells. This suggests that the cytotoxic effect of gamma-linolenic acid in malignant glioma may be due, in part at least, to its inability to upregulate CAT (Preuss et al, 2000).

Preliminary evidence indicates that essential fatty acid administration is not associated with significant toxicity to non-neoplastic brain tissue. Although *cis*-parinaric acid was cytotoxic to human and rat glioma cell lines, this C18 PUFA did not cause significant toxicity to foetal rat astrocytes (Traynelis et al, 1995). Similarly, while gamma-linolenic acid, docosahexaenoic acid and eicosapentaenoic acid administration was associated with cytotoxicity and radiosensitisation in the rat 36B10 astrocytoma cell line, these essential fatty acids were not toxic to normal rat astrocytes (Vartak et al, 1998). It has been postulated that this selective cytotoxicity is associated with increased oxidative stress in glioma cells (Vartak et al, 1999). This may be due to the inability of gamma-linolenic acid to upregulate glioma cell CAT,

and selective uptake of essential fatty acids by glioma cells (Preuss et al, 2000). Docosahexaenoate, palmitate and arachidonate preferentially penetrate cerebrally implanted tumours once they have gained access to the brain (Naraia et al, 1993), and although the PUFA content of glioma cells was significantly lower than that of normal astrocytes, both cell types contained similar levels of PUFAs following a 24 hour supplementation with gamma-linolenic acid (Preuss et al, 2000).

(b) Additional evidence supporting a potential clinical role for essential fatty acids in glioma therapy

Immunological, biochemical and neuropathological studies have indicated that essential fatty acids possess biological actions which may augment their cytotoxic potential *in vivo*. Both n-3 and n-6 essential fatty acids readily cross the blood brain barrier (Abumrad et al, 1981, DeGrella and Light, 1985 and Spector, 1988), where in addition to mediating direct cytotoxicity they may inhibit glioma invasion (Jiang et al, 1995a, Jiang et al, 1995b, Jiang et al, 1995c, Jiang, 1996 and Mareel et al, 1996) and blood vessel formation (Ito et al, 1993, Byres et al, 1995 and Cai et al, 1999), both of which are associated with malignant progression (Louis, 1997). Additionally, pathogenesis of glial tumours is associated with local immunosuppression (Roszman et al, 1991 and Tada and deTribolet, 1993). Essential fatty acids including arachidonic acid and gamma-linolenic acid have specific activities on macrophages and T-cells including NADPH activation and stimulation of the respiratory burst (Henderson and Chappell, 1992, Henderson et al, 1993, Aebischer et al, 1993, Sumimoto et al, 1994 and Sawai et al, 1993) (Chapter 3).

Octadecenoic, myristic and oleic acid enter the brain via a saturable, probenecid/phoretin sensitive mechanism (Abumrad et al, 1981 and Spector, 1988), which may be located in cerebral capillaries (Spector, 1988). This uptake is dependent on the ratio of fatty acid:plasma protein in the periphery and the CNS. Linoleic acid also readily gains access to the CNS, and there is evidence that this transfer is largely probenecid insensitive (Spector, 1988). The lack of interaction between linoleic acid and the probenecid sensitive carrier may be due to the presence

of two double bonds on the hydrocarbon chain which alter its shape and solubility. It has been proposed that linoleic acid and certain other essential fatty acid preparations enter the CNS by diffusion (Abumrad et al, 1981). As there is little or no net extraction of linoleic acid from the blood during a passage through the brain (Pardridge and Mietus, 1980) and phoretin can inhibit both the influx and efflux of fatty acids from the isolated adipocyte (Ambumrad et al, 1981) it has been suggested that there is a specialised exchange mechanism for fatty acids at the blood brain barrier.

Studies on cerebral microvasculature indicate that arachidonic acid increases membrane permeability in a dose dependant manner. This effect was inhibited by co-administration of the anti-oxidants superoxide dismutase and catalase, and by the iron chelator desferrioxamine, suggesting a role for reactive oxygen species in the regulation of the blood brain barrier (Easton and Fraser, 1998). Although the integrity of the blood brain barrier is compromised during the pathological progression from grade I astrocytoma to grade IV glioblastoma, the poor penetration of chemotherapy agents into the brain remains a barrier to cure (Brandes and Pasetto, 2000 and Olivi et al, 2000). This activity may therefore have therapeutic relevance.

The development of intratumoural microvascluture is involved in the progression of low grade astrocytoma to high grade glioblastoma (Louis, 1997). Inhibitors of arachidonic acid metabolism inhibit angiogenesis in human microvascular endothelial cells *in vitro* (Ito et al, 1993). Gamma-linolenic acid inhibits vessel formation *in vitro* (Cai et al, 1999), possibly by reducing vascular endothelial cell motility (Jiang et al, 1997) and modifying the expression of cell adhesion molecules (Jiang et al, 1995a, Jiang et al, 1995b, Jiang et al, 1995c and Byers et al, 1995). This action may be due, in part at least, to the formation of metabolites (Ito et al, 1993). However, arachidonic acid may stimulate angiogenesis by stimulating the production of tumour necrosis factor (TNF) (Sakata et al, 1987, Chaudhri and Clark, 1989 and Carman-Krzan and Wise, 1993). TNF initiates vascular endothelial cell growth in human malignant glioma by increasing expression of the vascular endothelial growth

factor gene (Ryuto et al, 1996). The potentiation of TNF production by arachidonic acid and reactive oxygen species may therefore contribute to vessel formation *in vitro*.

1.4 Current Opinions on the Biochemistry of Essential Fatty Acid-Mediated Tumour Cytotoxicity

(a) Essential fatty acids and apoptosis

Although there is evidence that essential fatty acids are associated with tumour cytotoxicity (Section 1.2), relatively few studies have analysed the molecular pathways mediating these cell death responses. However, preliminary evidence suggests that stimulation of apoptosis is the principal cause of essential fatty acid-mediated cytotoxicity. Gamma-linolenic acid stimulates apoptosis in human cervical carcinoma (deKock et al, 1996), arachidonic acid, gamma-linolenic acid, docosahexaenoic acid and eicosapentaenoic acid stimulate apoptosis in Hep2 human larynx tumour cells (Colquhoun et al, 1998), arachidonic acid stimulated apoptosis in GH3 cells (Yasuda et al, 1999) and eicosapentaenoic acid stimulates apoptosis in pancreatic tumour tissue (Lai et al, 1996). There is also evidence that linoleic acid induces apoptosis in endothelial cell cultures (Meerarani et al, 2000), although linoleic acid and oleic acid had little effect on cell death in Hep2 human larynx tumour cells (Colquhoun et al, 1998).

The stimulation of apoptosis by essential fatty acids may be due to potentiation of reactive oxygen production. *In vitro* evidence has indicated that the pro-apoptotic activity of conjugated eicosapentaenoic acid and docosahexaenoic acid in various transformed cell lines involved potentiation of lipid peroxidation, and it has been proposed that the increase in apoptotic activity observed in human glioma cell lines following addition of selenium is attributable to stimulation of cellular oxidative activity (Nakatsu et al, 1996 and Zhu et al, 1996). Hydrogen peroxide, a reactive oxygen species formed during the oxidation of highly unsaturated membrane lipids, has also been shown to stimulate apoptosis in GH3 and hepatoma cell lines (Yasuda et al, 1999 and Li et al, 2000), and the induction of apoptosis in human

retinoblastoma Y79 cells involved oxidative stress (Vento et al, 2000). Additionally, time-dependent episodes of increased apoptotic activity have been observed in dopamine secreting neurons and human colon carcinoma cell lines. This may correlate with peaks of oxidative activity in these cells (Shirvan et al, 1997). It has also been proposed that gamma-linolenic acid may stimulate apoptosis *in vivo* by disrupting integrin association with matrix proteins (Frisch et al, 1994 and Frisch et al, 1996), as an association between cell-matrix adhesion and cell cycle progression has been characterised (Assoian and Zhu, 1997).

The anti-apoptotic activity of various anti-oxidant enzymes provides additional evidence for a role of reactive oxygen species in the potentiation of programmed cell death. Alpha-tochopherol suppressed the pro-apoptotic activity of conjugated eicosapentaenoic acid and docosahexaenoic acid in cultured human tumour cells (Igarashi and Miyazawa, 2000), arachidonic acid-induced apoptosis in HepG2-MV2E1-9 cells was inhibited by the iron chelator desferioixamine (Chen et al, 1998) and depletion of cellular glutathione was associated with increased toxicity of arachidonic acid in HepG2-MV2E1-9 cells (Chen et al, 1998). Glutathione depletion has also been implicated in the initiation of apoptosis in rat C6 glioma cells (Higuchi and Matsukawa, 1999). However, the observation that vitamin E prevented eicosapentaenoic acid-induced apoptosis in Raji cells but not Ramose cells lead to the proposal that the cytotoxic activity of essential fatty acids *in vitro* is dependant on the cell type used, and that lipid peroxidation may not be the only pathway mediating essential fatty acid-induced stimulation of tumour cell death (Finstad et al, 1998 and Das, 1999).

Preliminary results indicate that unsaturated fatty acids may inhibit apoptosis in non-transformed cells. Addition of docosahexaenoic acid prevented the formation of apoptotic nuclei and increased cell survival in rat retinal cells stimulated into apoptosis through serum deprivation (Rotstein et al, 1997). It has also been demonstrated that *cis*-oleic acid prevented the loss of cardiomyocyte viability induced by the saturated fatty acid 16:0 (deVries et al, 1997). This implies essential

fatty acids may have a protective effect on tumour-associated normal brain tissue.

(b) Essential fatty acids and oncogene expression

Essential fatty acids modify the activity of genes implicated in tumour proliferation and death (Tebbey and Buttke, 1993, Clarke and Jump, 1994 and Sen and Packer, 1996). Linoleic acid regulated the expression of HER-2/neu levels in MCF-7 cells (Tiwari et al, 1991), eicosapentaenoic acid up-regulated expression of the tumour supressor gene nm23-H1 (Jiang et al, 1997), the activity of c-myc was regulated by linoleic acid (Earashi et al, 1996) and n-3 fatty acids induced apoptosis during colon carcinogenesis by reducing activation of the ras oncogene (Chapkin et al, 1997). There is also evidence that expression of the pro-apoptotic oncogene Fas is increased following stimulation of lipid peroxidation (Halder et al, 1995), and that jun activity is sensitive to redox regulation *in vitro* (Abate et al, 1990).

Other putative molecular targets of essential fatty acids and their metabolites include the anti-apoptotic oncogene bcl_2. The observation that bcl_2 protein possessed anti-oxidant activity lead to the proposal that i) activation of this oncogene inhibited apoptosis by reducing the expression or action of reactive oxygen species (Buttke and Sandstrom, 1995, Epperly et al, 1994, Sarafian and Bredessen, 1994 and Steinman, 1995), and ii) that essential fatty acid-mediated stimulation of cellular oxidative activity may represent a mechanism to subvert bcl_2-mediated inhibition of tumour cell death (Das et al, 1999). Although other reports suggest reactive oxygen species are not required for the initiation of programmed cell death (Hug et al, 1994, Muschel et al, 1995 and Jacobson, 1996), there is evidence that bcl_2 is not capable of inhibiting apoptosis induced by lipid hydroperoxides (Sandstrom et al, 1995). It has also been proposed that essential fatty acids may inactivate bcl_2 protein through phosphorylation (Halder et al, 1995 and Das, 1999), although additional studies are required to confirm this hypothesis.

There was no evidence of a correlation between bcl_2 expression and the presence or degree of malignancy in reactive and neoplastic astrocytes (Krishna et al, 1995).

However, expression of wild type p53 was associated with expression of bcl_2 in an immunohistochemical study of a series of human gliomas (Alderson et al, 1995). This may represent a mechanism to subvert p53 mediated apoptosis (Alderson et al, 1995). Recent evidence suggests that the induction of apoptosis by p53 involves the activity of reactive oxygen species (Polyak et al, 1997). In addition, docosahexaenoic acid-mediated inhibition of mammary tumour proliferation has been associated with up-regulation of p53 gene transcription (Tillotson et al, 1993).

1.5 Essential Fatty Acids and Conventional Cancer Therapy

Essential fatty acids may potentiate cytotoxic responses to chemotherapy and radiotherapy. Radiotherapy and certain chemotherapy drugs stimulate the generation of reactive oxygen species (Barber and Wilbur, 1959, Yamanaka et al, 1979, Sodhi and Gupta, 1986, Kanofsky, 1986, Dennis and Shibamoto, 1990, Yamaguchi et al, 1994 and Bordoni et al, 1999), which may be involved in modulating cell proliferation and/or apoptosis (George et al, 1983, Schlager et al, 1983, Ramu et al, 1984, Ewing and Jones, 1987, Ma et al, 1991 and Alaoui et al, 1992). It has been proposed that exogenous essential fatty acids may potentiate radiation-induced reactive oxygen species formation, and hence tumour cell cytotoxicity, by providing substrates for lipid peroxidation upon which radiation can act (Yamamoto et al, 1985, Porter, 1986 and Krinsky, 1992).

Vincristine-resistant human cervical carcinoma cell lines contain low levels of alpha-linolenic acid, gamma-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid in comparison to vincristine sensitive cells (Das et al, 1998), and pre-incubation of vincristine resistant cells with sub-optimal doses of these essential fatty acids increased the cytotoxic action of vincristine (Das et al, 1998). In addition, gamma-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid increased the radiation sensitivity of malignant astrocytoma cell lines (Vartak et al, 1997 and Vartak et al, 1998). This effect was blocked by antioxidants and was not observed in response to oleic acid (Vartak et al, 1997).

There is also evidence that gamma-linolenic acid potentiates chemosensitivity to placitaxel. In human breast cancer cells, placitaxel-mediated inhibition of cell proliferation was significantly greater following exposure to gamma-linolenic acid in comparison with the non-peroxidisible fatty acid oleic acid (Menendez et al, 2001). However, as gamma-linolenic acid-induced chemosensitivity was only partially abolished by the lipid peroxidation inhibitor vitamin E, the influence of oxidative metabolism on placitaxel cytotoxicity may be limited (Menendez et al, 2001).

In addition to potentiating therapeutic response, it has been postulated that essential fatty acids reduce the occurrence of pathological side effects associated with conventional cancer therapy (Horrobin, 1991). It has been proposed that these pathological side effects occur, in part at least, due to elevated free radical production in non-malignant tissue (Freeman and Crapo, 1982, Slater, 1984a, Slater 1984b, Galeotti et al, 1986, Halliwell and Gutteridge, 1986, Halliwell and Gutteridge, 1989 and Cadenas et al, 1989). This theory would predict that provision of polyunsaturated fatty acids should potentiate radiation induced damage by making available additional substrate for free radical formation. However, preliminary evidence suggests that dietary gamma-linolenic acid inhibits radiation-induced damage to porcine epidermis (Hopewell et al, 1993). *In vivo* studies have also demonstrated that gamma-linolenic acid reduces central nervous system damage associated with radiotherapy (Liebel and Sheline, 1991). It has therefore been proposed that pathological side effects associated with exposure to radiation are not caused by free radical activity *per se*, but rather by chain reaction-mediated loss of essential fatty acids from cell membrane phospholipids (Horrobin, 1991).

Essential fatty acids may also reduce the occurrence of certain pathological side effects associated with chemotherapy. Fatty acid metabolites associated with lung inflammation and fibrosis, including hydroxyproline (a marker for collagen synthesis) and the pro-inflammatory leukotriene LTB_4, are reduced by dietary gamma-linolenic acid (Ziboh et al, 1997). Additional *in vitro* evidence suggests that both n-3 and n-6 fatty acids with two or more unsaturated sites containing 18-22

carbon atoms in the hydrocarbon chain are potent antagonists at the LTB_4 receptor (Yagaloff et al, 1995).

Recent *in vitro* evidence also indicates that the cardiotoxicity of doxorubicin is associated with the formation of conjugated dienes, impairment of fatty acid desaturation and elongation and a reduction in cellular essential fatty acid content (Bordoni et al, 1999). Additionally, at therapeutic concentrations doxorubicin induces membrane peroxidation of the endoplasmic reticulum where fatty acid metabolism occurs, further reducing the rate at which essential fatty acid accumulation can occur (Bordoni et al, 1999). These observations are consistent with the hypothesis that provision of exogenous essential fatty acids may protect against certain side-effects of chemotherapy.

Essential fatty acids may inhibit the formation of radiation induced tumours (Horrobin, 1990 and Horrobin, 1991). Protein kinase C is rapidly and transiently activated following exposure to ionising radiation (Hallahan et al, 1991b, Woloschak et al, 1990 and Uckun et al, 1993) resulting in increased expression of the proto-oncogenes c-jun, Egr-1 and zif-268 (Hallahan et al, 1991a, Hallahan et al, 1991b and Hallahan et al, 1992). Radiation induced transformation and proliferation can be reduced by inhibitors of protein kinase C (Hallahan et al, 1991a, Hallahan et al, 1991b and Hallahan et al, 1992 and Umans and Kennedy, 1992). It has also been demonstrated that protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines (Couldwell et al, 1994 and Ikemoto et al, 1995). There is evidence that n-3 and n-6 essential fatty acid are able to inhibit activated protein kinase C (Chen and Murakami, 1992, Holian and Nelson, 1992 and May et al, 1993).

1.6 Current investigations

(a) Hypotheses to be investigated

The purpose of this study was to investigate the pro-oxidative and cytotoxic activity of arachidonic acid and gamma-linolenic acid in human glioma. Human malignant glioma is a highly invasive tumour which is refractory to all currently available

treatment options (Berens and Giese, 1999 and Neider et al, 2000). However, *in vitro* evidence has indicated that essential fatty acid-mediated stimulation of glioma oxidative activity is associated with tumour cytotoxicity, both alone and in conjunction with radiation (Das, 1990a, Das, 1990b, Hrelia et al, 1996, Vartak et al, 1997 and Vartak et al, 1998).

These results are consistent with a potential clinical role for arachidonic acid and gamma-linolenic acid in the clinical management of human malignant glioma. However, the kinetics of reactive oxygen formation in fresh human tissue are not well documented. Additionally, most of these investigations have been carried out on established cell lines, and use of these cells is associated with experimental limitations (see Section 1.6b). This study addressed these issues by investigating the following primary hypotheses:-

1) Oxidative activity is impaired in human glioma tissue in comparison with tumour-associated normal brain

2) Addition of exogenous essential fatty acids stimulates tumour reactive oxygen species generation

3) Potentiation of tumour reactive oxygen species generation is associated with tumour cytotoxicity, in part at least through stimulation of apoptosis

4) Stimulation of tumour oxidative and apoptotic activity by arachidonic acid and gamma-linolenic acid is potentiated by simultaneous exposure to therapeutic doses of radiation

(b) Choice of tissue

Fresh human glioma tissue was used for this analysis, after first establishing appropriate experimental conditions using human lymphocyte preparations. Fresh human glioma tissue was used because of limitations associated with established cell

lines. Cell culture is associated with loss of phenotypic markers, and this increases with passage frequency (Pilkington, 1992). Additionally, relatively homogeneous cell lines do not contain cell populations often present in biopsy samples which may influence tumour growth and proliferation, for example infiltrating immune cells (Pilkington, 1992 and Thomas and Graham, 1995, Chapter 1).

The use of established cell lines may also explain, in part at least, the discrepancy between the reported pro-oxidative and cytotoxic activities of arachidonic acid, gamma-linolenic acid, docosahexaenoic acid and eicosapentaenoic acid. As the growth inhibitory actions of essential fatty acids are dependent upon pre-existing levels of agents known to modulate cellular oxidative activity, for example anti-oxidants and bivalent cations, it is possible that intracellular variations between these factors are responsible for the differences in essential fatty acid activity which have been observed. This suggests that studies carried out on fresh tissue may predict the response of human malignant glioma to essential fatty acid therapy *in vivo* more accurately than those performed on established cell lines, where the native tumour phenotype is less well conserved.

As the human glioma samples received for analysis contained cell populations other than transformed glial cells, it was possible to analyse individual glioma sub-populations. Sub-population oxidative activity and essential fatty acid sensitivity were correlated with expression of the glial cell marker glial fibrillary acidic protein (GFAP). Additionally, the pro-apoptotic activities of arachidonic acid and gamma-linolenic acid in GFAP-positive cell populations were investigated. GFAP is an intermediate filament protein expressed in brain and spinal cord. It is present in astrocytomas and ependymomas, but not tumours of neural origin including neuroblastoma and Schwannoma (Debus et al, 1983, Miettinen et al, 1984, Coakham et al, 1985 Garson et al, 1985 and Royds et al, 1986). It can therefore be used as a marker of tumours of glial origin.

Essential fatty acid-mediated radiosensitisation of glioma cells was also investigated. Insufficient quantities of human tissue were available for this analysis. Additionally, it was predicted that cellular heterogeneity would make tumour-specific responses difficult to interpret. The rat C6 cell line was therefore used for this investigation. The C6 cell line is a clonal line which was developed in randomly bred adult Wistar rats from a glioblastoma induced through transplacental exposure to N-nitrosomethylurea (Benda et al, 1963). It was selected because of its well characterised astrocytic morphology, and its possession of glial markers such as GFAP and S-100 protein (Benda et al, 1963, Pfeiffer et al, 1971, Cravioto et al, 1973, Cravioto et al, 1974 and Weiss et al, 1976).

(C) Choice of reagents

In these studies, the activity of the essential fatty acid arachidonic acid and its n-6 precursor gamma-linolenic acid were examined. Arachidonic acid is an important metabolic and structural polyunsaturated fatty acid in CNS phospholipids (Chan and Fishman, 1980 and Chan and Fishman, 1982) which has been shown to induce the transient formation of superoxide radicals and lipid peroxides in brain cortical slices (Chan and Fishman, 1980). There is evidence that human malignant glioma has deficits in the n-6 products of delta 6-desaturation including arachidonic acid and gamma-linolenic acid (Martin et al, 1996).

The uptake of arachidonic acid into the brain is up to twenty times greater than that of docosahexaenoic acid (Easton and Fraser, 1998) and arachidonic acid preferentially penetrates intracerebrally implanted carcinosarcoma *in vivo* in comparison with surrounding normal brain (Nariai et al, 1993). There is also evidence that fatty acids from the n-6 series, including linoleic acid, arachidonic acid and gamma-linolenic acid modulate leukocyte activity (Bromberg and Pick, 1984, Poulos et al, 1991 and Braun et al, 1993). These cells are present in human glioma tissue and are involved in modulating tumour oxidative activity and apoptosis (Roszman et al, 1991 and Tada and deTribolet, 1993).

Docosahexaenoic acid is also an important metabolic and structural component of the brain. Docosahexaenoic acid was not chosen for analysis in these studies because of its involvement in brain specific biochemical pathways not associated with tumour metabolism (Uauy et al, 1996 and Kaplan and Greenwood, 1998). These include cognitive development in neonates, photoreceptor function (Uauy et al, 1989, Uauy and Hoffman, 1991, Carlson et al, 1994, Uauy et al, 1996 and Birch et al, 1998) and sleep regulation (Warne et al, 1979). Additionally, high concentrations of docosahexaenoic acid are localised in specific brain regions including the cerebral and occipital cortex and retina (Greiner et al, 1997 and Martinez, 2001). As the biopsy samples received for analysis had different cellular and anatomical origins, this non-uniform distribution of docosahexaenoic acid would have made tumour specific responses to docosahexaenoic acid difficult to analyse.

Gamma-linolenic acid, which is commercially available and currently licensed for human therapeutic use, may have potentially therapeutic activities in human malignant glioma. *In vivo* and *in vitro* studies have demonstrated that gamma-linolenic acid induces tumour cell cytotoxicity in established brain tumour cell lines (Das, 1990a, Das, 1990b and Hrelia et al, 1996), induces tumour regression and improves patient survival in a small clinical trial of human glioma patients (Das et al, 1995). Gamma-linolenic acid also inhibits glioma motility and invasion *in vivo* (Jiang, 1996). Western blot and immunohistochemical studies have demonstrated that gamma-linolenic acid increases expression of the cell adhesion molecule E-cadherin and the desmosonal adhesion complex in lung, colon, breast melanoma and liver cancer cells (Jiang et al, 1995a, Jiang et al, 1995b and Jiang et al, 1997). This is associated with increased cell aggregation *in vitro*. Studies in lung, colon, breast, melanoma and liver cancer cells *in vitro* suggested arachidonic acid was not able to induce alterations in E-cadherin expression (Jiang et al, 1995a and Jiang et al, 1995b). There is also evidence that gamma-linolenic acid is more effective than eicosapentaenoic acid and docosahexaenoic acid in selectively increasing the

sensitivity of the rat 36B10 astrocytoma cell line to radiation-induced tumour cell kill (Vartak et al, 1997).

(d) Investigations undertaken

(i) Stimulation of tumour reactive oxygen species production by essential fatty acids

This study examined the stimulation of reactive oxygen production by arachidonic acid and gamma-linolenic acid in single cell preparations of explants of human glioma tissue obtained at biopsy. These cells were characterised in terms of their basal oxidative activity i.e. reactive oxygen generation in the absence of exogenous essential fatty acid stimulation, and sensitivity to exogenous essential fatty acid stimulus. Differences between glioma tissue and tumour-associated normal brain were analysed, and tumour oxidative activity was correlated with tumour grade and cell membrane permeability. This analysis was carried out using novel statistical parameters derived in association with the University of Edinburgh Department of Statistics.

(ii) Investigation of glioma heterogeneity and its relation to oxidative metabolism

As human glioma preparations are characterised by cellular heterogeneity (Louis, 1997) analysis of the oxidative responses of these tumours was complex. In order to establish the experimental and dose response conditions required for the study of human glioma preparations, the oxidative activity of relatively homogeneous fresh human leukocyte preparations acquired from patients undergoing pulmonary resection for bronchogenic carcinoma was investigated. These cells were characterised in terms of their basal oxidative activity and sensitivity to exogenous arachidonic acid and gamma-linolenic acid, both before and after surgery. This analysis also provided information on the previously uncharacterised effects of surgery on human leukocyte reactive oxygen production and facilitated identification of a sub-population thought to represent leukocytes in human glioma preparations.

Cell size and DNA content were used to identify additional cell types present in human glioma. These sub-populations were characterised in terms of their basal oxidative activity, sensitivity to exogenous n-6 essential fatty acids, cell membrane integrity and GFAP expression. This study was used to confirm the cellular heterogeneity associated with malignant progression, and provided additional information on tumour-specific responses to exogenous arachidonic acid and gamma-linolenic acid.

(iii) Investigation of the stimulation of glioma apoptosis by essential fatty acids

It is recognised that dysregulation of apoptosis is implicated in the pathogenesis of neoplasia. This has prompted the development of new therapeutic strategies which may increase cellular apoptosis in tumour tissue (Bellamy et al, 1995). This study investigated the hypothesis that exogenous arachidonic acid and gamma-linolenic acid stimulate apoptosis in fresh explants of human glioma obtained at biopsy. The biochemical pathways which mediate the cytotoxic responses to essential fatty acids and their metabolites are incompletely understood and it is not known whether apoptosis or necrosis are the principal causes of essential fatty acid-mediated stimulation of cell death. This study examined the stimulation of apoptosis in fresh human glioma tissue removed at biopsy in response to stimulation by exogenous arachidonic acid and gamma-linolenic acid. This was correlated with basal apoptotic activity observed in fresh human glioma tissue and paraffin-fixed sections, cell membrane integrity and tumour grade. These studies also examined the kinetics of apoptotic activity in fresh human glioma tissue and their relation to tumour grade. The effect of arachidonic acid and gamma-linolenic acid on cell proliferation in the rat C6 cells line was also examined and correlated with apoptotic activity.

(iv) Investigation of the interaction between radiation and essential fatty acids

It has been demonstrated that arachidonic acid and gamma-linolenic acid increase the radiosensitivity of the rat 36B10 malignant astrocytoma cell line (Vartak et al, 1997 and Vartak et al, 1998). However, the kinetics of reactive oxygen species formation in glioma cells following simultaneous exposure to essential fatty acids and radiation has not been investigated. Additionally, the mode of cell death resulting from this interaction has not been characterised. The purpose of this study was to address these issues by investigating the interaction of arachidonic acid and gamma-linolenic acid with radiation. The rat C6 glioma cell line was used to examine the effects of arachidonic acid and gamma-linolenic acid on cellular oxidative activity, apoptosis and cell membrane permeability in the presence or absence of a therapeutically relevant dose of irradiation. These studies were carried out to investigate a potential therapeutic role for arachidonic acid and gamma-linolenic acid as radiosensitisers.

Summary of the Aims of the Work

The principal aims of this research were as follows:-

1) To establish the conditions required for analysis of glioma peroxidative activity by investigating the oxidative responses of relatively homogenous phagocyte preparations (Chapter 3).

2) To analyse basal oxidative activity and the kinetics of the response to exogenous arachidonic acid and gamma-linolenic acid in more heterogeneous samples of human glioma tissue, to establish any differences between tumour tissue and normal brain and to correlate WHO tumour type and grade of malignancy with peroxidative activity (Chapter 4).

3) To differentiate between the cell populations present in human glioma preparations and to characterise them in terms of their basal oxidative activity, sensitivity to arachidonic acid and gamma-linolenic acid and expression of the glial cell marker GFAP (Chapter 5).

4) To investigate the effects of exogenous arachidonic acid and gamma-linolenic acid on apoptosis and proliferation in human glioma explants and cell lines, and to correlate apoptotic activity with tumour peroxidation and WHO tumour type and grade of malignancy (Chapter 6).

5) To investigate the interaction between arachidonic acid, gamma-linolenic acid and radiation on the oxidative and apoptotic responses in the rat C6 glioma cell line (Chapter 7).

Chapter 2:

Materials and Methods

2.1 Introduction

All investigations were carried out on lymphocyte preparations, human glioma tissue or the rat C6 cell line. Fresh lymphocytes, obtained from patients undergoing pulmonary resection, were used to investigate lymphocyte reactive oxygen species generation in the presence and absence of exogenous arachidonic acid and gamma-linolenic acid. This initial study was undertaken to help characterise experimental and dose response conditions appropriate for subsequent analysis of glioma oxidative activity. Human glioma tissue, obtained at the time of surgery, was either preserved in paraffin for routine diagnostic procedures or digested into single cell suspensions using collagenase. Single cell suspensions were used to investigate oxidative or apoptotic activity, or were preserved in ethanol for analysis of GFAP expression.

Experiments on the rat C6 cell line were conducted on adherent cells or on single cell suspensions obtained through trypsinisation. Adherent cells were used for analysis of apoptotic and proliferative activity. Single cell suspensions were used to investigate oxidative and apoptotic activity in the presence and absence of exogenous arachidonic acid and gamma-linolenic acid, sodium nitroprusside and radiation. Cell viability, determined through analysis of membrane permeability to vital dyes, was routinely measured following each analysis of metabolically active cells.

Table 2.1. Overview of the Experimental Analysis Undertaken

tissue	preparation	experimental analysis
lymphocytes	ortholyse purification	oxidative activity viability
human glioma	collagenase digestion	oxidative activity apoptotic activity viability GFAP expression
	paraffin fixation	routine diagnosis GFAP expression
C6 cell line	adherent cells	apoptotic activity proliferative activity
	trypsin digestion	oxidative activity apoptotic activity viability radiosensitisation

2.2 Initial Preparation of Fresh Tissue

(a) Human Phagocytes

Peripheral blood lymphocytes were withdrawn between 8.00a.m. and 9.30a.m from patients undergoing pulmonary lobectomy for peripheral pulmonary opacity consistent with peripheral bronchogenic carcinoma. Additional blood samples were taken post-operatively and 2 and 7 days after surgery. Blood withdrawn from healthy donors at similar time intervals was used as a contemporary control. Lymphocytes were purified using Ortholyse solution within 2 hours of venupuncture, and the cells were resuspended at a density of 10^6 cells/ml in phosphate buffered saline.

(b) Human Glioma Tissue

Tumour samples obtained at the time of surgery were placed in Hams F-10 medium containing 20mM HEPES buffer pH 7.5, 10% foetal calf serum (FCS), 200μg/ml penicillin, 200μg/ml streptomycin, 100μg/ml kanamycin and 2.5μg/ml amphoterecin within 15 minutes of removal. Obvious non-tumour material was removed and the remaining tissue chopped into 1-2mm fragments with sterile disposable scalpels. The tissue was resuspended in Hams F-10 medium containing 200 units/ml collagenase (clostidopeptidone A; EC 3.4.24.3. Type 1 Lot 113H1019, Sigma, Poole, U.K.) for 4-18 hours at 37°C, and agitated regularly to facilitate the formation of a single cell suspension. Once tumour dissagregation was complete, the cells were centrifuged and resuspended at a density of 10^6 cells/ml in collagenase-free Hams F-10 medium containing 10% FCS.

(c) The Rat C6 Glioma Cell Line

C6 glioma cells, obtained initially from Porton Down Laboratories, Sussex, U.K., were grown from samples stored at -70°C in 10% dimethylsulphoxide in Hams F-10 medium containing 10% FCS. The frozen cells were thawed in a water bath at 37°C, centrifuged with 10-12ml of Hams F-10 medium containing 10% FCS and 5% penicillin and streptomycin, and grown in 15ml of Hams F-10 complete medium at 37°C and 5% CO_2 for 2-3 days or until confluent.

Single cell suspensions of rat C6 glioma cells were obtained by briefly incubating adherent C6 cells with 10ml trypsin diluted 1:10 with Hams F-10 solution containing 10% FCS. Excess solution was removed, and the cells were incubated at 37^{0}C for 5 minutes. Trypsinisation was terminated by the addition of 10ml Hams F-10 solution containing 10% FCS. The cells were centrifuged and resuspended at a density of 10^5 cells/ml for analysis of apoptotic activity, or 10^6 cells/ml for analysis of oxidative activity.

2.3 Experimental Analysis of Oxidative Activity

Oxidative activity was investigated using flow cytometric analysis of the intracellular accumulation 2',7'-dichlorofluorescein. 2',7'-dichlorofluorescein is fluorescent oxidation product derived from the non-fluorescent precursors 2'7'-dichlorofluorescin diacetate and 2'7'-dichlorofluorescin (Figure 2.1). As a result of its high lipid solubility, 2'7'-dichlorofluorescin diacetate readily traverses cell membranes, and is converted to 2'7'-dichlorofluorescin by non-specific intracellular esterases. In the presence of intracellular peroxides and reactive oxygen species, 2'7'-dichlorofluorescin is converted to 2'7'-dichlorofluorescein, whose green fluoresence can be measured at 515-545nm. 2'7'-dichlorofluorescein-associated fluorescence is therefore directly proportional to cellular oxidative activity.

2',7'-dichlorofluorescein was chosen for this analysis because of its rapid cellular uptake and well characterised metabolism and stochiometry (Cathcart et al, 1983 and Leaver et al, 1995). However, it has the disadvantage that certain tumour cells may metabolise diacetete esters more slowly than non-transformed cells (Watson et al, 1978). Additionally, intracellular fluorescein degradation has been demonstrated, and the probe slowly leaks from cells (Watson et al, 1978). Therefore the endogenous leakage and degradation of the probe was corrected by expressing stimulated metabolism as a ratio of the unstimulated rate. In order to correct for variations in uptake, 2',7'-dichlorofluorescein fluorescence was expressed in terms of the change in initial fluorescence.

Figure 2.1. Intracellular oxidation of 2'7'-dichlorofluorescin.

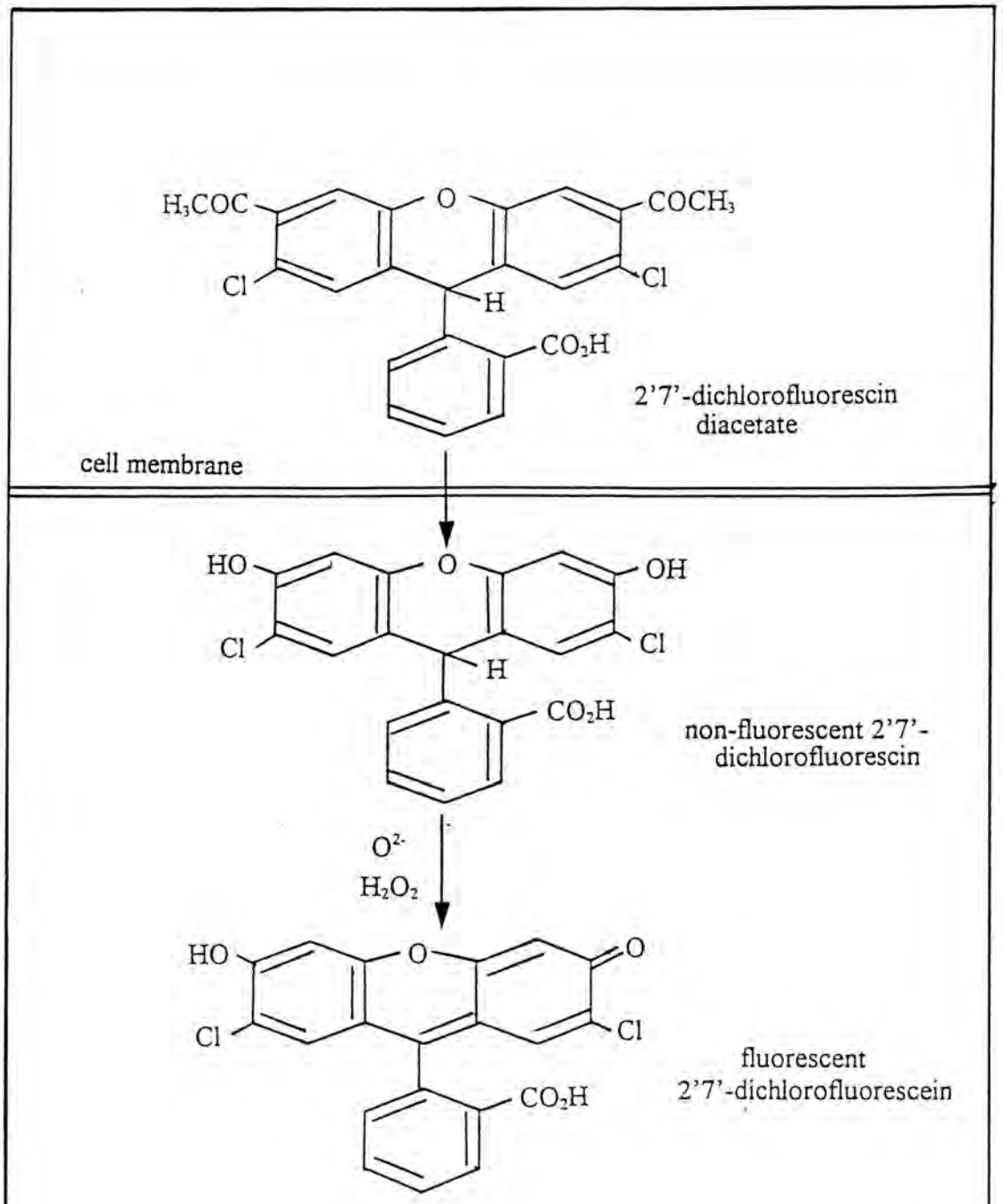


Figure 2.1. The intracellular oxidation of the fluorescent probe 2'7'-dichlorofluorescein. The non-fluorescent substrate 2'7'-dichlorofluorescin diacetate traverses the cell membrane and is converted to non-fluorescent 2'7'-dichlorofluorescin by non-specific intracellular esterases. 2'7'-dichlorofluorescin can then interact with peroxides and oxygen based free radicals to form the fluorescent product 2'7'-dichlorofluorescein. 2'7'-dichlorofluorescein-associated fluorescence is therefore proportional to cellular oxidative activity.

(a) Phagocyte Preparations

Purified leukocyte preparations were incubated with 5μM 2',7'-dichlorofluorescin diacetate (Kodak, Harrow, U.K.) for 10 minutes at 37°C, and analysis of 2',7'-dichlorofluorescin oxidation was carried out using an Ortho flow cytometer with Immunocount software and Orthocount calibration.

The kinetics of reactive oxygen intermediate production were analysed in duplicate samples of mononuclear and polymorphonuclear lymphocytes at 30 second intervals. Oxidative activity was analysed for 7 minutes in the absence of exogenous arachidonic acid or gamma-linolenic acid. The stimulated rate of reactive oxygen intermediate generation was then measured following the addition of arachidonic acid (sodium salt, Sigma) or gamma-linolenic acid (lithium salt, Scotia Pharmaceuticals, Stirling, U.K.) for 13 minutes.

(b) Human Glioma Preparations

Cell suspensions of 10^6 cells/ml in Hams F-10 medium containing 10% FCS were centrifuged and loaded with 2',7'-dichlorofluorescin by incubating with 5ml 5μM 2'7'-dichlorofluorescin diacetate (Kodak, Harrow, U.K.) for 10 minutes at 37°C. The cells were washed and resuspended at approximately 10^6 cells/ml in collagenase free medium containing 10% FCS. Flow cytometric determination of oxidative activity was carried out by analysing 2'7'-dichlorofluorescin-associated fluorescence at 515-545nm using LYSIS software on a FACScan flow cytometer.

Basal reactive oxygen intermediate formation was determined for up to 7 minutes. The sample was then treated with 4-40μM arachidonic acid (sodium salt, Sigma) or gamma-linolenic acid (lithium salt, Scotia Pharmaceuticals, Stirling, U.K.), and intracellular 2',7'-dichlorofluorescin oxidation monitored as described above for up to 3 hours. Basal and stimulated oxidative activity was assessed on 5 000 cells every 10 seconds. Statistical analysis of human glioma peroxidation is described in Section 2.4b.

(c) Human Glioma Sub-Populations

Although macroscopically normal and necrotic tissue was removed prior to oxidative analysis, the cellular composition of the tumours analysed was highly heterogeneous, containing varying proportions of transformed glial cells at various stages of differentiation, reactive astrocytes, vascular cells and normal brain (Darling, 1990 and Thomas and Graham, 1995, Chapter 1). To differentiate between these various populations, up to 6 sub-populations within each tumour analysed was identified on the basis of their laser scatter characteristics. The oxidative activity of these sub-populations was calculated using flow cytometric analysis of stored data obtained following the initial analysis of whole tumour reactive oxygen species formation.

2.4 Statistical Analysis of Reactive Oxygen Species Generation

(a) Human Phagocyte Preparations

Analysis of 2',7'-dichlorofluorescin-associated oxidation in monocyte and neutrophil populations indicated a normal distribution of fluorescence. Analysis of variance was therefore used to compare treatment groups (Ratkowsky, 1989).

(b) Human Glioma Preparations

The kinetics glioma reactive oxygen intermediate production did not fit any well established linear model well. Nonlinear reaction kinetics were therefore required to calculate the rate of 2',7'-dichlorofluorescin oxidation with time (Ratkowsky, 1989). This was achieved using the exponential function:-

$$y = I + \beta[1-\exp(-\kappa t)] \quad \text{.....(Equation 1)}$$

where y=the ratio of the stimulated/unstimulated reactive oxygen intermediate formation, I=the intercept on the y axis i.e. the value of y at t=0, β=the range of the response between t0-∞, i.e. the total extent of stimulated oxidation at the x asymptote and κ=the rate of stimulated oxidation, i.e. the rate at which y changed from its initial value I at t=0, to its final value at t=∞.

The rate of 2',7'-dichlorofluorescin oxidation in the absence of essential fatty acid stimulation was analysed using the equation:-

$$y = \sigma\log(-\rho) + \sigma\log(\log \text{time} + 200\text{sec}) \quad \text{.....(Equation 2)}$$

where y=mean cell fluorescence in unstimulated cells compared with cells immediately after loading with 2',7'-dichlorofluorescin, i.e.(mean fluorescence at x seconds)/(mean fluorescence at 0 seconds)x100. The y intercept $I=\sigma\log(-\rho)$ indicates probe metabolism per cell at 200 seconds and σ is the rate constant describing the change in cell associated metabolism per second. All statistical analysis of human glioma preparations was carried out on a Sun SPARC computer station using the statistical analysis software program (SAS Institute Inc. N. Carolina,1991).

(c) Human Glioma Sub-Populations

As a result of the relative heterogeneity and small population size of the human glioma sub-populations, oxidative activity could be described using linear reaction kinetics (Ratkowsky, 1989). Basal oxidative activity in human glioma sub-populations was analysed using the equation:-

$$\Delta BF_{(0\text{-}200)} - \Delta BF_{(200\text{-}400)} \text{ x100} \quad \text{.....(Equation 1)}$$

where ΔBF= mean basal 2',7'-dichlorofluorescein-associated fluorescence. The time periods in seconds are shown as subscripts in parentheses. For each determination of mean basal fluorescence over a 200 second interval, 10 values of mean 2',7'-dichlorofluorescein- associated fluorescence were used.

The rate of increase of cellular oxidation in human glioma sub-populations following the addition of exogenous arachidonic acid or gamma-linolenic acid was analysed using the equation:-

$$F_{(0\text{-}200)} - \Delta F_{(200\text{-}400)} / \Delta F_{(0\text{-}200)} \text{ x } 100 \quad \text{..... (Equation 2)}$$

where ΔF=the mean % increase in 2',7'-dichlorofluorescein-associated fluorescence over the time periods shown in parentheses. Arachidonic acid or gamma-linolenic acid was added at time t=0.

2.5 Analysis of GFAP Expression

Flow cytometric analysis of GFAP staining intensity was carried out on human glioma cells which had been fixed in 100% ethanol and stored at -20°C for 2-8 weeks. Glioma sections were used for the positive control and the primary antibody was omitted for the negative control. The ethanol preserving process did not alter the characteristic laser scatter profile which was observed in samples of the fresh tissue.

(a) Monoclonal GFAP staining

Monoclonal rabbit anti-cow GFAP (protein concentration 14.0 g/L, Dako) was diluted 1:5, 1:10 and 1:20 and phycoerythrin was diluted 1:10, in phosphate buffered saline (PBS) containing 20% FCS and 5% tween (Bio-Rad, Watford, U.K., CAS 9005-64-5, batch 126714A). Ethanol preserved cells were washed twice with 1ml PBS and resuspended at a density of approximately 10^6 cells in 1ml PBS containing 20% FCS and 5% tween. 100µl of antibody solution was added to each cell pellet and incubated at room temperature for 30 minutes. The cells were centrifuged, resuspended in 1:10 phycoerythrin and incubated at 4°C for 2 hours. GFAP labelled cells were suspended in 1ml PBS containing FCS and tween, centrifuged and resuspended in 1ml PBS for flow cytometry at 564-607nm using a Becton-Dickinson FACScan flow cytometer and LYSIS software. Control samples were incubated with the fluorochrome alone. This method was adapted from standard techniques applied in flow cytometric immunohistochemistry (Ormerod, 1990 and Darzynkiewicz and Crissman, 1990).

(b) polyclonal GFAP Staining

FITC-conjugated polyclonal swine anti-rabbit GFAP (protein concentration 0.5g/L, Dako) was diluted 1:5, 1:10 and 1:20 in PBS containing 20% FCS and 5% tween. 1ml aliquots of ethanol preserved cells containing 10^6 cells/ml were washed twice in

1ml PBS-FCS-tween solution and incubated with 100µl of the antibody solutions at 4°C for 2 hours. GFAP labelled cells were suspended in 1ml PBS-FCS-tween solution, centrifuged and resuspended in 1ml PBS for flow cytometry at 564-607nm using a Becton-Dickinson FACScan flow cytometer and LYSIS software. Control samples were incubated with the fluorochrome alone. This method was adapted from standard techniques applied in flow cytometric immunohistochemistry (Ormerod, 1990 and Darzynkiewicz and Crissman, 1990).

2.6 Analysis of Cell viability

Loss of cell viability is associated with reduced cell membrane integrity and increased permeability to vital dyes. In these studies, leukocyte and human glioma cell viability was assessed by analysing the cellular uptake of ethidium homodimer and propidium iodide respectively, both of which are excluded from cells whose membrane is intact. If membrane integrity is compromised and these compounds gain access into the cell, they bind with high affinity to nuclear DNA, and form a fluorescent product which can be detected using flow cytometry at 564-607nm. The fluorescence associated with ethidium homodimer and propidium iodide is therefore inversely proportional to cell viability (Ormerod, 1990 and Darzynkiewicz and Crissman, 1990).

Human glioma cell viability was also determined by analysing cellular uptake of the hydrophilic dye trypan blue. Light microscopy was used to determine the proportion of cells which were stained with this dye. Staining was associated with reduced cell membrane integrity and hence loss of viability.

(a) Analysis of lymphocyte and human glioma cell viability

Lymphocyte and glioma cell viability was determined at the end of each analysis of oxidative activity. Human glioma cells were suspended at a density of 10^6 cells/ml, incubated with 1mg/ml propidium iodide at 20°C for 10 minutes, and analysed with LYSIS software on a FACScan flow cytometer. In lymphocyte preparations, viability was determined by assessing cell permeability to ethidium homodimer.

2.7 Analysis of Apoptosis

Apoptotic activity was determined flow cytometrically using the TUNEL assay. During the process of apoptosis, Ca^{2+} activated endonucleases are responsible for the induction of double strand breaks in chromosomal DNA (Arends et al, 1990). The TUNEL assay uses the enzyme deoxyuridine-5'-triphosphate (dUTP) to label these oligonucleotide fragments. Flow cytometric detection of dUTP binding, which is proportional to cellular apoptotic activity, is made possible by the covalent addition of the fluorescent tag fluorescein isothiocyanate (FITC) to dUTP. This reaction is catalysed by the enzyme terminal deoxynucleotidly transferase (TdT) (Gorczyca et al, 1992, Gavrieli et al, 1992 and Gorczyca et al, 1993).The major advantage of this method is its ability to reveal early DNA breaks during apoptosis (Vermes et al, 2000). However, since DNA strand breaks are not unique to apoptosis (Darzynkiewicz et al, 1992), it is important that cell viability is determined simultaneously.

(a) Analysis of Apoptotic Activity in Human Glioma Preparations

Collagenase dispersed human glioma cells were resuspended at a density of 10^6 cells/ml in Hams F-10 medium, and incubated in the presence or absence of 20-30μM arachidonic acid or gamma-linolenic acid for up to 36 hours at 20°C. At intervals of 0, 15, 30, 45 minutes and at hourly intervals for up to 36 hours, 1ml aliquots of human glioma cells were fixed in ethanol and stored at -20°C.
These ethanol preserved preparations were washed twice in TBS and resuspended in 40μl cacodylate terminal deoxynucleotidyl transferase reaction buffer (Promega, Madison, U.S.A.) diluted 1:5 with distilled water. The cells were incubated at room temperature for 10 minutes, centrifuged and resuspended in 25μl of reaction buffer containing 19.5μl of distilled water, 5μl of terminal deoxynucleotidyl transferase reaction buffer, 0.25μl of terminal deoxynucleotidyl transferase Co^{2+} (20 units/ml, Promega, Madison, U.S.A.) and 0.25μl of fluorescein-12-2'-deoxyuridine-5'-triphosphate (Fluorescein-12-dUTP), and incubated at 37°C for 2 hours. The reaction was terminated by centrifuging the cells twice with TBS. The cells were left to stand at room temperature for 15 minutes before analysing 2 000-10 000 cells on a

FACScan flow cytometer using LYSIS software. This identified apoptotic cells on the basis of dUTP/FITC-associated fluorescence and side scatter.

At the same time as glioma cells were fixed in ethanol, membrane integrity was measured by analysing trypan blue permeability. Approximately $0.5x10^6$ cells were incubated with 100µl trypan blue for 2 minutes at 20^0C, and viewed on a modified Fuchs Rosenthal haemocytometer (Webber Scientific International) at magnification x40. Cell viability was expressed as the percentage of non-staining cells in a random field containing 100-200 cells. Paired data sets comparing apoptotic activity and cell viability were compared using Students t-test. Results were calculated as the mean ± SEM of n determinations.

(b) Analysis of Apoptotic Activity in Rat C6 Glioma Cells

Pre-confluent C6 cells were incubated with Hams F-10 medium containing 10% FCS, penicillin, streptomycin, glutamine and gamma-linolenic acid at concentrations 0µM, 1µM, 5µM, 10µM, 20µM and 40µM. The medium bathing the cells was harvested and replaced every 6 hours, and centrifuged at 2 500rpm. The resulting pellet contained C6 cells which had detached from culture and was stored in ethanol for subsequent assessment of apoptotic activity using the TUNEL method (Section 2.7).

After 7 days, adherent C6 cells were harvested using 10% trypsin digestion (Section 2.2c). The cells were resuspended at a density of 10^6 cells/ml, and their viability was measured using flow cytometric analysis of propidium iodide uptake. The remaining adherent cells were stored in ethanol for TUNEL analysis of apoptotic activity (Section 2.7).

2.8 Analysis of Cell Proliferation

Cells proliferation was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Cells were incubated with MTT, which was converted to formazan crystals by intracellular dehyrogenases. The formazan crystals

were solubilised using dimethyl sulphoxide, and absorbance of the resulting solution was measured at 540nm. As cell proliferation in each of the C6 populations analysed was directly proportional to the activity of intracellular dehydrogenases, absorbance at 540nm gave an indication of the overall rate of proliferative activity.

Confluent C6 cells were harvested using trypsin digestion (Section 2.2c) and suspended in Hams F-10 medium containing 0μM, 1μM, 5μM, 20μM and 40μM gamma-linolenic acid (lithium salt, Scotia Pharmaceuticals, Stirling, U.K.) at a density of 10^3 cells/100μl of medium.100μl of each solution was added to seven 96 well plates, which were left overnight to allow the cells to adhere to the surface of the well. 50μl of MTT solution at a concentration 1.5mg per ml of culture medium was aliquoted into each test well and the cells were incubated at 37°C for 7 days. 150μl of dimethylsulphoxide containing 0.5% FCS was added to each well, and the plates were shaken at room temperature on an orbital shaker for 30 minutes. Absorbance was measured at 540nm on a microplate reader (model 450; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, U.K.).

2.9 The Interaction of Glioma Cell Preparations with Sodium Nitroprusside and Radiotherapy

(a) Sodium Nitroprusside

Single cell suspensions of human glioma tissue and the rat C6 cell line were resuspended at a density of 10^6 cells/ml and incubated in the presence or absence of arachidonic acid or gamma-linolenic acid and the nitric oxide donor sodium nitroprusside (Sigma). Immediately, and at hourly intervals for 10 hours, 1ml aliquots were withdrawn for analysis of peroxidative metabolism (Section 2.3), apoptosis (Section 2.7) and viability (Section 2.6).

(b) Irradiation

Approximately six 250ml flasks of rat C6 glioma cells at different phases of growth and development were harvested with trypsin to form a single cell suspension (Section 2.2c). The cells were centrifuged and resuspended at a density of 10^6

cells/ml in the presence or absence of exogenous arachidonic acid or gamma-linolenic acid. Care was taken to exclude all air gaps, and the cells were irradiated with a single dose of irradiation at 2Gy. Immediately, and at hourly intervals for 10 hours, 1ml aliquots were withdrawn for analysis of peroxidative metabolism, apoptosis and viability using flow cytometry, and proliferation using the MTT assay.

2.10 Analysis of Paraffin Fixed Human Glioma Tissue

The WHO classification of each tumour analysed for peroxidative and apoptotic activity was assessed using standard histopathological techniques (Liebovitz, 1990) Paraffin-fixed tissue sections were stained with haematoxylin and eosin to determine diagnostic indicators such as necrosis, vascularity, the presence of mitotic bodies and cellular differentiation. Where required, GFAP expression was also analysed in order to confirm the glial origin of the tumour. The diagnosis was recorded in a definitive histopathology report whose conclusions were confirmed by a second independent observer. In addition, the expression of bcl_2 and p53 was determined by immunocytochemistry in a limited series of tumours.

(a) Pathological and Diagnostic analysis

Pathological and diagnostic analysis was carried out on each of the human glioma samples analysed for oxidative activity. This was achieved using standard histopathological and immunohistochemical techniques (Liebovitz, 1990). Tumour diagnosis was determined following analysis of glioma sections stained with haematoxylin and eosin. Where appropriate, GFAP expression was also investigated.

(i) Haematoxylin and Eosin

Paraffin embedded tumours were cut into 5μm sections, floated onto poly-L-lysine (PLL) coated microscope slides and allowed to dry at 80°C for at least 60 minutes. The sections were rehydrated by sequential exposure to xylene, ethanol, picric acid and hydrogen peroxide as shown in Table 2.1, and incubated with Meyer's haematoxylin solution for 5 minutes. The sections were rinsed in tap water, placed in lithium carbonate for 30 seconds and rinsed in tap water. The sections were

dehydrated according to the protocol shown in Table 2.2 and fixed with clean microscope slides.

(ii) Glial Fibrillary Acidic Protein

Paraffin embedded tumours were cut into 5μm sections, floated onto PLL coated microscope slides and allowed to dry at 80°C for at least 60 minutes. The sections were rehydrated by sequential exposure to xylene, ethanol, picric acid and hydrogen peroxide as shown in Table 2.1, incubated with 3% aqueous hydrogen peroxide for 10 minutes and rinsed with tap water. The sections were incubated with 20% normal swine serum for 10 minutes, followed by polyclonal GFAP (Dako) diluted 1:200 in 20% normal swine serum for 30 minutes. The sections were washed in TBS for 5 minutes, incubated with ABC reagent containing 100μl streptavidin (Dako) for 30 minutes and rinsed in tap water, and incubated with 3,3-diaminobenzidine tetrahydrochloride (DAB) solution for 5 minutes. After being rinsed in tap water, the sections were incubated with lithium carbonate solution for 30 seconds, rinsed in tap water and dehydrated according to the protocol shown in Table 2.2. The sections were mounted on a clean microscope slide. A section of normal brain was used as a positive control, and the primary antibody was omitted for the negative control.

Reagent	Time of Exposure
xylene	5 minutes
95% ethanol	3 minutes
70% ethanol	3 minutes
picric acid	15 minutes
hydrogen peroxide	5 minutes

Table 2.2. Rehydration procedure for paraffin embedded tumours used for histopathological analysis of tissue ultrastructure (haematoxylin and eosin) and glial fibrillary acidic protein (Section 2.10). The sections were exposed sequentially to the reagents shown below for the time periods stated.

Reagent	Time of Exposure
70% ethanol	2 minutes
74% ethanol	2 minutes
100% ethanol	2 minutes
100% ethanol	2 minutes
xylene	2 minutes
xylene	2 minutes

Table 2.3. Dehydration procedure for paraffin embedded tumours used for histopathological analysis of tissue ultrastructure (haematoxylin and eosin) and glial fibrillary acidic protein (Section 2.10). The sections were exposed sequentially to the reagents shown below for the time periods stated.

Chapter 3:

The Effects of Arachidonic Acid and Gamma-Linolenic Acid on Monocyte and Neutrophil Reactive Oxygen Species Production

3.1 Introduction

The main purpose of this study was to use flow cytometry to i) investigate the effect of exogenous arachidonic acid and gamma-linolenic acid on cellular reactive oxygen species formation in human phagocytes and ii) investigate the phenotype of these cells. This was undertaken using phagocyte preparations acquired from lung cancer patients undergoing pulmonary resection. Initially, these cells were purified and their forward and side scatter characteristics determined. Basal and stimulated reactive oxygen species formation was then investigated by analysing phagocyte oxidative activity in the presence and absence of exogenous arachidonic acid and gamma-linolenic acid. Cell viability was assessed by measuring permeability to ethidium homodimer. These parameters were analysed pre and post-operatively to evaluate the effect of surgery and essential fatty acid sensitivity on phagocyte activation.

These studies were carried out primarily to assist in subsequent flow cytometric analysis of human glioma cells. Although a number of studies have analysed glioma cell lines using flow cytometry (Knott, 1990, Pellicciari et al, 1995, Yahanda et al, 1995, Li et al, 1997, Rooprai et al, 1999 and Ly et al, 2001) fresh human cells acquired from biopsy samples have not been studied extensively using this technique. Consequently, it was necessary to optimise experimental and dose response conditions using cell types whose flow cytometric properties were better characterised. Phagocyte preparations were chosen for this analysis because of their well established laser scattering properties, relative homogeneity and ready availability (Ormerod, 1990 and Darzynkiewicz and Crissman, 1990).

In addition to establishing appropriate conditions required for the analysis of human glioma reactive oxygen species formation, the involvement of arachidonic acid and

gamma-linolenic acid in phagocyte activity is of interest because essential fatty acid mediated stimulation of leukocyte reactive oxygen species production may be associated with tumour cytotoxicity (Cornwell and Morisaki, 1984 and Gonzalez, 1992). It is known that patients with primary intracranial tumours have altered immune function, due, in part at least, to inhibition of T-cell function (Bullard et al, 1986). However, there is evidence that certain fatty acids, including arachidonic acid, gamma-linolenic acid and docosahexaenoic acid potentiate superoxide formation in human neutrophils and cell free extracts (Badwey et al, 1981 and Poulos et al, 1991). This may be associated with generation of the cellular immune response against malignant glioma (Holladay and Wood, 1993).

The generation of reactive oxygen species by lymphocytes which have infiltrated tumour tissue involves activation of NADPH oxidase (Badwey et al, 1981), and specific roles for arachidonic acid have been identified at several molecular sites associated with NADPH oxidation (Henderson and Chappell, 1992 and Henderson et al, 1993). These include i) the interaction of the NADPH oxidase component rap 1A with rap-GTPase activating protein (Mohazzab et al, 1994) ii) the activation of rap-GTPase activating protein by arachidonic acid (Ligeti et al, 1993) resulting in iii) the exposure of the p47-SH3 region crucial for oxidase assembly (Sumimoto et al, 1994) and iv) the binding of cytochrome b protein p22 phox and the p67 phox protein (Sumimoto et al, 1994). Additionally, arachidonic acid induces translocation of rac p21s to the membrane and the activation of NADPH oxidase (Sawai et al, 1993).

The involvement of arachidonic acid in physiological changes associated with phagocyte activity are less well characterised. However, an arachidonic acid-specific phospholipase A2, which is sensitive to nanomolar Ca^{2+} and responds to the chemotactic peptide fMet-Leu-Phe and the g-protein agonist guanosine 5'[y-thio]-triphosphate has been identified in neutrophils (Bauldry et al, 1996). Additionally, recent evidence indicates that arachidonic acid is involved in phagocyte NADPH-oxidase activation through induction of a conformational change to p47phox (Shiose and Sumimoto, 2000). The release of arachidonic acid by cytoplasmic PLA_2 has also

been implicated in the activation of an NADPH-oxidase-associated H^+ channel (Levy et al, 2000). These specific activities of arachidonic acid on phagocyte activation may contribute to tumour cytotoxicity *in vivo*.

In order to investigate the role of arachidonic acid and gamma-linolenic acid in phagocyte oxidative activity, individual cell types, specifically lymphocytes, monocytes and granulocytes, were identified using sub-population analysis. Reactive oxygen species formation in each population was then investigated in the presence and absence of exogenous arachidonic acid and gamma-linolenic acid, both before and after surgery. These studies established suitable parameters for the investigation of human glioma cell oxidative activity, while simultaneously providing information on the previously uncharacterised effect of arachidonic acid and gamma-linolenic acid in phagocyte activation.

3.2 Materials and Methods

Blood samples were withdrawn between 8.00 and 9.30a.m.from patients undergoing pulmonary lobectomy for peripheral bronchogenic carcinoma (Section 2.2a). All patients underwent bronchoscopy, media stenoscopy and computed axial tomography of the thorax and upper abdomen. Routine haematological and biochemical profiles were performed, and isotopic bone scans were carried out when indicated. All patients had pulmonary function adequate for lobectomy and stable cardiac status. Blood samples were taken pre-operatively, 2 and 7 days after surgery. Informed consent and local ethical board permission was obtained for this study. Blood withdrawn from healthy blood donors at the same time as patient blood samples were collected were used as contemporary controls.

Leukocyte preparations were purified using ortho-lyse solution within 2 hours of venepuncture. The cells were loaded with 2',7'-dichlorfluorescin (Section 2.3a), and patient and control phagocytes were analysed using an Ortho flow cytometer with Immunocount software and Orthocount calibration. Oxidative activity was determined in duplicate mononuclear and polymorphonuclear phagocytes derived

from the same individual. The rate of reactive oxygen species formation was analysed at 30 second intervals for 7 minutes before that addition of arachidonic acid and gamma-linolenic acid and for 13 minutes after.

3.3 Results

(a) Effect of Arachidonic Acid and Gamma-Linolenic Acid on Phagocyte Reactive Oxygen Species Generation

Preliminary analysis indicated a normal distribution of fluorescence in gates B (monocytes) and C (neutrophils) but not A (lymphocytes) (Figure 3.1). Mean fluorescence associated with phagocyte populations B and C was therefore used to estimate reactive oxygen species generation. Analysis of variance (ANOVA) was used to compare treatment groups.

The rate of reactive oxygen species generation in peripheral mononuclear and polymorphonuclear phagocytes was significantly stimulated ($P<0.05$-$P<0.001$) following addition of 1-40μM arachidonic acid and gamma-linolenic acid (Figure 3.1). In both patient and control phagocytes, this stimulation was associated with increased dichlorofluorescein-associated fluorescence and a broadening of the fluorescence distribution describing reactive oxygen species formation (Figure 3.1). Cell viability was greater than 58%, and was not significantly different in patients and control phagocytes.

(b) The Kinetics of Arachidonic Acid and Gamma-Linolenic Acid Induced Activation of Phagocyte Reactive Oxygen Species Generation

Arachidonic acid and gamma-linolenic acid increased reactive oxygen species formation in each peripheral leukocyte category (Figure 3.2). Similar kinetics were observed in response to arachidonic acid and gamma-linolenic acid, although arachidonic acid had the greatest stimulatory effect. Essential fatty acid-mediated stimulation of oxidative activity followed a linear regression curve during the first 10 minutes of analysis, even at the lower concentration of the weaker agonist (6μM

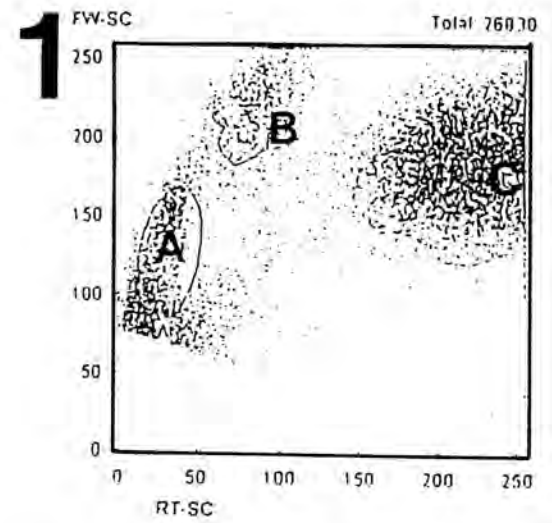
gamma-linolenic acid). Correlation coefficients for the rate of increase of reactive oxygen species with time in phagocytes treated with 6μM gamma-linolenic acid were 0.685 in patient monocytes, 0.839 in patient neutrophils, 0.862 in control monocytes and 0.862 in control neutrophils.

(c) Comparison of Gamma-Linolenic Acid and Arachidonic Acid Stimulation in Patient and Control Phagocytes

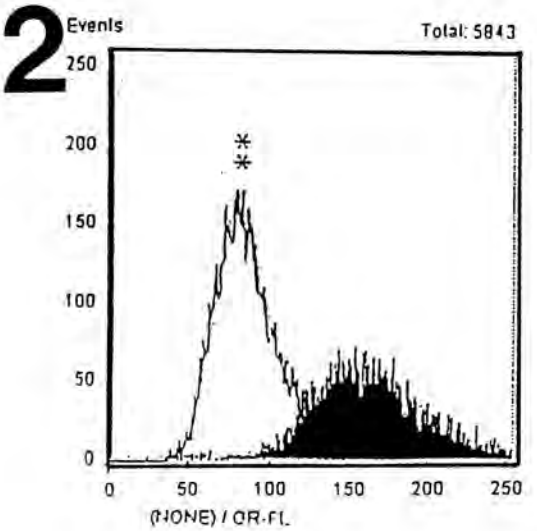
Essential fatty acid-mediated stimulation of phagocyte reactive oxygen species generation was significantly less in patient groups in comparison with control cells ($p<0.005$, $n=15$), although this difference was more apparent in neutrophils than monocytes (Figure 3.2). Stimulation of control neutrophils was significantly greater than patient neutrophils at 15uM ($p<0.05$, $n=15$) and 30uM ($p<0.05$, $n=16$) arachidonic acid. The mean increase in reactive oxygen species production over 5.5 minutes ranged from 1.5± 2.7% for patient neutrophils treated with 8μM gamma-linolenic acid to 50.1±15.0% for control neutrophils stimulated with 15μM arachidonic acid.

(d) Generation of Reactive Oxygen Species in Patient Phagocytes Before and After Surgery

The effect of surgery on reactive oxygen species generation was compared by pairing pre-operative and post-operative levels of reactive oxygen species production, and analysing differences between these groups using ANOVA. Significantly greater reactive oxygen species generation was detected in post-operative patient phagocytes ($p<0.05$-$p<0.01$) (Figure 3.3). Additionally, analysis of the pre-operative group suggested that the neutrophils of lung cancer patients were activated ($p<0.001$, $n=21$).



region	% total	events	mean x	mean
A	6.9	1840	27.1	121.9
B	1.5	407	77.8	203.9
C	21.8	5843	207.6	167.3



gated by: C

% total	events	mean
100.0	5843	174.4

**gated by: C'

% total	events	mean
100.0	8058	100.0

Figure 3.1. The effect of 40μM gamma-linolenic acid on granulocyte peroxidation in a peripharal blood leukocyte preparation from a patient with lung cancer. Diagram 1 indicates the forward and side scatter of (A) lymphocytes, (B) monocytes and (C) granulocytes in peripharal blood leukocytes previously incubated with 5μM 2'7'-dichlorofluorescein diacetate. Diagram 2 is a sub-population histogram of the intensity of dichlorofluorescein associated fluorescence (x-axis) *vs.* cell number (y-axis) in granulocytes (region C of the cytogram) in cells incubated with 40μM gamma-linolenic acid for 8 minutes at 20°C (■) compared with cells from the same region and individual in the absence of exogenous essential fatty acid stimulus (□). **The mean intensity of fluorescein in control cells (granulocytes without exogenous gamma-linolenic acid).

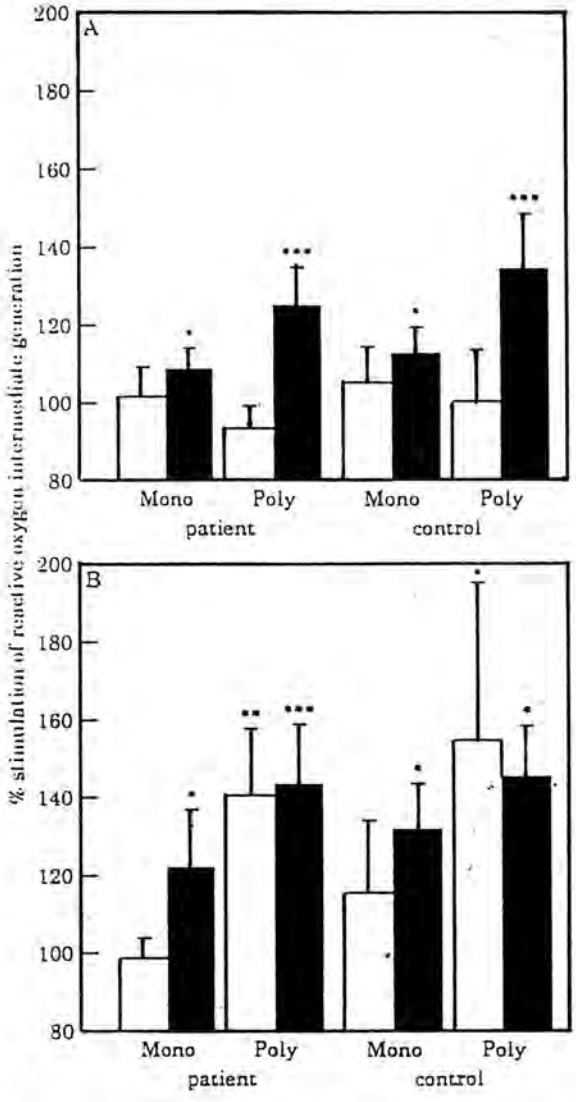


Figure 3.2. The effects of arachidonic acid and gamma-linolenic acid on mononuclear and polymorphonuclear reactive oxygen production in lung cancer patients and healthy controls. The percentage of pre-stimulated reactive oxygen intermediate production in patient phagocytes loaded with 2',7'-dichlorofluorescein and paired normal control phagocytes were analysed simultaneously using flow cytometry of mean 2',7'-dichlorofluorescein fluorescence in mononuclear (mono) and polymorphonuclear (poly) cells from the same individual. Results describe mean ± SEM reactive oxygen intermediate production in phagocytes exposed for 5.5 minutes *in vitro* to either: (A) gamma-linolenic acid, lithium salt 6μM (□) or 30μM (■) or (B) arachidonic acid, sodium salt, 4μM (□) or 15μM (■). Mean reactive oxygen intermediate production in the presence of n-6 essential fatty acids was expressed as a percentage of unstimulated reactive oxygen intermediate production in the same individual. *reactive oxygen intermediate production significantly different from pre-stimulation reactive oxygen intermediate production $P<0.05$, **$P<0.01$, ***$P< 0.001$ using ANOVA to compare stimulated values with pre-stimulated values in the same individual. In (A), sixteen leukocyte samples from the control group and 16 leukocyte samples from the patient group and sixteen samples from the control group were incubated with 6μM (□) and 30μM (■) respectively. In (B), 16 samples in the patient and control groups were incubated with 4μM arachidonic acid (□) and 5 samples in the patient and control groups were incubated with 30μM arachidonic acid (■).

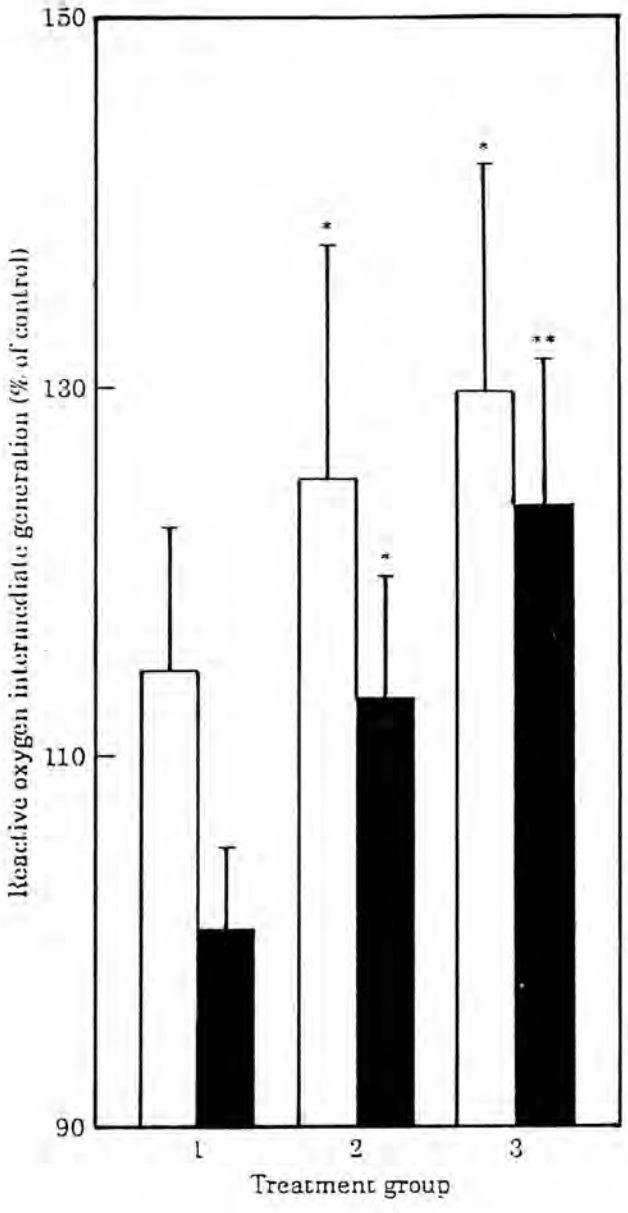


Figure 3.3. The generation of reactive oxygen intermediate production in patient phagocytes expressed as a percentage of mean control phagocyte production before (group 1) and after (groups 2 and 3) pulmonary resection. Group 2 leukocytes were taken 48 hours after surgery and group 3 leukocytes 7 days after surgery. Mean reactive oxygen intermediate production (± SEM) was analysed by monitoring 2',7'-dichlorofluorescein associated fluorescence in 20 patients and 20 paired controls in monocytes (■) and neutrophils (□) after 5.5 minutes incubation. Post-operative monocyte and neutrophil reactive oxygen intermediate (groups 2 and 3) were compared with pre-operative reactive oxygen intermediate in the same patient. Significant increases in reactive oxygen intermediate after surgery *($P<0.05$), **($P<0.01$) are indicated in monocytes and neutrophils in both surgical groups.

3.4 Discussion

The main purpose of this study was to characterise experimental and dose response conditions suitable for analysis of human primary glioma. This was achieved using flow cytometric analysis of the oxidative activity of phagocyte preparations acquired from lung cancer patients undergoing pulmonary resection. The morphological properties of these cells were investigated, and were found to correlate well with previously established forward and side-scatter characteristics (Ormerod, 1990 and Darzynkiewicz and Crissman, 1990). Dose response characteristics were analysed, and a significant stimulation of reactive oxygen species formation in patient and control phagocytes was observed at 1-40uM arachidonic acid and gamma-linolenic acid (Figures 3.1 and 3.2).

The kinetics of reactive oxygen species formation following addition of arachidonic acid and gamma-linolenic acid were similar in monocytes and neutrophils, although 2-3 fold greater concentrations of gamma-linolenic acid were required to produce equivalent stimulation (Figure 3.2). This suggests that gamma-linolenic acid may act as a partial agonist at the molecular sites where arachidonic acid induces stimulation of phagocyte reactive oxygen species production. It is unlikely that differences in cell viability accounted for the relatively low sensitivity of patient phagocytes to exogenous essential fatty acid stimulus, as no significant differences in phagocyte permeability to ethidium homodimer were detected.

Differences were observed between basal oxidative activity and essential fatty acid sensitivity in i) patient and control phagocytes, and ii) phagocytes obtained pre and post-operatively. Reactive oxygen species production was elevated in neutrophils acquired from lung cancer patients, and when stimulated with exogenous arachidonic acid and gamma-linolenic acid these cells responded with low reactive oxygen species formation in comparison with those obtained from healthy donors. Similarly, monocytes and neutrophils acquired post-surgically were characterised by elevated basal reactive oxygen species formation. These differences are consistent with partial

activation of phagocytes by lung cancer and its surgical treatment via arachidonic acid-dependant pathways.

These results suggest that phagocytes previously activated by lung cancer or surgery are activated less by exogenous n-6 essential fatty acids. This lack of hyperstimulation may be important as the antiproliferative properties arachidonic acid and gamma-linolenic acid may be pertinent to bronchogenic carcinoma. Although there has been little improvement in long term survival following resection of bronchogenic carcinoma over the last 20 years (Shields et al, 1994), recent evidence suggests that arachidonic acid and gamma-linolenic acid are associated with inhibition of tumour proliferation and viability in a human lung carcinoma grown in nude mice(deAntueno et al, 1998). Additionally, there is evidence that phagocytes are associated with tumour cytotoxic reactions in human lung cancer (Braun et al, 1993), and that stimulation of peripheral blood monocytes of human lung tumour patients may be involved in the control of metastasis (Braun et al, 1993, Gjomarkaj et al, 1994 and Karmali et al, 1986).

The stimulation of phagocyte reactive oxygen by n-6 essential fatty acids may also be relevant to malignant glioma therapy. Although it is well established that glioma patients are immunosupressed, especially in their T-cell function (Roszman et al, 1991 and Tada and Tribolet, 1993), there is often evidence of a rudimentary attempt of the host to reject the tumour (Jaeckle, 1994). Approximately 30-60% of primary human gliomas have mononuclear cell infiltrates (Bullard et al, 1986 and Stevens et al, 1988), and antigen-antibody complexes have been observed in gliomas (Wood and Morantz, 1979 and Wood et al, 1980). In addition, activated microglia have been observed surrounding invading tumour cells (Streit, 1994).

The suppression of T-cell function observed in glioma patients may arise from the release of immunosuppressive factors such as transforming growth factor-beta (TGFβ) from transformed glial cells (Weller and Fontana, 1995). TGFβ1 inhibits the proliferation of certain cancer cells *in vitro*, and there is evidence that this activity is

increased by polyunsaturated fatty acids (Newman, 1990). Similar results have been obtained with other cytokines including tumour necrosis factor (TNF), and certain interleukins and growth factors (Puntis and Jiang, 1994). It has been hypothesised that this action is due to the conversion of essential fatty acids to a common lipid mediator which acts as a second messenger for these cytokines and their receptors (Begin and Horrobin, 1985, Peppelenbosch et al, 1993 and Mollerup and Haugen, 1996).

Potentiation of reactive oxygen in CNS leukocytes may therefore induce inhibition of tumour proliferation through potentiation of the immune response. However, additional studies are required to evaluate the effect of exogenous essential fatty acids on oxidative activity in glioma-associated leukocytes, and to characterise the contribution of phagocyte reactive oxygen species generation to glioma cytotoxicity.

3.5 Conclusions

This study established experimental conditions appropriate for the evaluation of human glioma oxidative activity. In addition, this analysis provided information on reactive species generation in phagocytes, its stimulation in response to malignant transformation and surgery, and its regulation by arachidonic acid.

Elevated reactive oxygen species formation was detected in the neutrophils of lung cancer patients and in the monocytes and neutrophils of patients after surgery. Further stimulation of oxidative activity was observed following the addition of arachidonic acid and gamma-linolenic acid, with gamma-linolenic acid being the weaker agonist. However, phagocytes activated by malignant transformation were characterised by low sensitivity to exogenous essential fatty acid stimulation. These differences are consistent with activation of phagocyte reactive oxygen species formation by arachidonic acid-sensitive pathways in response to lung cancer and its surgical treatment.

Chapter 4:

The Stimulation of Cellular Oxidative Activity in Human Glioma by Arachidonic Acid and Gamma-Linolenic Acid

4.1 Introduction

The purpose of this study was to analyse the oxidative activity of human glioma cell preparations in the presence and absence of exogenous arachidonic acid and gamma-linolenic acid. Differences were investigated between i) the basal oxidative activity of tumour tissue and tumour-associated normal brain, ii) the kinetics of reactive oxygen species formation in normal and tumour tissue and iii) the essential fatty acid sensitivity of tumours of different grades.

These studies were undertaken to investigate a potential clinical role for n-6 essential fatty acids in glioma therapy. The mean adult survival for patients suffering from malignant glioma is typically less than one year following diagnosis (Thomas and Graham, 1995, Chapter 1, Berens and Giese, 1999 and Neider et al, 2000), and it is unlikely that this poor prognosis can be improved through increasing the efficacy of existing therapeutic procedures (Darling, 1990). The elucidation of novel therapies with the potential to increase tumour cytotoxicity, either alone or in conjunction with conventional treatments, would therefore have important clinical implications.

The pro-oxidative activities of essential fatty acids may be pertinent to malignant glioma therapy. *In vitro* and *ex vivo* studies have demonstrated an inverse association between basal reactive oxygen species formation and malignant progression of glioma (Levcheko and Demchuk, 1991 and Louwe et al, 1997), supporting the hypothesis that oxygen-based free radicals are involved in the regulation of tumour proliferation and death. Oxidative activity in a series of astrocytoma cell lines was inversely proportional to the grade of tumour from which the cell lines were derived, thus it was observed that the oxidative activity of human glioma tissue was lower than that of associated normal brain (Louw et al, 1997). Additionally, the oxidative activity of human astrocytoma and meningioma cells has been shown to be lower than that of non-transformed brain tissue (Levcheko and Demchuk, 1991). As it has been demonstrated that human glioma is deficient in the products of delta-6-desaturation,

including arachidonic acid, gamma-linolenic acid and docosahexaenoic acid (Martin et al, 1996), this supports the hypothesis that the low levels of basal oxidation observed in transformed glial cells are due to deficiency of essential fatty acids (Bartoli and Galeotti, 1979 and Cheeseman et al, 1984).

These observations suggest that addition of exogenous essential fatty acids may inhibit glioma proliferation by providing substrate for the production of potentially cytotoxic oxygen-based free radicals and lipid peroxides (Cornwell and Morisaki, 1984 and Gonzalez, 1992), and evidence supporting this hypothesis has been acquired from *in vitro* investigations. Arachidonic acid-mediated inhibition of cell division in a human glioma cell clone was associated with increased oxidative activity (Liepkalns et al, 1982), and inhibition of cell proliferation in glioma cell lines by gamma-linolenic acid was associated with potentiation of free radical activity (Das et al, 1990a).

Although these observations are consistent with a therapeutic role for arachidonic acid and gamma-linolenic acid in the management of malignant glioma, there is limited information on a number of parameters of clinical importance. A correlation between tumour grade and essential fatty acid sensitivity has not yet been established, and the kinetics of reactive oxygen species formation in human glioma tissue in response to exogenous essential fatty acid stimulation have not been described. The purpose of this study was to address these issues by investigating the effect of exogenous arachidonic acid and gamma-linolenic acid on reactive oxygen intermediate generation in short-term primary cultures of fresh human glioma tissue removed at biopsy.

However, the kinetics of oxidative activity in human glioma explants were not described well by existing statistical methods (Ratkowsky, 1989). This was due predominantly to cellular heterogeneity which is a characteristic of glial tumours, particularly those of high grade. Consequently, it was necessary to derive novel statistical parameters which more accurately described the complexity of the reaction kinetics observed in mixed cell populations.

Using these novel statistical models, the dynamics of tumour reactive oxygen intermediate generation in the presence and absence of arachidonic acid and gamma-linolenic acid was investigated, and the dependence of reactive oxygen intermediate formation on essential fatty acid type and concentration was determined. To further characterise the association between cellular oxidative activity, essential fatty acid sensitivity and malignant progression, reactive oxygen species formation in human glioma tissue was compared with that of associated normal brain. Additionally, the kinetics of glioma cell oxidation in the presence of exogenous arachidonic acid and gamma-linolenic acid was compared with that of tumour-associated normal brain and areas of focal tumour necrosis. The results from this study provide further information on the control of reactive oxygen intermediate formation in human primary brain tumours and the scientific rationale behind putative therapies for brain tumours using arachidonic acid and gamma-linolenic acid.

4.2 Materials and Methods

(a) Tissue Preparation

Explants of fresh glioma, and, if available, tumour-associated normal and necrotic tissue, were obtained at the time of surgery. Informed consent and local ethical board permission was obtained. The tissues were chopped using sterile disposable scalples, digested with collagenase, washed and resuspended at a density of 10^6 cells/ml in Hams F-10 medium. The cells were loaded with 2'7'-dichlorofluorescin diacetate, the intracellular probe of oxidative activity (Section 2.2b and 2.3b).

(b) Analysis of Cellular Oxidative Activity

The kinetics of 2'7'-dichlorofluorescin oxidation were determined in the presence and absence of exogenous essential fatty acids at 10 second intervals. The pre-stimulated rate of reactive oxygen intermediate generation was determined for 7 minutes, and the essential fatty acid-stimulated rate of reactive oxygen intermediate production was determined in the presence of 4-40μM arachidonic acid or gamma-linolenic acid for up to 3 hours (Section 2.3b). At each time point, 5 000 or 10 000 cells were analysed.

2'7'-dichlorofluorescin was chosen for analysis of oxidative activity because of its rapid cellular uptake and well characterised metabolism and stochiometry (Cathcart et

al, 1983 and Leaver et al, 1995). However, use of this probe was associated with a number of problems: probe leakage following cellular uptake has been reported, intracellular fluorescin degradation can take place (Makrigiorgos et al, 1996) and the activity of intracellular esterases, which are responsible for the metabolic conversion of dichlorofluorescin to dichlorofluorescein, may be low in tumour cells (Watson et al, 1978). To compensate for this, the rate of endogenous probe leakage and degradation was corrected by expressing oxidative metabolism in the presence of exogenous essential fatty acid as a ratio of the unstimulated rate. In unstimulated cells, the fluorescence of 2'7'-dichlorofluorescein was expressed in terms of the change in fluorescence of the initial 2',7'-dichlorofluorescin load per cell at the beginning of the incubation, thus correcting for variations in uptake.

(c) Statistical Analysis of Essential Fatty Acid Induced Stimulation of Tumour Oxidation

Reactive oxygen intermediate production was analysed using the exponential function:-

$$y = I+\beta[1-\exp(kt)] \quad \text{.....(Equation 1)}$$

where y=the ratio of the stimulated/unstimulated reactive oxygen intermediate production, I=the intercept on the y axis when t=0 seconds, β= the range of the response between t_0 seconds and t_∞, and k= the rate at which y changed from its initial value I at t=0 seconds to its final value as it approached its asymptotic maximum at t= ∞ seconds.

(d) Statistical Analysis of Tumour Oxidation in the Absence of Essential Fatty Acid

Tumour oxidation in the absence of essential fatty acid was analysed using the equation:-

$$y=\sigma\log(-\rho) + \sigma\log(\log \text{time} + 200 \text{ seconds}) \quad \text{.....(Equation 2)}$$

where y=mean cell fluorescence in unstimulated cells compared with cell fluorescence immediately after loading cells with 2'7'-dichlorofluorescin. The y intercept $I=\sigma\log(-\rho)$ indicates probe metabolism per cell at 200 seconds and σ is the rate constant describing the change in cell associated fluorescence per second. The goodness of fit of this model was estimated using the residual means squared value (Section 2.4b).

(e) Analysis of Cell Viability

Cell viability was determined in the presence and absence of arachidonic acid or gamma-linolenic acid at the end of each analysis of oxidative activity. 1ml aliquots of collagenase-dispersed glioma cells were incubated with 1mg/ml propidium iodide. Propidium iodide-associated fluorescence, which is inversely proportional to cell viability, was determined using flow cytometry (Section 2.6).

(f) Analysis of Tumour Pathology

The WHO classification and grade of each tumour analysed for peroxidative activity was determined using standard histopathological techniques (Section 2.10). Where appropriate, the glial origin of the tumour was investigated using immuno-histochemical analysis of GFAP expression.

4.3 Results

(a) The Effects of Arachidonic acid and Gamma-Linolenic Acid on the Kinetics of Reactive Oxygen Intermediate Generation

The brain tumours analysed in this study were obtained from 30 patients undergoing surgical resection or biopsy. Median age was 51 years (range 5-74) and 20 of the 30 patients were male. 18 of the 30 tumours received were glioblastomas (grade IV), and within this group mean patient age was 58.5 years. Three of these GBM patients were below the age of 35 and 12 were male. 9 of the remaining 12 tumours were astrocytomas (grades I-II), 3 were anaplastic astrocytomas (grade III), one was an anaplastic ependymoma (grade III) and one was an anaplastic oligodendroglioma (grade III). Five samples of peritumoural normal brain and one sample of tumour-associated necrotic tissue, each derived from biopsy specimens of glioblastoma, were also available for analysis.

Collagenase-dispersed cells derived from these tissues were used to investigate the effect of arachidonic acid and gamma-linolenic acid on tumour cell peroxidation. This was achieved by analysing the stimulation ratio, i.e. the ratio of basal reactive oxygen species formation to reactive oxygen species formation in the presence of exogenous essential fatty acid, in 5 000 or 10 000 cells at 10 second intervals for up to three hours. High, intermediate and low doses of arachidonic acid and gamma-linolenic acid were randomised after the dose response characteristics of essential fatty acid associated stimulation of reactive oxygen intermediate production were established. These concentrations were selected to fall within a range known to elicit a significant ($p<0.005$) stimulation of reactive oxygen intermediate production within 10 minutes, while having minimal effects on cell membrane integrity (Section 4.3e).

The stimulation ratio was used to derive two kinetic constants: the asymptotic maximum β and a rate constant κ. A good estimate of the asymptotic maximum β, which indicated the total extent of stimulated oxidation, was obtained when the stimulation ratio approached the asymptote during the course of the experiment. However, the estimate of β was poor for the four curves whose stimulation ratios continued to rise at a more uniform rate. When analysing the kinetic constant κ, the inverse of κ was plotted, as lower values of κ indicated a more rapid rate of increase of the stimulation ratio with time (Figure 4.1, Table 4.1 and Table 4.2).

A significant correlation between arachidonic acid and gamma-linolenic acid concentration, asymptotic maximum β ($p<0.001$) and the rate constant κ were detected in the 38 samples analysed using multivariate ANOVA. An effect of fatty acid type on the maximum stimulation β was also detected ($p<0.003$), with gamma-linolenic acid being more active than arachidonic acid in stimulating tumour peroxidation (Tables 4.1 and 4.2). The goodness of fit of this model was estimated using the residual means squared value was $r^2=0.977\pm0.00469$, range 0.837-0.998. The rate of unstimulated 2',7'-dichlorofluorescin oxidation in these samples therefore showed a good fit to equation 2. A compartmental model using equation 1 for the initial t=0-200 second stage did not give a better fit to the unstimulated rate curve.

Basal oxidative metabolism was analysed using equation 2. Dichlorofluorescein-associated fluorescence was expressed as a proportion of initial cell associated probe because deficits in tumour cell esterase activity compared with normal cells, which may result in impaired cell loading of the probe, have been reported (Watson et al, 1978). The y axis intercept I at 200 seconds was used to analyse probe metabolism and the rate constant σ was used to investigate probe leakage from cells. The two stimulation ratio kinetic constants β and κ, and the unstimulated kinetic constants σ and I for each of the 38 cell preparations are shown in Tables 4.1 and 4.2. The goodness of fit of this model was estimated by analysing the y axis intercept and the residual means squared (r^2). The y intercept i.e. the ratio of the stimulated/unstimulated reactive oxygen intermediate production at t=0 seconds was close to the predicted value of 1, being 1.019±0.0229 (mean± SEM of 38 data sets, range 0.783-1.528). The residual means squared was r^2=0.9719-0.00476 (mean± SEM of 38 data sets, range 0.902-0.998).

(b) The Effect of Tumour Grade on Essential Fatty Acid Induced Stimulation of Cellular Oxidation

A correlation was found between WHO tumour grade and the stimulation constant β, with high grade tumours generally exhibiting the highest overall level of cellular oxidation in the presence of exogenous n-6 essential fatty acid (Figures 4.2 and 4.4). This association was not linear, and was greatest in grade I-III tumours (Figure 4.4). The initial rate of essential fatty acid-mediated stimulation of oxidative activity, which was proportional to 1/k, also tended to be greater in high grade tumours. These observations suggest that high grade tumours are characterised by high sensitivity to exogenous essential fatty acid stimulus.

The linear (Pearson) correlation coefficient between tumour grade and logβ was 0.614 for the 12 grade I-III tumours analysed in this study. This was calculated by dividing the stimulation constant β by essential fatty acid concentration. When the stimulus concentration was not corrected, the correlation coefficient for tumour grade versus logβ was 0.543. The correlation coefficient between tumour grade and the rate constant κ was 0.408. This was also calculated by dividing κ by stimulus

concentrations, as essential fatty acid concentration has been shown to influence the kinetic rate constant κ.

An increase in the variability of β was observed in high grade tumours. This may be due to cellular heterogeneity, which increases with malignant progression of human glioma. The oxidative responses measured in this study were those of mixed cell populations, and immunohistochemical analysis indicated a greater variability of cell types present in tumours of high grade. A significant inverse relationship between tumour grade and propidium iodide permeability was also detected (Figure 4.4A). This observation is consistent with histological features of these tumours, as areas of necrosis are most commonly associated with grade IV tumours.

(c) Reactive Oxygen Intermediate Production in Normal and Tumour Cells

Basal oxidative activity and sensitivity to exogenous arachidonic acid were analysed in cell preparations obtained from 5 human primary gliomas and associated peritumoural normal brain (Table 4.3). During each investigation, up to 95 measurements of basal and stimulated oxidative activity were made at 10 second intervals in samples containing 5 000 or 10 000 cells. Basal oxidative activity was significantly lower in tumour tissue in comparison with normal brain (Figure 4.3). Differences were also observed between the responses of normal and tumour tissue to exogenous essential fatty acid stimulation. The stimulation ratio of 71 paired 10 000 cell samples was significantly greater in tumour tissue following a 0-20 minute incubation with arachidonic acid or gamma-linolenic acid ($p<0.0000017$). These differential responses were detected in the initial phase of the stimulation curve (3 - 6 minutes), and also at the later phase (10 - 20 minutes) when most curves were approaching their asymptotic maximum β.

(d) Reactive Oxygen Intermediate Production in Necrotic and Viable Tumour Cells

One glioblastoma sample received for analysis possessed a large visible region of tumour-associated necrosis. The necrotic region was dissected, and used for analysis of oxidative activity in the presence and absence of 12μM gamma-linolenic acid. A

significantly lower rate of basal oxidation was observed for the dissected necrotic region in comparison with viable tumour cells ($p<0.0001$), and the stimulation ratio was significantly higher in non-necrotic tissue. These differences were observed throughout the course of the investigation (Figure 4.5).

(e) Cell Viability

Cell viability was determined using flow cytometric analysis of PI uptake immediately following analysis of oxidative activity. Mean viability±SEM of the 30 tumour cell preparations was 91.2±1.56% (Tables 4.2 and 4.3). This was not significantly different from the viability of tumour cells incubated in the presence of 4-40μM arachidonic acid and gamma-linolenic acid (90.3±2.45%). Tissue storage did not significantly increase cell permeability to propidium iodide. Mean viability of 25 tissue preparations analysed less than 24 hours following resection was 91.0±1.92% compared with 91.6±2.81% 3 days after surgery (n=13).

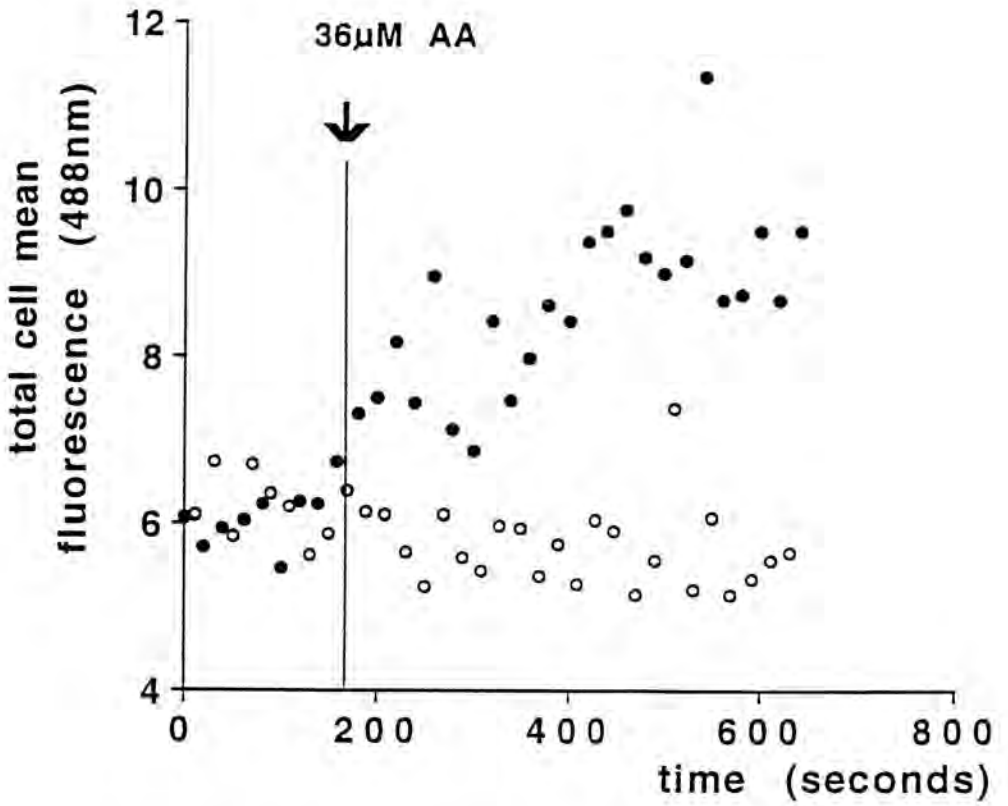
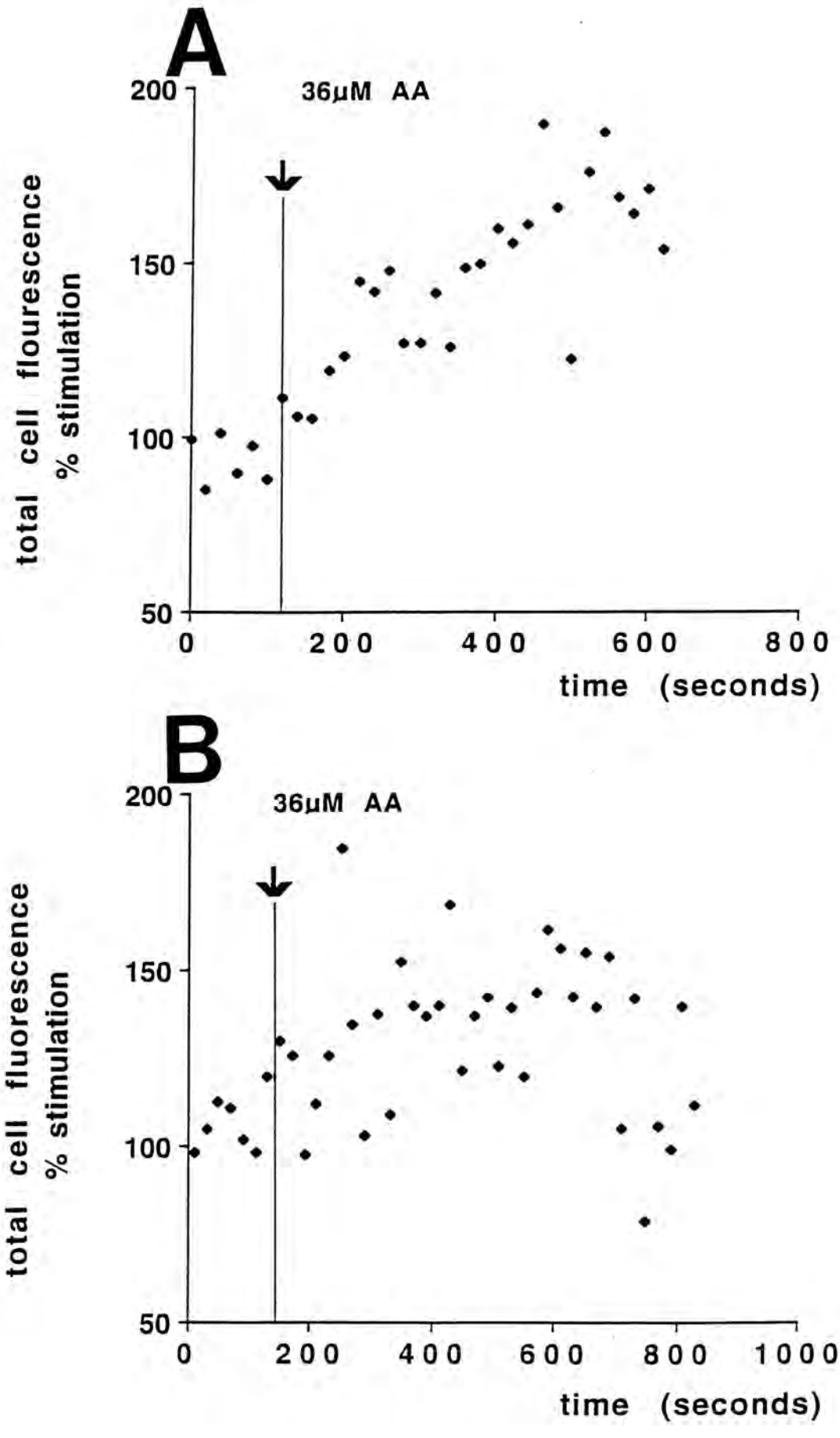


Figure 4.1. The rate of reactive oxygen intermediate formation in cells derived from a glioblastoma tumour incubated in the presence (closed circles) or absence (open circles) of 36µM sodium arachidonate, as measured by 2',7'-dichlorofluorescein oxidation. The y-axis indicates mean 2',7'-dichlorofluorescein oxidation per cell at 515-545nm. Total cell fluorescence at 488nm was proportional to the mean cellular content of oxidised probe.

Grade	FA type	μM	% V	Days	β	κ	α	I
0	AA	4	97.81	0.771	0	1	NA	NA
0	AA	18	96.235	0.781	0.191	1.442	33.07	66.14
0	AA	18	94.81	3	0.4	0.0058	100	112.48
0	GLA	40	99.14	3	4.175	0.0071	100	112.74
1	AA	4	60.09	0.771	0	1	NA	NA
1	AA	18	87.99	2	0.301	0.0049	61.47	90.3
1	AA	36	84.61	2	0.077	0.0084	98.21	114.18
2	AA	4	98.31	2.813	0.118	1	168.67	166.74
2	AA	18	93.27	3	1.198	0.0028	77.18	97.96
2	AA	18	70.28	0.104	0.583	0.0212	100	124.43
3	AA	18	86.99	0.781	0.062	0.0529	36.81	73.62
3	AA	18	96.53	0.781	0.074	0.008	52.82	80.23
3	GLA	12	91.06	3	0.449	1	40.34	72.41
3	GLA	27	97.98	1	2.143	0.0023	50.33	81.8
3	GLA	27	98.77	4	3.676	0.0013	97.45	104.49
3	GLA	40	95.45	3	5.746	0.0093	79.27	84.56

Table 4.1. Kinetic rate constants of oxidative metabolism in cells derived from explants of human primary gliomas. **Column 1:** grade: WHO grade I-III, 0=normal brain, stimulated with either arachidonic acid or gamma-linolenic acid. **Column 2:** fatty acid type. **Column 3:** fatty acid concentration (μM). **Column 4:** The overall viability (%V) of cells acquired from tumour explants. **Column 5:** the period of tissue storage in days in Hams F-10 medium. **Columns 6 and 7:** the kinetic rate constants β and κ of stimulated cells (see Figure 4.3). **Columns 8 and 9:** the kinetic rate constant δ and the intercept I of unstimulated cells.

Grade	FA Type	μM	% V	Days	β	κ	α	l
4	AA	4	84	1	0	0.0941	34.53	69.06
4	AA	18	87.36	0.7604	0.264	0.0178	126.15	135.17
4	AA	18	90.4	0.792	0.8	0.0004	141.97	142.52
4a	AA	18	86.76	0.833	0.747	0.0036	100	199.17
4b	AA	18	93.76	0.833	0.838	0.0164	89.93	114.83
4	AA	18	94	0.9375	0.058	1	34.76	69.52
4e	AA	18	95	0.8125	0.385	1	56.93	84.46
4f recurr	AA	18	79.96	0.8125	0.468	0.0019	44.27	72.7
4	AA	18	98.08	1	0.485	0.0061	125.43	132.95
4c	AA	18	98.81	1.03	0.102	1	41.13	82.26
4d stor	AA	18	99.11	4.0313	0.436	0.0127	53.75	78.52
4	AA	36	93.86	0.948	0.75	0.0036	50.04	77.22
4a'	GLA	12	92.17	1.885	1	0.0014	100	117.11
4b' necr	GLA	12	94.65	1.885	1	0.0005	100	119.42
4	GLA	12	98.59	0.917	0.326	0.011	99.1	101.77
4	GLA	12	98.35	0.917	0.11	0.0132	100	115.77
4a	GLA	20	83.99	0.125	0.127	0.0028	49.48	77.69
4b	GLA	20	94.32	0.125	6.883	0.0001	100	123.83
4	GLA	20	97.69	1	0.622	0.0024	70.21	88.12
4a	GLA	20	97.21	1	0.524	0.0095	100	123.12
4b	GLA	20	97.72	1	0.556	1	82.88	95.8
4	GLA	27	61.24	6	0.394	0.0069	100	117.35


A
36µM AA
200
150
100
50
0
200
400
600
800
time (seconds)
total cell flourescence
% stimulation
B
36µM AA
200
150
100
50
0
200
400
600
800
1000
time (seconds)
total cell fluorescence
% stimulation

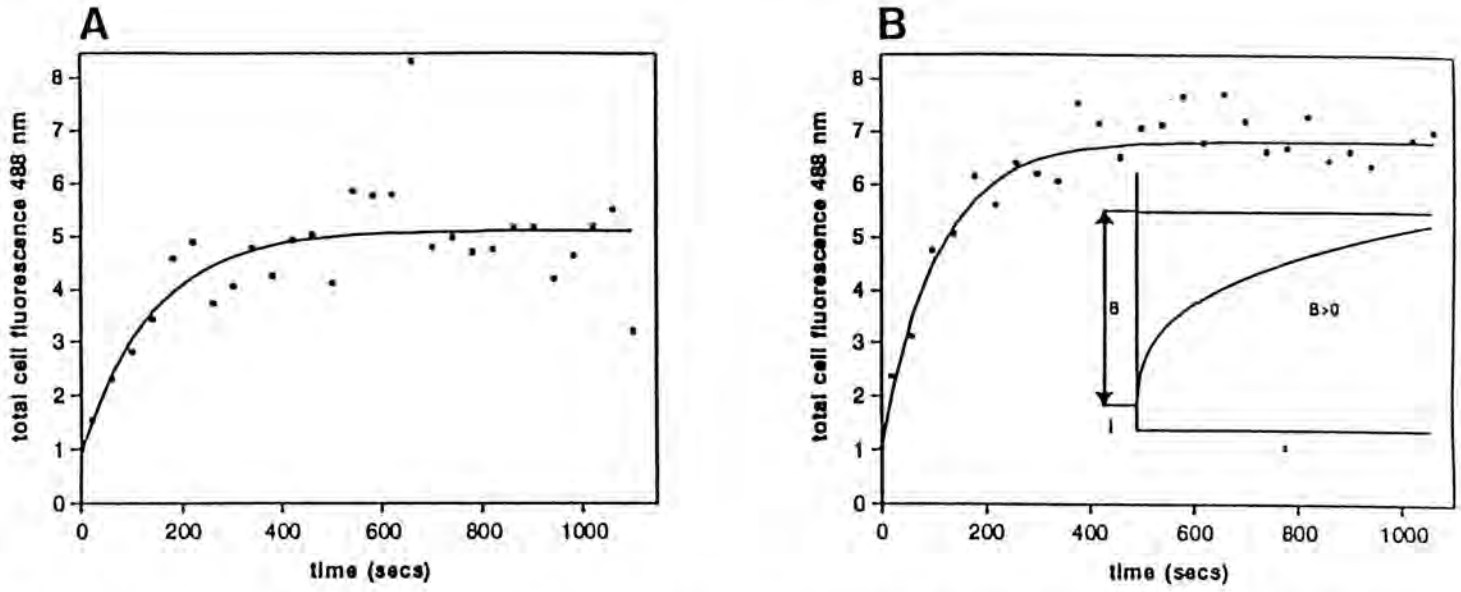


Figure 4.3 Comparison of the rate and extent of gamma-linolenic acid stimulation of reactive oxygen intermediate formation in tumour tissue and surrounding normal brain. The rate of stimulated reactive oxygen intermediate formation is shown in cells derived from A: normal brain tissue or B: gemistocytic astrocytoma tissue acquired from the same individual. Both preparations were incubated in the presence or absence of 40μM gamma-linolenic acid, and their reactive oxygen intermediate generation was assessed by measuring 2',7'-dichlorofluorescein associated oxidation. The rate of increase of 2'7'-dichlorofluorescein production with time in the total tumour and normal cell populations was analysed using the exponential function $y=I+\beta[I-\exp(-\kappa t)]$, where y= the ratio of stimulated:unstimualted reactive oxygen intermediate formation, i.e. mean 2',7'-dichlorofluorescein associated fluorescence in the stimulated cell preparation divided by mean 2',7'-dichlorofluorescein associated fluorescence in the unstimulated cell preparation in cells derived from the same tumour explant. The insert in Figure 4.4B illustrates the derived constants β (maximum asymptotic increase in the rate of oxidative activity) and I, the intercept of the rate curve. Results are expressed as the stimulation ratio of stimulated divided by unstimulated cell fluorescence.

Table 4.3. Comparison of arachidonic acid stimulated oxidation in normal and tumour cells

time (minutes)	number of paired samples	mean stimulation ratio (mean oxidation in stimulated/unstimulated cells±SEM)	
		normal	tumour
0-2.67	13	1.44±0.153	1.68±0.310
2.67-6.67	21	2.17±0.300	2.68±0.468*
6.67-10.00	14	2.70±0.454	3.46±0.765
10.00-15.00	12	2.62±0.588	3.07±0.917*
15.00-20.00	11	4.41±0.376	6.15±0.504**

The mean stimulation ratio (mean roi in arachidonic acid stimulated/unstimulated cells from the same individual±SEM) for 71 paired samples each containing 10 000 cells derived from the same individuals. The 5 tumour samples were astrocytoma (grades I-III), oligoastrocytoma (grade II), gemistocytic astrocytoma (grade III) and glioblastoma multiforme (grade IV). The basal unstimulated roi in 95 paired normal and tumour cell preparations from the same patient before arachidonic acid addition was significantly ($p<0.00002$) lower in tumour cells (49%±5.72 the roi of paired normal control cells derived from adjacent non-tumour tissue). After exposure to exogenous arachidonic acid for the time periods indicated in column 1, the stimulation ratios of cell roi in normal and tumour cells was analysed (columns 2 and 3). The tumour stimulation ratios were significantly greater than normal control cell stimulation ratios are indicated *$p<0.05$, **$p<0.0005$ (paired t-test)

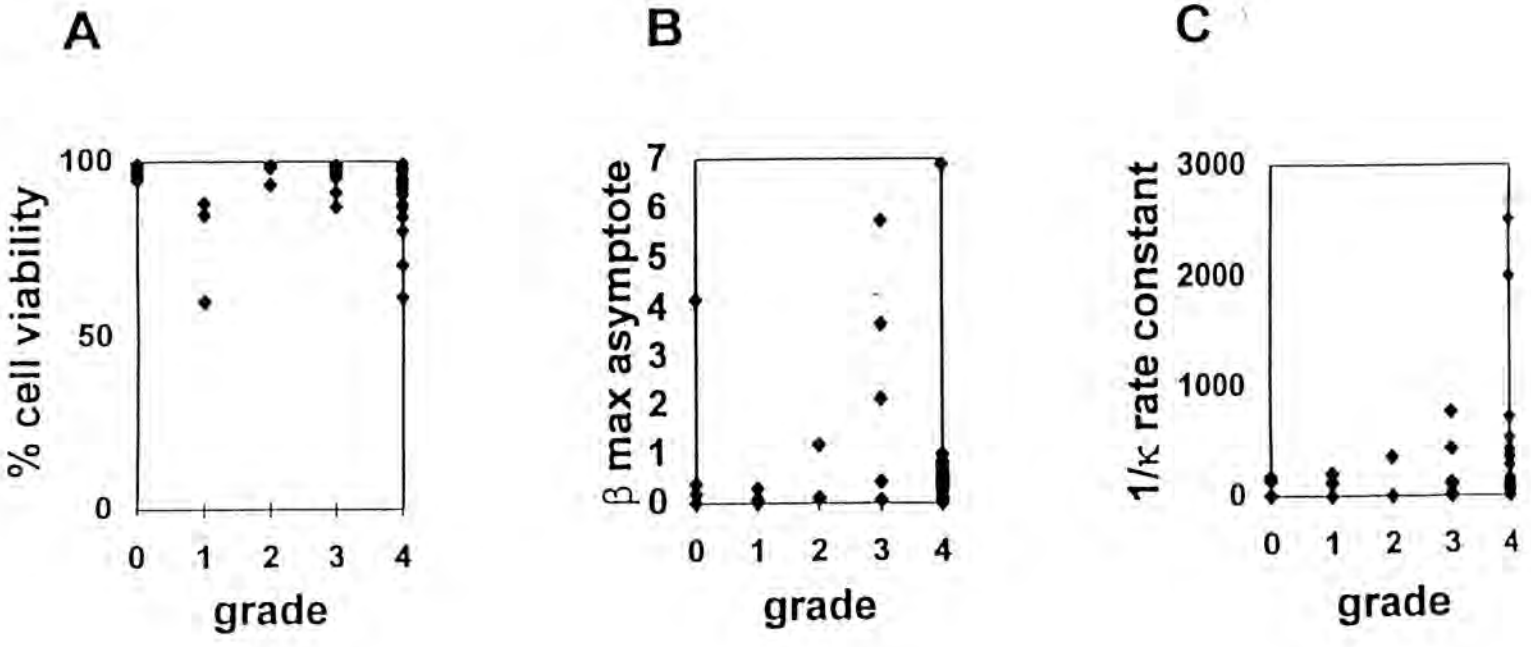


Figure 4.4 The influence of tumour grade on A: percent cell viability, as determined by the uptake of propidium iodide, B: the maximum asymptotic increase in the stimulation ratio β following essential fatty acid stimulation and C: the kinetic rate constant I/κ All 39 tumours analysed were WHO grade I-IV, with grade 0 representing tumour-associated normal brain.

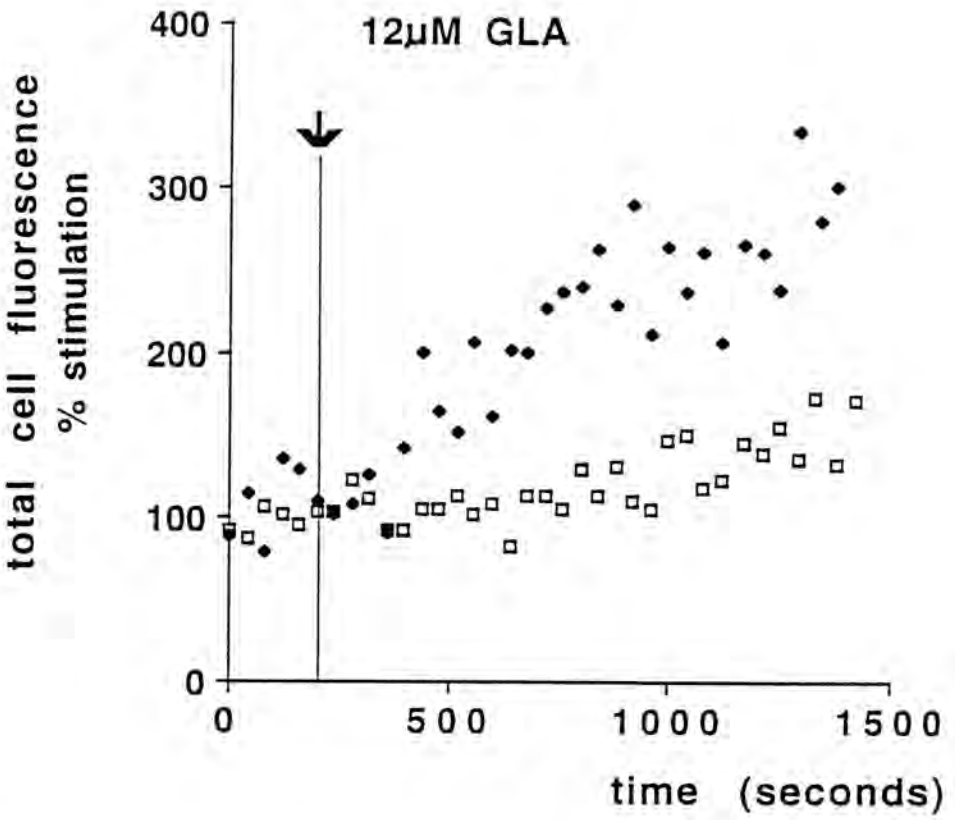


Figure 4.5. Comparison of the stimulation of reactive oxygen intermediate formation in viable and necrotic regions of an explant of glioblastoma multiforme by the addition of 12μM gamma-linolenic acid at 270 seconds. The rate of 2'7'-dichlorofluorescein formation is shown in black diamonds for the viable cells and open squares for necrotic cells. Results are expressed as % stimulation of mean basal cell fluorescence.

4.4 Discussion

This study describes oxidative metabolism in the presence and absence of arachidonic acid and gamma-linolenic acid in 36 collagenase-dispersed explants of human primary brain tumours. In each of the samples analysed, arachidonic acid and gamma-linolenic acid rapidly increased intracellular reactive oxygen intermediate production (Figure 4.1). The initial increase in peroxidation 0-200 seconds after addition of arachidonic acid or gamma-linolenic acid was greater than any subsequent increase in peroxidative activity, and in most tumour samples the rate of peroxidation approached an elevated steady state after 10 minutes incubation (Tables 4.1 and 4.2). All the tumour cell preparations analysed responded to exogenous arachidonic acid and gamma-linolenic acid in a grade dependent manner, with high grade tumours tending to have higher values of the asymptotic maximum β and the rate constant k. The stimulation of reactive oxygen intermediate generation by arachidonic acid was significantly greater in tumour tissue in comparison with normal human brain in the five paired samples for which non-transformed tissue was available ($p<0.0000017$, paired t-test, $n=71$).

In both normal and tumour tissue, the stimulation of reactive oxygen intermediate formation was proportional to essential fatty acid type and concentration. Although the kinetics of the reactive oxygen intermediate response to arachidonic acid and its C18 precursor gamma-linolenic acid were similar, the extent of gamma-linolenic acid-induced stimulation of reactive oxygen intermediate production was greater at equimolar concentrations. This suggests that the ability of long chain PUFA to stimulate reactive oxygen species formation is not proportional to hydrocarbon chain saturation, and that essential fatty acids may potentiate glioma oxidative activity by modulating molecular pathways other than lipid peroxidation.

In each of the thirty tumour preparations, the lower rate of reactive oxygen intermediate production by necrotic cells was confirmed by sub-population analysis. The laser scatter profile of necrotic cells is characterised by low forward angle and right angle light scatter. A significantly ($p<0.0001$) lower response was obtained when necrotic cells of a mixed tumour population were gated (see also Chapter 5). An association was also observed between cell membrane permeability and tumour grade

with grade IV tumours exhibiting greater membrane permeability to vital dyes. This correlates with histochemical features used in the diagnosis of malignant gliomas, as glioblastoma is characterised by areas of focal necrosis.

Evidence for an anti-proliferative effect of exogenous gamma-linolenic acid at the concentrations used in this study has been demonstrated in a number of tumour types (Karmali et al, 1985, Begin et al, 1986, Hyashi et al, 1990 and Pritchard and Mansell, 1990). Although the pathways by which arachidonic acid and gamma-linolenic acid exert their tumour cell toxicity are poorly characterised, there is evidence that reactive oxygen intermediates can diffuse rapidly across membranes during lipid peroxidation (Slater, 1984a and 1984b) and that the metabolic flux of arachidonic acid and gamma-linolenic acid in membranes is involved in cell signalling and cell death (Merrill and Schroeder, 1993 and Leaver et al, 1995). This may involve modulation of the activity of the transcription factors NF-κβ and bcl_2 associated pathways (Abate et al, 1990, Schreck et al, 1992 and Kane et al, 1993). In addition, arachidonic acid directly activates c-jun, resulting in cellular apoptosis (Rizzo and Carlo-Stella, 1996), and p53 involvement in reactive oxygen intermediate metabolism has recently been demonstrated (Polyak et al, 1997). However, additional studies are required to further characterise the molecular events associated with essential fatty acid-mediated induction of tumour cytotoxicity.

4.5 Conclusions

The results of this study support the hypotheses that i) reactive oxygen intermediate generation is impaired in human malignant glioma in comparison with associated normal brain and that ii) exogenous arachidonic acid and gamma-linolenic acid stimulate glioma peroxidative activity. Analysis of tumour-associated normal brain indicated that human glioma tissue was characterised by low basal oxidative activity and high sensitivity to exogenous essential fatty acid stimulus in comparison with non-transformed cells. Reactive oxygen species formation in the absence of arachidonic acid or gamma-linolenic acid was significantly lower in tumour tissue in each of the 5 samples available for analysis. Although both normal and tumour cells responded to exogenous essential fatty acid stimulus with increased reactive oxygen

species formation, this increase was significantly greater in cells derived from transformed tissue ($p<0.0000017$).

The magnitude of essential fatty acid-mediated stimulation of glioma oxidative activity was a function of tumour grade, with more malignant tumours responding with higher sensitivity to exogenous n-6 essential fatty acids. Both the rate (defined by the constant k) and extent (defined by the asymptotic maximum β) of stimulated oxidative metabolism were greater in high grade tumours, although this association was not linear for β. The kinetics of oxidative metabolism observed in response to exogenous arachidonic acid and gamma-linolenic acid were similar, although gamma-linolenic acid was the more potent agonist at equimolar concentrations. Neither arachidonic acid nor gamma-linolenic acid was associated with loss of cell membrane permeability at the concentrations used in this study.

Chapter 5:

The Stimulation of Reactive Oxygen Intermediate Production in Glioma Populations by Arachidonic Acid and Gamma-Linolenic Acid

5.1 Introduction

The purpose of this study was to provide preliminary information on the flow cytometric characteristics and oxidative activities of human glioma sub-populations. Sub-populations were selected on the basis of their forward scatter, which is proportional to cell size, and side scatter, which is proportional to granularity which is related to DNA content. These regions were analysed in terms of i) basal oxidative activity, ii) oxidative activity following stimulation by exogenous arachidonic acid and iii) propidium iodide permeability. Following this analysis, additional studies were undertaken to investigate the oxidative activity of cells of glial origin present in tumour biopsy samples. This was achieved using flow cytometric analysis of GFAP distribution.

This sub-population analysis was undertaken because it is known that glial tumours, particularly those of high grade, are characterised by cellular heterogeneity (Figure 5.1) (Darling, 1990 and Thomas and Graham, 1995, Chapter 1). This heterogeneity is due in part to pathological processes associated with malignant progression, for example cellular dedifferentiation, angiogenesis and necrosis (Greaber et al, 1996 and Louis, 1997). Additionally, glial tumours rapidly invade surrounding normal brain, resulting in diffuse boundaries between neoplastic and non-neoplastic tissue (Turazzi and Licata, 2000). For these reasons, cell preparations derived from biopsy specimens contain varying proportions of tumour cells at various stages of dedifferentiation, normal brain cells, erythrocytes, reactive and non-reactive leukocytes, blood vessel endothelium and tumour cells characterised by low viability (Darling, 1990, Thomas and Graham, 1995, Chapter 1 and Berens and Giese, 1999) (Figure 5.1).

Although the cellular heterogeneity of human glioma is well established, the various cell types present in primary tumour samples have not been studied extensively

using flow cytometry. However, these considerations are important as the sensitivity of individual reactive populations to exogenous n-6 essential fatty acids will contribute to the overall level of reactive oxygen species formation observed. These investigations were undertaken to provide further information on the dynamics of reactive oxygen species formation in human glioma tissue. Analysis of the glioma sub-population characterised by GFAP-positivity was undertaken to test the hypothesis that malignant glial cells are characterised by impaired basal reactive oxygen species formation, but high sensitivity to exogenous arachidonic acid and gamma-linolenic acid.

Preliminary observations indicated that unlike the phagocyte preparations analysed in Chapter 3, cells derived from glioma explants were not sorted into discrete sub-populations. In addition, variability of cellular composition meant that a cell population which occurred in an individual tumour did not necessarily occur in all glioma preparations. It was therefore necessary to select tumours whose laser scatter profiles were similar, so that gates selected for one tumour could readily be applied to the others being investigated. This selection provided 5 glioblastomas (including 1 which had recurred), and 1 anaplastic oligidendroglioma and 1 pilocytic astrocytoma. These tumours had all been treated with 18 or 36μM arachidonic acid, and their oxidative activity had been analysed at similar time intervals.

A total of 6 populations were analysed in each of these tumours, including a region along the x=y gradient characterised by low to medium forward and side scatter (G1), a region of low side scatter which occurred below this main population (G2), a region of high side scatter and medium forward scatter (G3), a region of medium forward and side scatter (G4) and cells possessing very high forward and side scatter (G5). In addition, a region close to the origin was selected (G6) (Figure 5.2).

Linear regression models were then used to define the following parameters: i) the rate constant κ, ii) the asymptotic maximum β, iii) basal oxidative activity, iv) % stimulation of reactive oxygen intermediate generation and v) the rate of probe efflux. The rate of probe influx was compared with the influx of the viability probe propidium iodide. Unlike the analysis of cellular oxidation in ungated glioma preparations (Chapter 4), it was not necessary to use complex statistical analysis in

this study. This was due in part to the relatively small population size and the more heterogeneous nature of the cell populations being investigated.

Analysis of the oxidative activity of cells of glial origin present in tumour biopsy samples was achieved using flow cytometric analysis of GFAP distribution. The sub-population of cells expressing GFAP was identified in ethanol-fixed cell preparations acquired from 17 tumours grades I-IV by i) the binding of a polyclonal anti-GFAP antibody and ii) the high forward angle and high side angle laser scatter characteristic of differentiated cells. This GFAP-positive cell population was gated and the stimulation of reactive oxygen intermediate generation in response to n-6 essential fatty acid was analysed in fresh cells.

GFAP was used in these studies because it is a well characterised astrocytic marker (Debus et al, 1983, Miettinen et al, 1984, Coakham et al, 1985 Garson et al, 1985 and Royds et al, 1986) with an established role in the routine diagnosis of the biopsy samples which were analysed for oxidative activity (Chapter 4). The previously established record of GFAP immunoreactivity made it possible to ensure that all the samples selected for flow cytometric analysis were GFAP-positive. This was necessary because of an important disadvantage of this marker, namely that it may not be present in all astrocytic tumours. This is particularly so for those characterised by a high degree of cellular dedifferentiation (Kurpad et al, 1994).

Other well established astrocytic markers are S-100 protein and vimentin. Vimentin is an intermediate filament protein found predominantly in immature glia (Yang et al, 1994). S-100 protein is a highly acidic protein which is also localised primarily in glial cells, although some may be present in neuronal nuclei or the plasma membrane (Moore, 1982). Vimentin and S-100 protein possess all the disadvantages associated with GFAP, and are less commonly used in routine diagnosis. Other tumour markers, e.g. nuclear proliferation markers (BrdU, PCNA, MIB-I), growth factors (EGF) and oncogene and tumour suppressor gene products (bcl_2 and p53) have limited value in predicting clinical outcome, and cannot be correlated precisely with WHO tumour type and grade of malignancy (Kurpad et al, 1995, Stemmer-Rachamimov and Louis, 1997, McKeever, 1998, Grzybicki and Moore, 1999, Morrison and Prayson, 2000 and Reavey-Cantwell et al, 2001).

The lack of specific markers for malignant astroglial cells remains an important problem in neuro-oncology research (Yang et al, 1994, Kurpad et al, 1995, Stemmer-Rachaminov and Louis, 1997 and Grzybicki and Moore, 1999). Despite recent advances in the profiling of gene expression and monoclonal antibody technology, little progress has been made in elucidating molecular markers of glioma diagnosis and prognosis (Kurpad et al, 1995 and Grzybicki and Moore, 1999). One exception may be the intermediate-filament-associated protein IFAP-300KDa (Yang et al, 1994). An immunofluorescence study has indicated that IFAP-300KDa is detectable in all astrocytic tumours, but not normal mature or reactive astrocytes. This protein may therefore represent a specific marker for transformed astrocytes, and it may have a role in subsequent characterisation of the tumour sub-population.

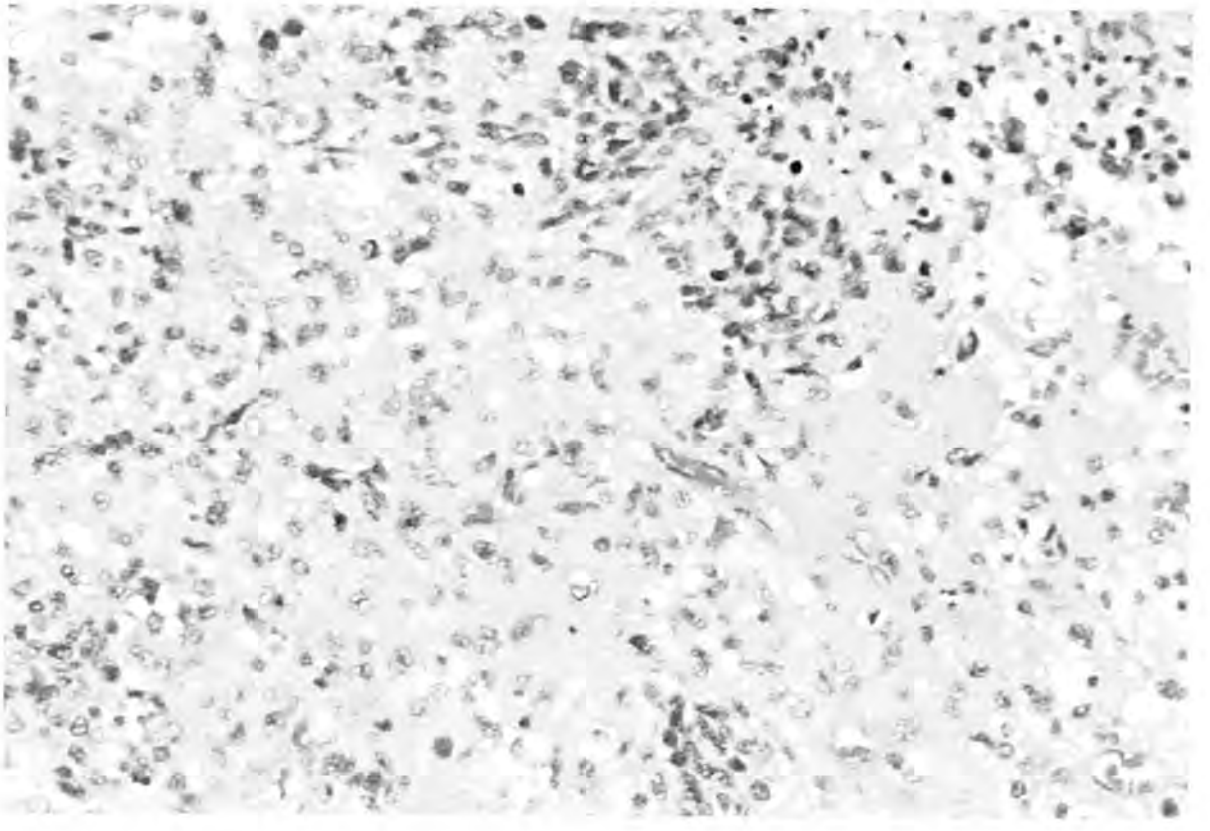


Figure 5.1. A histological section of glioblastoma showing regions of cellular heterogeneity. Apoptotic nuclei are visible in the viable glioblastoma tumour cells adjacent to an area of necrosis (right). Haematoxylin and eosin x200.

5.2 Materials and Methods

(a) Characterisation of Typical Laser Scatter Profiles

Tumour sub-population analysis was conducted retrospectively from stored data which was acquired during investigation of glioma oxidative activity (Chapter 4).

(b) Characterisation of the GFAP-Positive Population

(i) Monoclonal GFAP Staining

Ethanol-preserved glioma cells were washed, resuspended at a density of $1x10^6$ cells/ml and incubated with monoclonal rabbit anti-cow GFAP diluted 1:5, 1:10 and 1:20 in distilled water. The cells were incubated at room temperature for 30 minutes, centrifuged, resuspended in phycoerythrin diluted 1:10 in distilled water and incubated at 4^0C for 2 hours. GFAP-positive cells were identified using flow cytometry (Section 2.5a).

(ii) Polyclonal GFAP staining

1ml aliquots of ethanol preserved cells were washed and incubated for 2 hours with anti-GFAP-FITC at dilutions 1:5, 1:10 and 1:20. The cells were centrifuged and resuspended for flow cytometry at 564-607nm using a Becton Dickinson FACScan flow cytometer and LYSIS software (Section 2.5b).

(c) Statistical Analysis of Human Glioma Sub-Population Oxidation

Basal oxidative activity in human glioma sub-populations was analysed using the equation:-

$$\Delta BF_{(0\text{-}200)} - \Delta BF_{(200\text{-}400)} \times 100 \quad \text{.....(Equation 1)}$$

where ΔBF=the change in mean basal 2',7'-dichlorofluorescein-associated fluorescence over the time periods shown (in seconds) as subscripts in parentheses. For each determination of mean basal fluorescence over a 200 second interval, 6-10 values of mean 2',7'-dichlorofluorescein-associated fluorescence were used. The

rate of decrease of 2',7'-dichlorofluorescein-associated cellular oxidation gave an indication of the rate of probe efflux and hence loss of membrane permeability. This was compared with the extent of influx of the viability probe propidium iodide (Section 2.4c).

The rate of increase of cellular oxidation in human glioma sub-populations following the addition of exogenous arachidonic acid was analysed using the equation:-

$$\Delta F_{(0\text{-}200)} - \Delta F_{(200\text{-}400)} / \Delta F_{(0\text{-}200)} \times 100 \quad \text{.....(Equation 2)}$$

where ΔF=the mean % increase in 2',7'-dichlorofluorescein-associated fluorescence over the time periods shown in parentheses. Arachidonic acid was added at t=0. Further determinations of the increase in reactive oxygen intermediate generation every 200 seconds for up to 1 200 seconds were made if possible. The SEM of all determinations was calculated in analysis of both basal and stimulated reactive oxygen intermediate generation (Sections 2.4c).

5.3 Results

(a) Analysis Tumour Sub-Population Oxidative Activity

The six regions selected for analysis are shown in Figure 5.2 and their fluorescence distribution profiles in Figure 5.3. Their responses to exogenous essential fatty acid stimulus are described in Figure 5.4, Table 5.1 and Table 5.2. Sub-population analysis of cell membrane permeability is shown in Table 5.3, and the rate of probe efflux is described in Table 5.4. The morphological and oxidative characteristics of human glioma sub-populations are discussed in Section 5.3b.

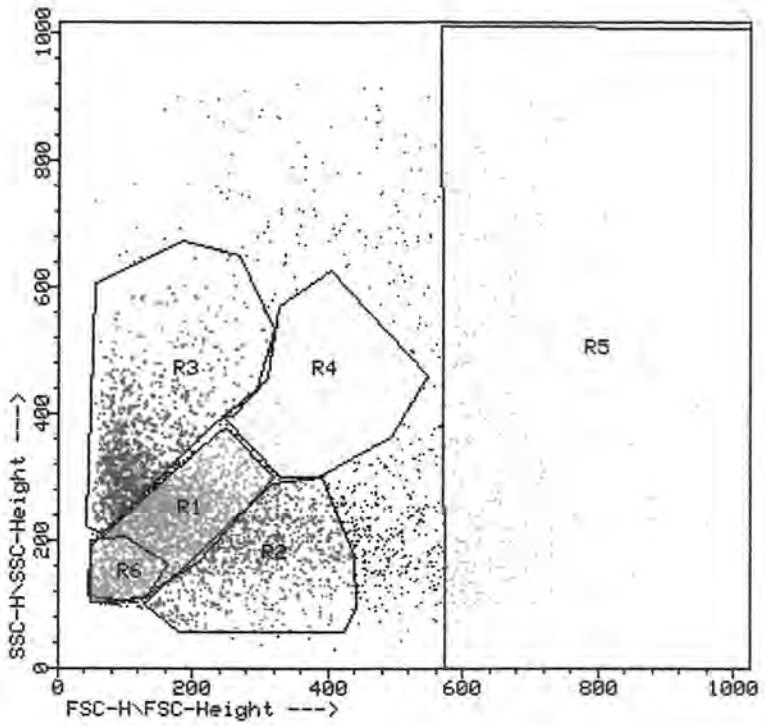


Figure 5.2. Dot plot of cells derived from a collagenase dispersed explant of human glioblastoma multiforme. Cells are separated on the basis of forward scatter (FCS) (x-axis), which is proportional to cell size, and side scatter (SSC) (y-axis), which is proportional to granularity. Similar regions were selected for an additional four glioblastoma tumours, one anaplastic astrocytoma and one pilocytic astrocytoma.

Figure 5.3. Fluorescence intensity distributions for the tumour sub-populations described by gates 1-6 in Figure 5.2. The y-axis indicates 2'7'-dichlorofluorescein-associated fluorescence, which is proportional to cellular peroxidation, and the x-axis indicates cell number.

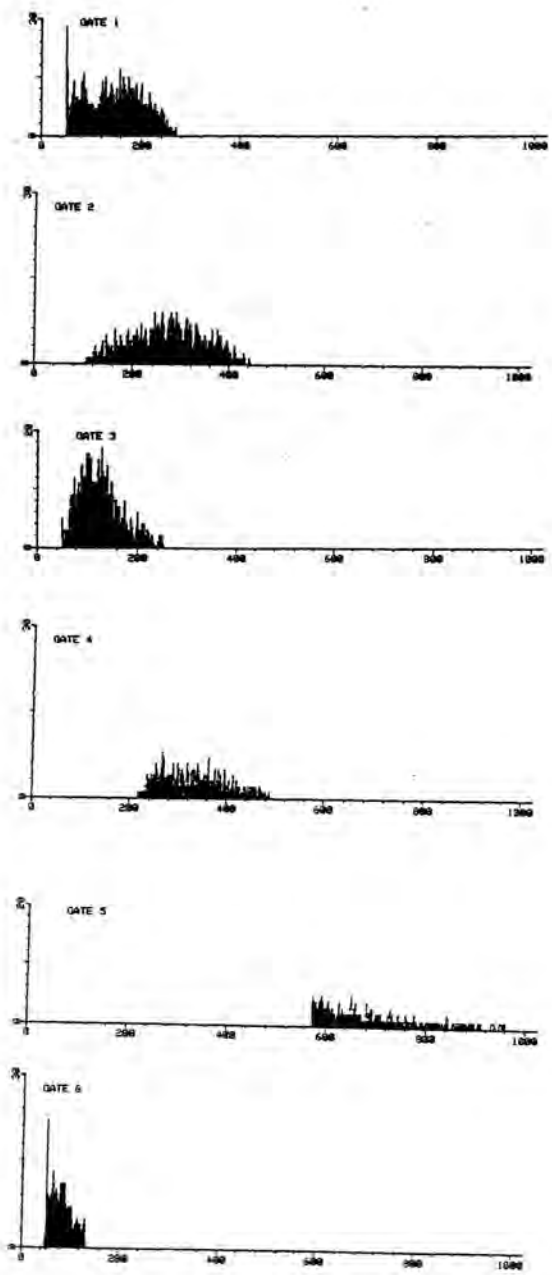
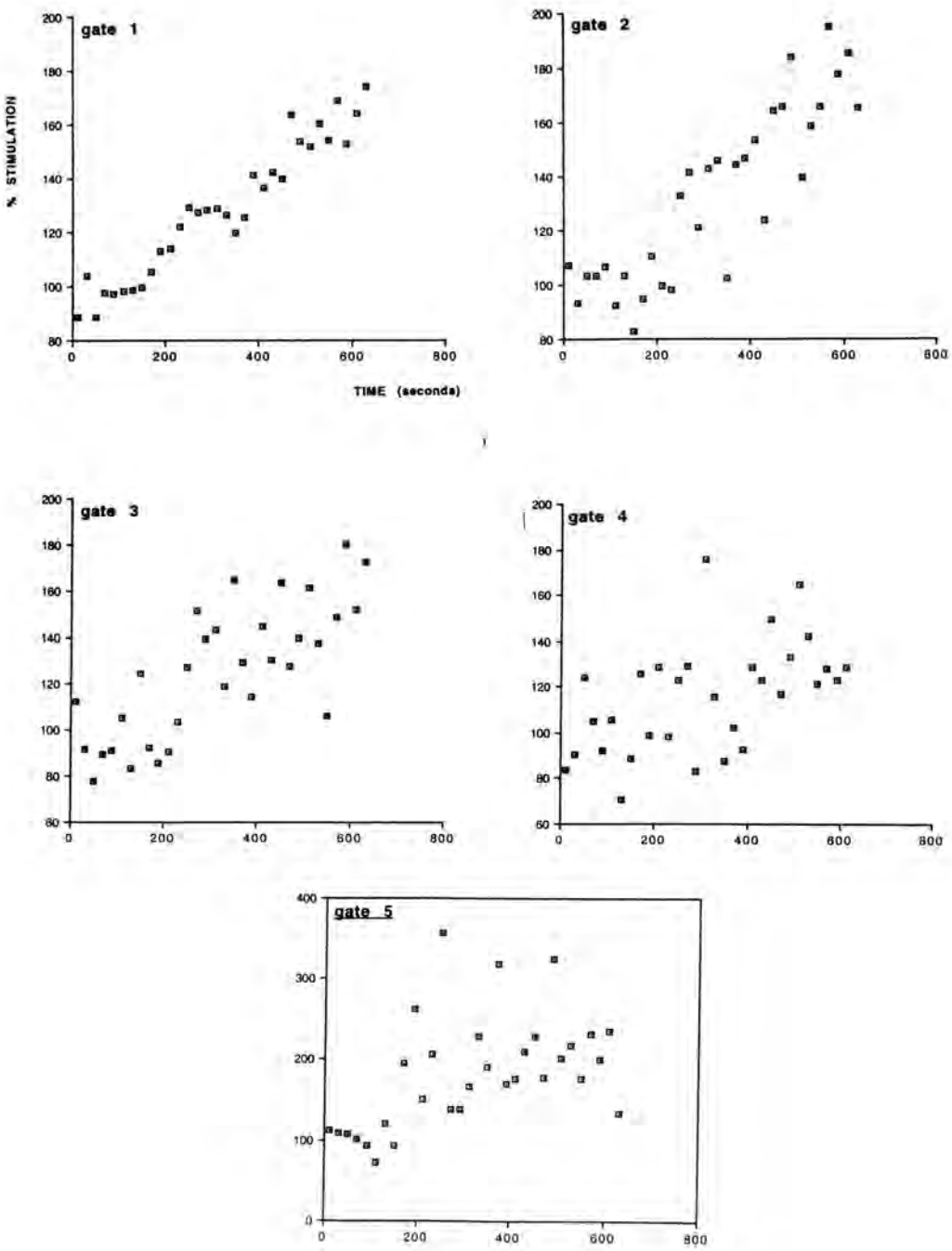


Figure 5.4. Rate curves describing the stimulation ratio of cellular peroxidation by 36μM arachidonic acid in human glioma sub-populations. The stimulation profiles shown for gates 1 to 6 indicate the oxidative responses of cells gated by the laser scatter regions illustrated in Figure 5.2. No significant stimulation of cellular peroxidation was observed in region 6 (data not shown).



diagnosis	region	time (s) 0-200		time (s) 200-400	
		mean	SEM	mean	SEM
astro.	G1	103.1946	0.023451	103.5295	0.083618
(grade I)	G2	106.4201	1353483	100.1448	2.545388
	G3	98.90995	1.241533	99.10099	4.123364
	G4	114.7819	2.518534	119.3178	3.198115
	G5	101.9837	3.3085	110.5634	3.562616
	G6	103.3532	-0.02854	103.8153	1.109435
		mean	SEM	mean	SEM
AA	G1	100.8479	-1.38526	102.4207	1.217805
(grade II)	G2	103.8893	1.428315	109.0909	1.55499
	G3	99.93717	1.83443	98.7568	3.900485
	G4	105.8254	1.623941	105.1085	2.594544
	G5	115.1428	1.587672	113.6377	2.783379
	G6				
		mean	SEM	mean	SEM
GBM	G1	118.949	3.055285		
(grade IV)	G2	117.8698	3.206817	154.9604	3.85537
	G3	122.3102	1.980024	128.289	1.208461
	G4	123.2211	16.68486	127.8485	10.33746
	G5	98.98336	14.86495	110.2033	8.461848
	G6	115.0281	2.941634	137.3116	3.915765
		mean	SEM	mean	SEM
GBM	G1	100.6305	-0.42859	107.2163	1.610611
(grade IV)	G2	107.8161	2.817088	113.6984	3.233605
	G3	105.58	1.464795	111.5144	2.86143
	G4	103.2048	3.517723	116.0883	4.323675
	G5	113.1336	12.34382	113.3629	18.58938
	G6	98.3345	-0.51521	106.1528	0.835862
		mean	SEM	mean	SEM
GBM	G1	120.0262	1.014269	145.4456	2.424145
(grade IV)	G2	117.631	5.261634	153.3514	3.985527
	G3	120.546	7.747371	134.3915	4.304141
	G4	115.4541	7.284966	126.348	6.210505
	G5	202.0921	19.33363	219.0529	15.96918
	G6	119.9983	1.471631	147.726	1.683237
		mean	SEM	mean	SEM
GBM	G1	115.0677	3.586096	119.4991	1.874596
(grade IV)	G2	115.077	4.151504	123.7289	6.852435
	G3	124.2637	10.07214	136.8946	6.0168
	G4	147.266	8.521032	149.968	7.652833
	G5	107.5625	4.856717	125.5983	1.14844
	G6	109.0644	2.008869		
		mean	SEM	mean	SEM
GBMrecurr	G1	110.8598	1.247929	118.3283	-0.02905
(grade IV)	G2	123.1584	5.604772	146.3973	5.363785
	G3	120.8266	4.294948	149.1854	2.945346
	G4	128.5069	0.999287	115.3945	6.39961
	G5	152.8398	22.29511	207.1123	21.85634
	G6				

Table 5.1. Sub-population analysis of arachidonic acid-mediated stimulation of reactive oxygen intermediate generation in the seven samples of human gliomas grades I-IV using equation 2. Results were calculated as mean percent stimulation of 2'7'-dichlorofluorescein-associated fluorescence over the time periods 0-200 seconds, 200-400 seconds and 400-600 seconds for each sub-population (gates 1-6) indicated in Figure 5.2. Results are expressed as the mean ±SEM.
astro.=astrocytoma, G=gate.

TIME (s)	ΔFl	GATE 1	GATE 2	GATE 3	GATE 4	GATE 5	GATE 6
0-200	mean	0.145822	0.192328	0.04363	0.12358	0.086534	0.168675
	SEM	0.035443	0.049153	0.034106	0.052914	0.022709	0.029032
	n	5	5	5	5		4
200-400	mean	0.03564	-0.05984	0.082228	-0.04377	0.136332	-0.01149
	SEM	0.028736	0.06627	0.043636	0.051911	0.174125	0.030942
	n	4	5	5	5	5	4

Table 5.2. Stimulation of reactive oxygen intermediate formation in 4 explants of human glioblastoma by 18μM arachidonic acid at 0-200 seconds, 200-400 seconds and 400-600 seconds in the cell sub-populations described by gates 1-6 in Figure 5.2. ΔFl indicates the increase in 2'7'-dichlorofluorescein-associated fluorescence. Results are expressed as the mean ±SEM

diagnosis	region	time (s) 0-200		time (s) 200-400	
		mean	SEM	mean	SEM
astro.	G1	0.054576	0.04374	0.065719	-0.46821
(grade I)	G2	0.097778	0.392128	0.143753	-0.39998
	G3	0.012988	0.14734	0.098002	0.078515
	G4	0.112528	-0.23839	0.11314	0.477271
	G5	0.021569	0.196143	0.104159	-0.6624
	G6	0.043409	-0.16825	0.055064	0.157876
		mean	SEM	mean	SEM
AA	G1	0.00249	0.120378	-0.00499	-0.66559
(grade II)	G2	-0.0023	0.414722	0	0.261451
	G3	-0.02253	-0.09224	0.015198	0.011119
	G4	-0.01675	-0.31143	0.05906	0.407942
	G5	0.005209	-0.09411	-0.05246	-1.31981
	G6	-0.0075	-0.92154	-0.00186	0.14001
		mean	SEM	mean	SEM
GBM	G1	0.196197	-0.55608		
(grade IV)	G2	0.208433	-0.10157	0.208232	0.17403
	G3	0.225658	0.300236	0.134088	0.162519
	G4	-0.12626	-1.25082	0.178815	-0.01972
	G5	-0.50092	-0.35039	0.127401	0.567403
	G6	0.160099	-0.06181	0.129766	0.54781
		mean	SEM	mean	SEM
GBM	G1	0.07396	-0.18457	0.058401	-0.03284
(grade IV)	G2	0.163628	0.09738	0.1834	0.515198
	G3	0.127864	-0.13268	0.101252	0.157431
	G4	0.092031	0.25103	0.121343	0.063568
	G5	-0.20051	-0.15537	0.188992	0.166304
	G6	0.060968	-0.15383	0.07708	0.119766
		mean	SEM	mean	SEM
GBM	G1	0.090211	0.515517	0.057866	-0.17932
(grade IV)	G2	0.010924	0.097557	0.062514	-0.57457
	G3	0.086132	-0.64214	0.01122	-0.56492
	G4	-0.00013	0.163884	0.001277	0.070949
	G5	0.186128	-0.09321	0.186224	0.367297
	G6	0.087107	-0.16	0.054903	-0.00803
		mean	SEM	mean	SEM
GBM	G1	0.046674	-0.32617	0.049572	0.347764
(grade IV)	G2	0.043758	0.262472	-0.04702	0.03893
	G3	-0.10337	-0.16746	0.088956	0.122716
	G4	-0.16547	0.12696	0.08759	-0.01114
	G5	0.091355	0.145202	0.102549	-0.11102
	G6	109.0644	2.008869		
		mean	SEM	mean	SEM
GBMrecurr	G1	0.017325	-0.84011	0.111042	0.537366
(grade IV)	G2	0.17606	0.184007	0.181325	0.34312
	G3	0.127174	0.240625	0.14176	0.453791
	G4	0.018799	-0.37522	0.070577	-0.09006
	G5	0.089455	0.456672	-0.13163	-0.44659
	G6	-3.9	-277.03	0.815537	0.99635

Table 5.3. Sub-population analysis of cell membrane permeability in the seven samples of human primary glioma grades I-IV using equation 1. Results were calculated as the mean decrease in 2'7'-dichlorofluorescein-associated fluorescence over the time periods 0-200 seconds and 200-400 seconds for each sub-population (gates 1-6) indicated in Figure 5.2. using equation 1. Results are expressed as the mean±SEM. astro=astrocytoma, G=gate.

TIME (s)	ΔFl	GATE 1	GATE 2	GATE 3	GATE 4	GATE 5	GATE 6
0-200	mean	-0.08478	-0.12056	-0.09269	0.036206	0.066896	0.898
	SEM	-0.08487	0.034972	0.04841	0.042704	0.113092	0.866794
	(n)	5	5	5	5	5	4
200-400	mean	-0.06922	-0.11769	-0.09546	-0.09192	-0.09471	-0.26932
	SEM	0.012199	0.043239	0.020792	0.026159	0.052772	0.158265
	(n)						
400-600	mean	-0.03885	-0.09922	-0.0266	-0.04971	-0.03306	-0.03906
	SEM	0.017225	0.023257	0.028358	0.008944	0.054922	0.003592
	(n)	3	4	4	4	4	3

Table 5.4. Rate of probe efflux in 4 explants of human glioblastoma multiforme at 0-200 seconds, 200-400 seconds and 400-600 seconds in the cell sub-populations described by gates 1-6 in Figure 5.2. ΔFl indicates the change in 2'7'-dichlorofluorescein-associated fluorescence in the absence of exogenous essential fatty acid stimulus. Results are expressed as the mean ±SEM.

(b) Morphological and Oxidative Characteristics of Human Glioma Sub-Populations

Gate 1: In each of the tumours analysed, the largest sub-population was represented by region 1. This region lay along the x=y gradient, and was characterised by low to medium forward and side scatter. Smaller sub-populations gated within G1 were did not possess normal fluorescence distributions, indicating the overall heterogeneity of cells within this region. However, it was observed that as individual regions selected within G1 moved further along the x=y gradient away from the origin, basal oxidative activity, essential fatty acid sensitivity and sub-population homogeneity increased.

Gate 2: The cell population defined by G2 was characterised by low forward and side scatter, and occurred slightly below the largest cell population G1. Low values of forward and side scatter are characteristic of small size and low DNA content respectively. Although it is not possible to accurately identify this population, it is possible that cells within this region represent red blood cells, as this would be consistent with the morphological properties described above. Additional evidence supporting the hypothesis that these cells may represent tumour-associated erythrocytes was acquired from flow cytometric analysis of high grade tumours. When the laser scatter profiles of glioblastoma explants possessing macroscopically visible erythrocyte contamination were compared with those of low grade astrocytomas free from ertythrocyte contamination, a cell population was observed in the high grade tumour whose morphological characteristics were similar to those observed in G2: both these populations were characterised by low forward and side scatter, and both had low basal oxidative activity and sensitivity to exogenous essential fatty acid stimulus. Preliminary investigations also suggested that the occurrence of this population increased with tumour grade. This is consistent with the observation that malignant progression is associated with the local release of angiogenic factors which increase vascular proliferation.

Gate 3: The cells in region 3 were characterised by high side scatter and low to medium forward scatter. Again, it is not possible not possible to accurately identify

this population, however, it is possible that this region represents polymorphonuclear leukocytes. These cells are characterised by high granularity, and previous studies indicated that these cells respond to exogenous n-6 essential fatty acids with increased reactive oxygen species formation (Chapter 3).

Additionally, it was demonstrated in Chapter 3 that leukocytes are activated by surgery. Consequently, the relatively high level basal oxidative activity observed in this sub-population may represent lymphocyte activation following glioma resection. These changes may also be due to local inflammatory reactions associated with tumour proliferation. However, further studies are required to confirm the identity of this population.

Gate 4: Gate 4 was characterised by high side scatter which separated it from the largest cell population G1, however, it was more difficult to distinguish this population from G3 and also from the large cells occurring in G5. Cells in G4 were characterised by low basal oxidative activity, low membrane permeability and high sensitivity to exogenous arachidonic acid. At concentrations of arachidonic acid and gamma-linolenic acid greater than 12μM, the stimulation profile of this population tended to follow linear kinetics.

Gate 5: The highest level of basal oxidative activity occurred in the relatively diffuse gate 5, which was characterised by large cells which possessed high forward and side scatter. This population also responded with increased reactive oxygen intermediate production to exogenous n-6 essential fatty acid stimulus. Although the relatively small number of cells in this population precluded further detailed sub-population analysis, there was limited evidence that basal oxidation became lower as side scatter was reduced, and that this was associated with an increase in sensitivity to exogenous arachidonic acid.

Gate 6: The population closest to the origin had the lowest basal oxidative activity and essential fatty acid sensitivity of all the populations studied, and analysis of the

propidium iodide permeability of the cells in this region suggested significant loss of membrane integrity. As the laser scatter profile of macroscopically necrotic tissue demonstrates that a high proportion of the cells analysed in this preparation were located towards the origin, it was concluded that this region is likely to represent non-viable cells and cell debris resulting from mechanical damage during tissue preparation.

(c) Characterisation of the Oxidative Response of the GFAP Positive Population

When ethanol-preserved human glioma preparations were labelled with monoclonal GFAP, the proportion of GFAP-positive cells was typically less than 5% of the total cell population. A normal distribution of GFAP-associated fluorescence was not observed, and the kinetics of reactive oxygen species formation in GFAP-positive cells was characterised by high scatter. In contrast, GFAP positivity was expressed in up to 15% of cells when polyclonal GFAP was used. These differences may have been due to higher affinity of the polyclonal antibody for GFAP epitopes, or the presence of larger numbers of polyclonal GFAP binding sites. For these reasons, polyclonal GFAP staining was used in all subsequent analyses of GFAP positivity and oxidative activity.

The sub-population of cells expressing GFAP in ethanol-fixed cell preparations was characterised by i) the binding of anti-GFAP antibody and ii) high forward and side scatter which characteristic of differentiated cells (Figure 5.5 and Table 5.5). This GFAP-positive cell population was gated, and used to identify a similar region in fresh cells derived from the same tumour. The kinetics of reactive oxygen intermediate generation in this sub-population was analysed in the presence and absence of exogenous n-6 essential fatty acid.

The GFAP-positive cells responded with high sensitivity to exogenous essential fatty acid stimulus, with the mean increase in 2'7'-dichlorofluorescein-associated fluorescence being greater than that of the ungated population (Figures 5.6).

However, the kinetics of this increase in oxidative activity were not described well by linear statistical models. This is a characteristic of heterogeneous cell populations, and it is possible that the GFAP-positive population represents a mixture of untransformed glial cells (possibly reactive astrocytes), and tumour cells with varying GFAP expression. The wide distribution of reactive oxygen intermediate within this sub-population may also be the result of the relatively small population size: approximately 125 cells/sample were present in the GFAP positive population compared with 5 000 cells in the ungated tumour population (Table 5.5).

There was evidence that GFAP expression was a function of tumour grade, however, this association was not linear (Figure 5.7). Highest GFAP positivity was observed in grade III tumours and lowest in grade II tumours. The low expression of GFAP in glioblastoma tumours may be due to loss of glial phenotype resulting from cellular dedifferentiation. Tumours analysed flow cytometrically for GFAP expression were also analysed using immunohistochemistry, and a correlation was observed between the GFAP staining intensity characterised by these two methods. However, numerical analysis of this association was difficult because of cellular heterogeneity and regional variation characteristic of these tumours.

GFAP positivity was also observed in the very large cells occurring in G5, and regions of G3 and G4 with high side scatter and intermediate forward scatter. Morphologically similar cell types were observed on laser scatter profiles of macroscopically normal brain, suggesting that these cells may represent untransformed neurones and glia. Additionally, cells in this region were characterised by lower granularity than those thought to represent malignant tumour, suggesting lower DNA content.

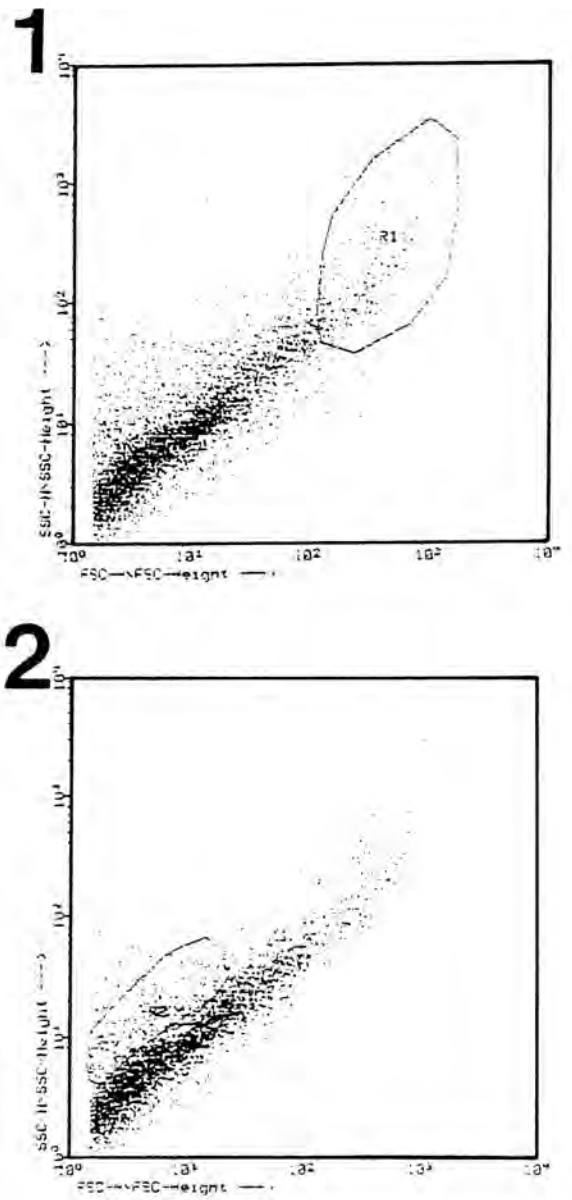


Figure 5.5. Flow cytometric analysis of polyclonal GFAP expression in a collagenase-dispersed explant of human glioblastoma. GFAP positivity is analysed in the ungated population (page 96) and in the populations expressing high (G1) (diagram 1 page 97) and intermediate (G2) (diagram 2 page 98) GFAP/FITC-associated fluorescence. GFAP positivity was analysed using FITC-conjugated polyclonal GFAP at dilutions 1:5, 1:10 and 1:20. Control samples were incubated with the fluorochrome alone.

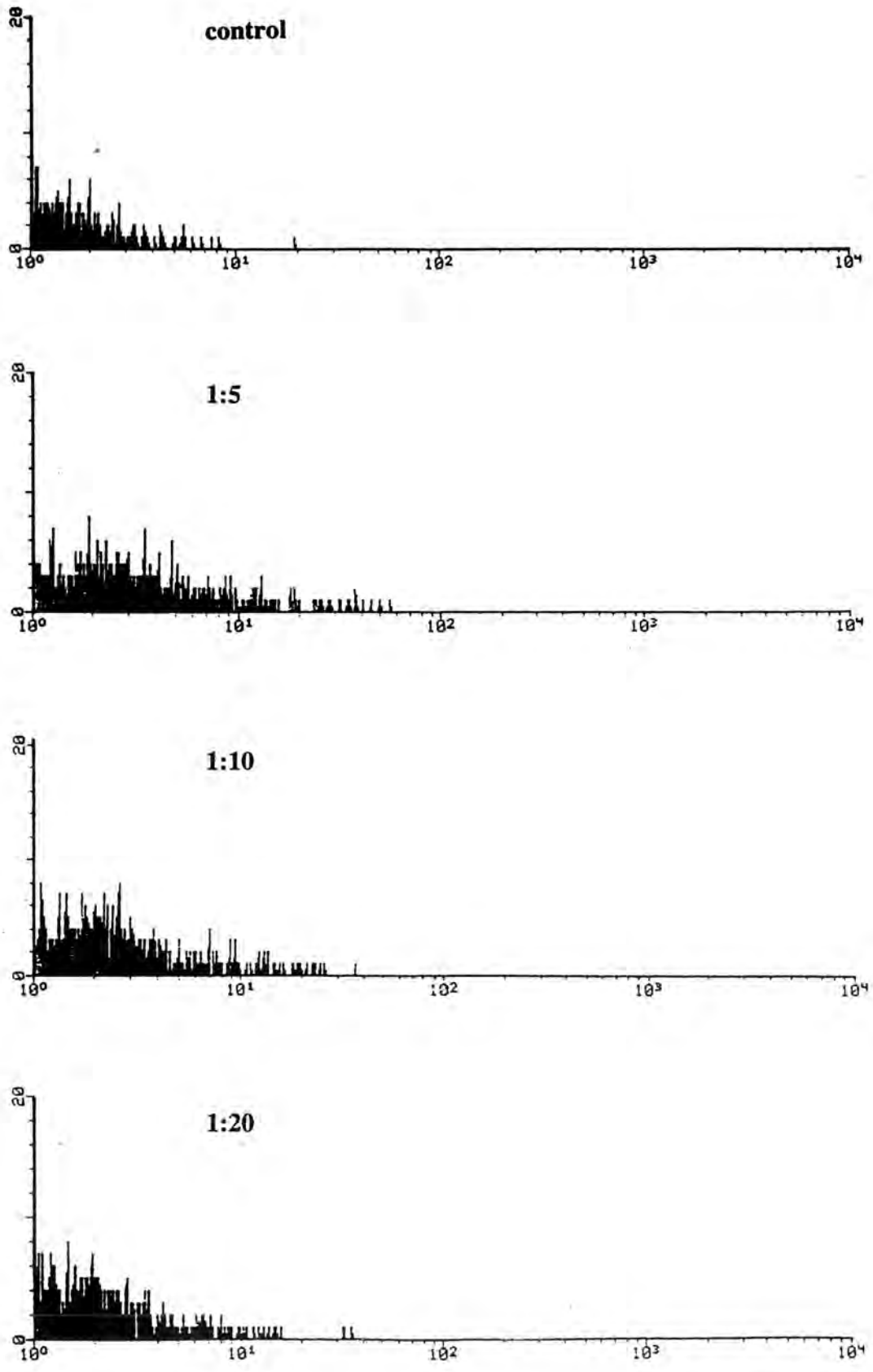


Figure 5.5 continued

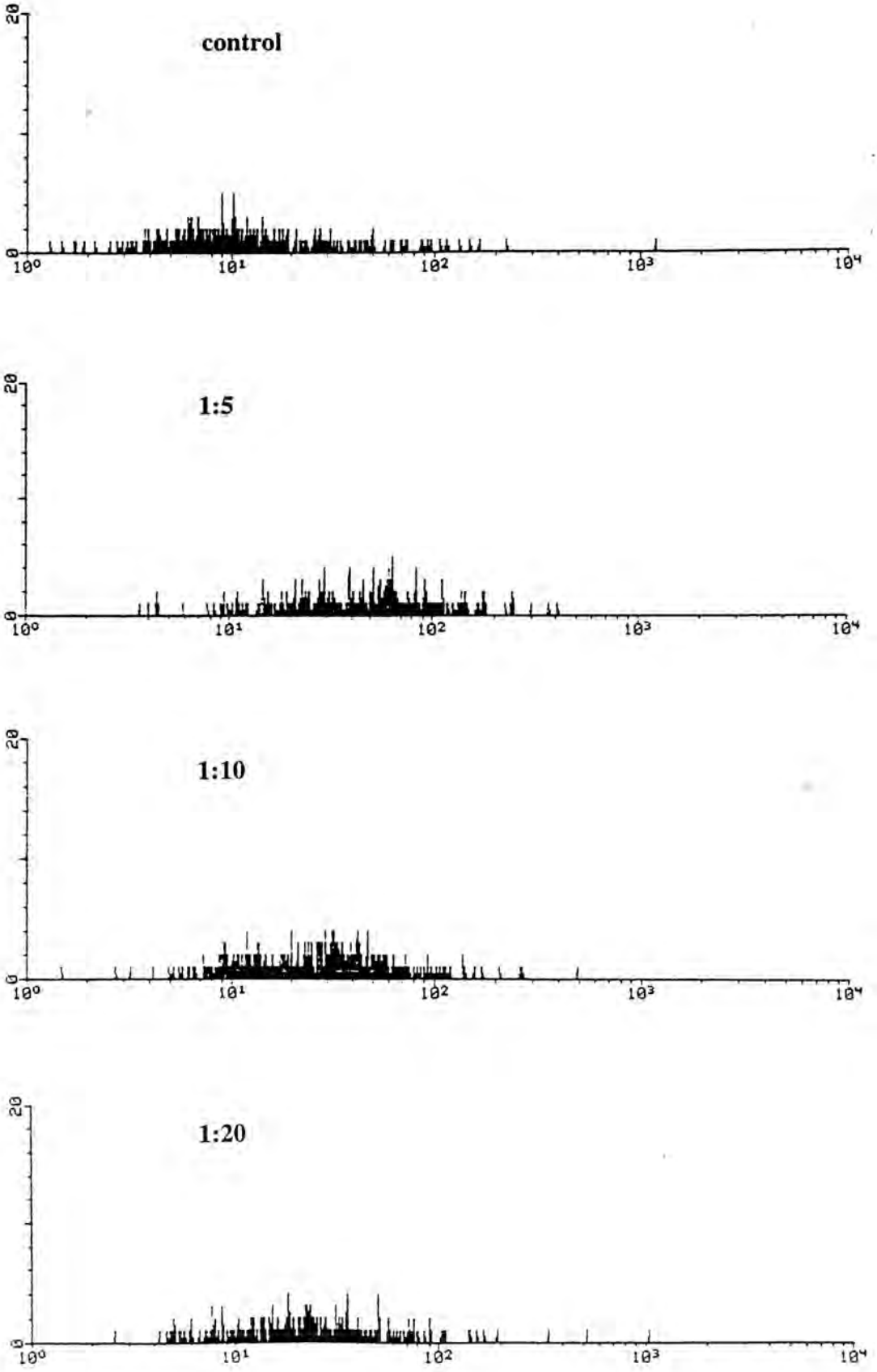


Figure 5.5 continued

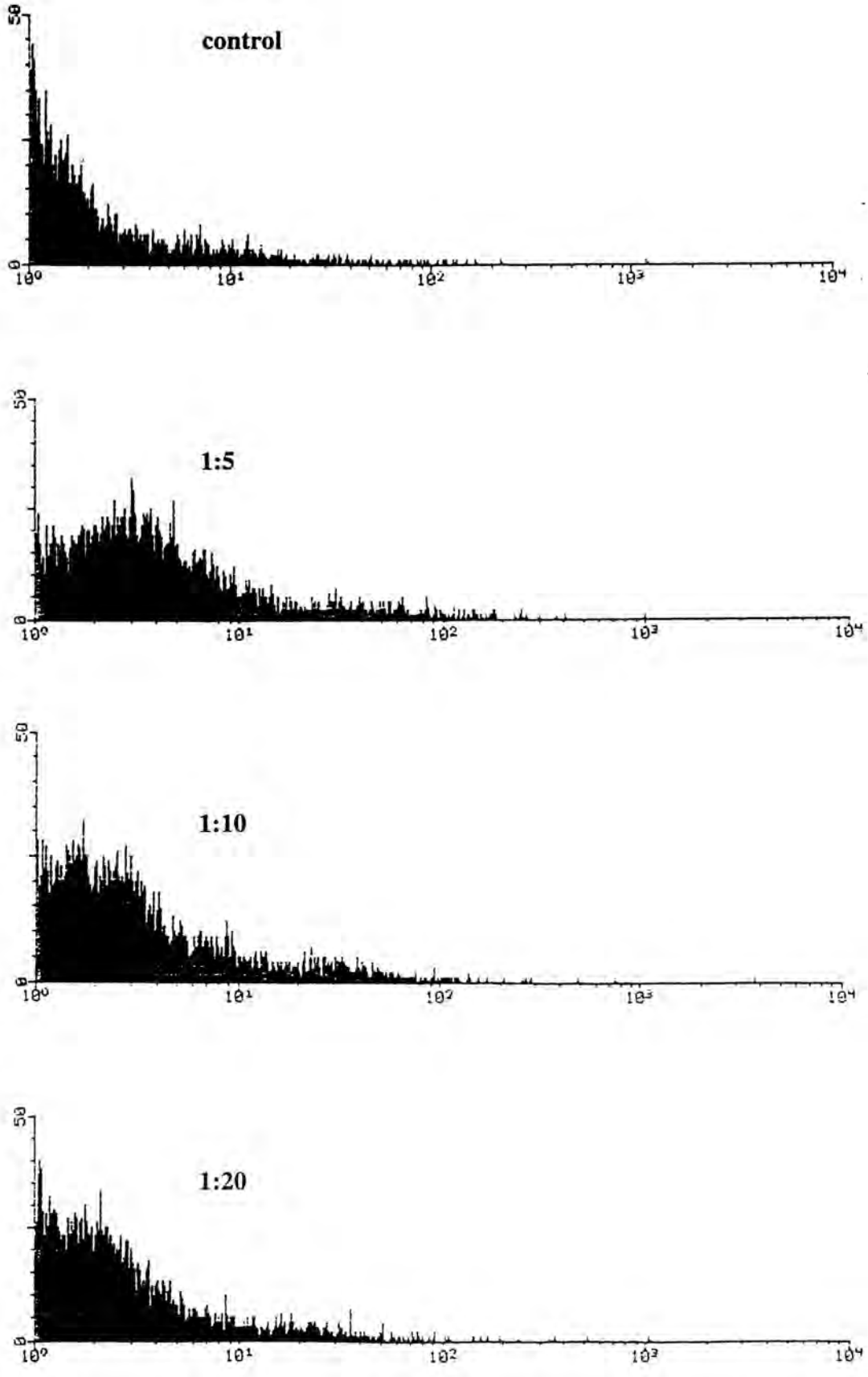


Figure 5.5 continued

DIAGNOSIS	GRADE	REGION	MEDIAN FLUORESCENCE				CELL NUMBER			
			control	1:5	1:10	1:20	control	1:5	1:10	1:20
glioma edge	II	WP	1	1	1	1	5000	5000	5000	5000
		G1	13.97	11.76	10.84	10.09	271	267	289	288
oligoastro.	II	WP	1.3	2.02	1.64	1.58	5000	5000	5000	5000
		G1	34.91	43.32	47.4	34.91	84	63	79	69
		G2	1.44	2.27	1.84	1.73	1206	1354	1130	1255
gem. astro.	III	WP	4.29	4.98	4.18	5.33	5000	5000	5000	5000
		G1	27.76	31.06	26.66	28.13	844	802	735	984
AO	III	WP	1.78	2.48	2.33	2.05	5000	5000	5000	5000
		G1	31.34	39.6	36.03	28.8	147	162	162	152
		G2	1.33	1.81	1.61	1.29	398	456	406	442
oligodendro.	III	WP	1	1.39	1.05	1	5000	5000	5000	5000
		G1	14.59	30.78	19.99	17.78	173	226	239	237
		G2	1	1.58	1.13	1	191	193	170	170
ependymoma	III	WP	1	1.23	1.24	1.01	5000	5000	5000	5000
		G1	9.2	15.26	14.46	11.34	499	431	499	406
		G2	1.01	1.68	1.45	1.2	416	405	378	431
GBM	IV	WP	1	1	1	1	5000	5000	5000	5000
		G1	6.92	11.04	12.75	10.94	127	90	78	88
GBM	IV	WP	1.05	2.29	1.72	1.33	5000	5000	5000	5000
		G1	11.34	23.71	15.96	13.7	440	323	390	317
		G2	1	1.84	1.38	1.13	1514	1629	1545	1698
GBM	IV	WP	1.33	2.39	1.93	1.61	5000	5000	5000	5000
		G1	17.88	27.88	28.13	20.54	557	535	562	526
		G2	1.06	1.72	1.43	1.27	948	744	804	791
GBM	IV	WP	1.03	2.21	1.73	1.49	5000	5000	5000	5000
		G1	3.22	12.41	8.98	8.98	896	738	700	758
		G2	1	1.42	1.28	1.06	332	421	362	393
GBM	IV	WP	1	1.72	1.04	1	5000	5000	5000	5000
		G1	4.22	14.79	7.77	5.88	357	372	393	354
		G2	1	1.35	1	1	745	786	751	711
GBM	IV	WP	1	1	1	1	5000	5000	5000	5000
		G1	3.23	5.73	4.22	3.52	278	303	278	244
GBM	IV	WP	1	2.76	1.95	1.63	5000	5000	5000	5000
		G1	10.27	48.7	28.01	22.07	255	242	320	223
		G2	1	2.23	1.79	1.49	611	692	700	671
GBM	IV	WP	1.37	2.97	2.19	1.7	5000	5000	5000	5000
		G1	12.69	21.67	16.25	13.22	312	297	297	320
		G2	1.1	2.21	1.75	1.41	860	865	774	995
GBMrecurr	IV	WP	1	1	1	1	5000	5000	5000	5000
		G1	7.37	14.2	10.65	8.9	155	202	198	152
GBMrecurr	IV	WP	1.26	1.78	1.72	1.53	5000	5000	5000	5000
		G1	13.22	17.15	15.82	14.33	249	297	297	262
		G2	1.68	2.07	1.89	1.75	272	305	305	304
glioma recurr	IV	WP	1	1.55	1.31	1.12	5000	5000	5000	5000
		G1	8.58	18.77	14.08	14.72	189	188	174	156
		G2	1	1.74	1.36	1.15	677	610	634	640

Table 5.5. Summary of GFAP positivity in populations expressing high (G1) and intermediate (G2) GFAP/FITC-associated fluorescence. 5 000 cells were analysed at each determination of GFAP expression in the ungated tumour population (WP). Oligoastro-oligoastrocytoma, gem. astro.-gemistocytic astrocytoma, AO-anaplastic oligodendroglioma, glioblastoma-glioblastoma, GBMrecurr- recurrent glioblastoma.

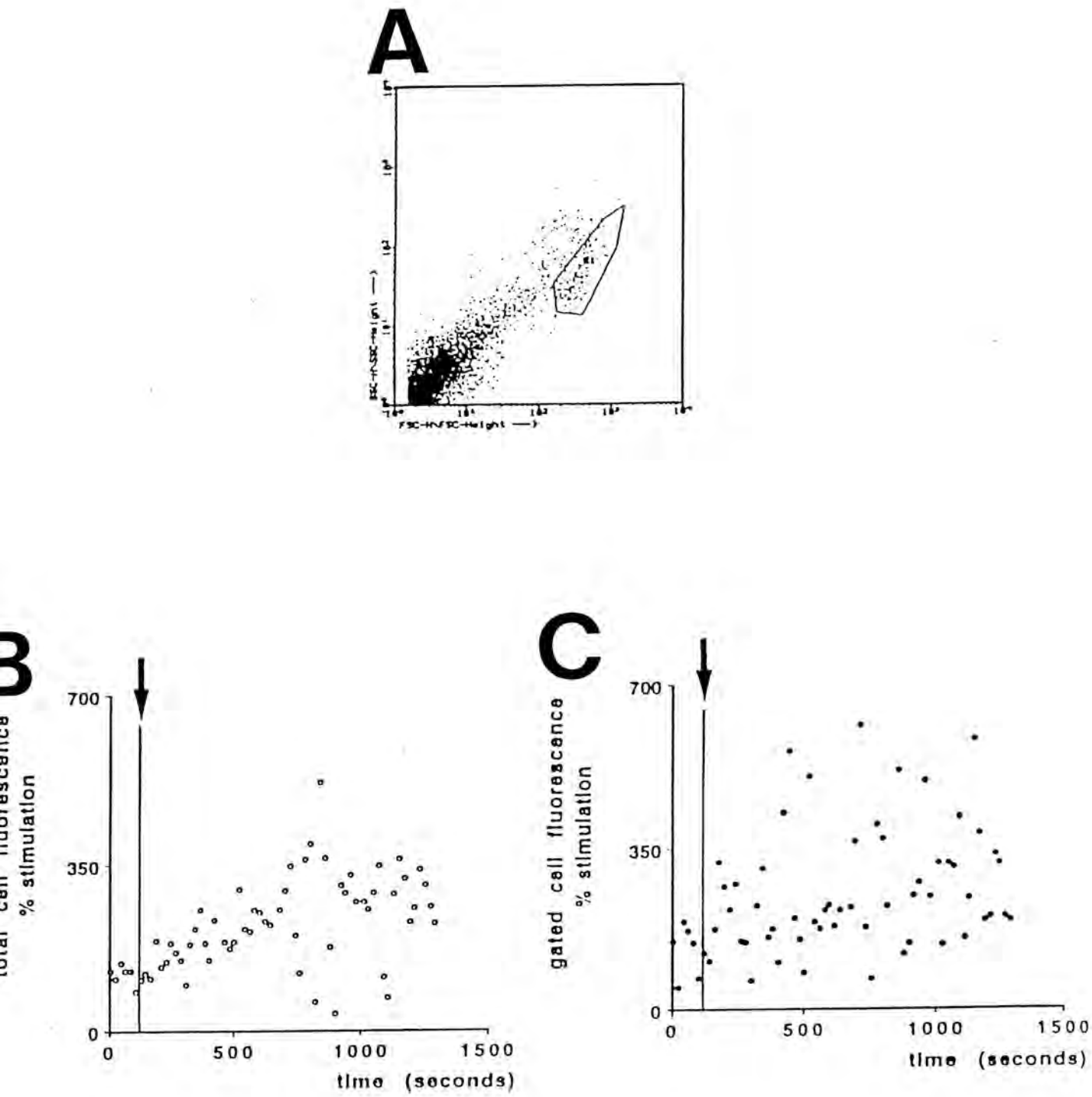


Figure 5.6. The stimulation of reactive oxygen intermediate formation in an explant of collagenase dispersed human ependymoma, WHO grade III, and a GFAP-positive sub-population. Graph A shows the forward (x-axis) (FSC) and side scatter (y-axis) (SSC) cytogram of the whole tumour population and G1 represents a cell population with high GFAP expression. The mean stimulation ratio of reactive oxygen intermediate formation by 27μM gamma-linolenic acid (added at 120 seconds) is shown in graph B for the whole tumour population and graph C for the GFAP-positive population.

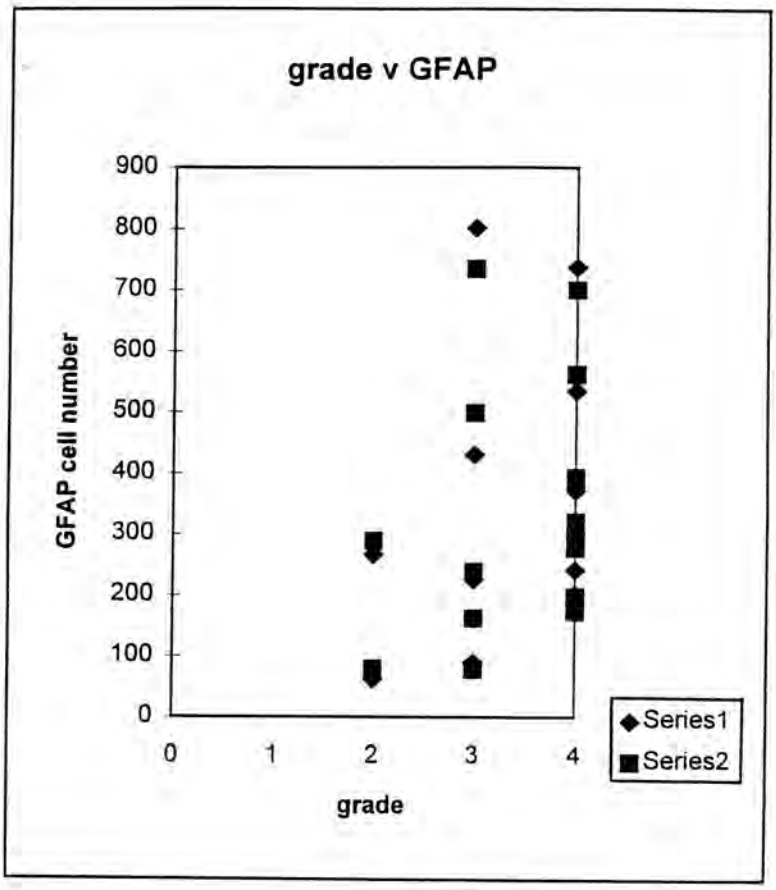


Figure 5.7. Flow cytometric comparison of the cell numbers exhibiting high GFAP/FITC-associated fluorescence (as indicated by gate 1 in Figure 5.5) in collagenase dispersed explants of human primary gliomas grades I-IV. Cells were incubated with polyclonal GFAP at dilutions 1:5 (black diamonds) and 1:10 (black squares) and analysed on a Becton Dickinson FASCsan flow cytometer. 5 000 cells were analysed at each determination of GFAP positivity.

5.4 Discussion

The characterisation of total glioma reactive oxygen intermediate generation in the presence and absence of exogenous arachidonic acid and gamma-linolenic acid was described in Chapter 4. However, the oxidative activity of the tumour cell preparations observed *in vitro* were those of mixed cell populations containing differing proportions of transformed glial tissue at varying stages dedifferentiation, erythrocytes, leukocytes, normal brain and vascular endothelium. The purpose of this study was to carry out preliminary differentiation between various cell types present in human glioma explants, and to characterise them in terms of basal oxidative activity, essential fatty acid sensitivity and cell membrane permeability.

Preliminary investigations confirmed the heterogeneity of the human glioma preparations analysed for oxidative activity. The variability of cellular composition generally increased with tumour grade, and this observation correlates with the occurrence of known diagnostic indicators (Darling, 1990, Thomas and Graham, 1995, Chapter 1, Berens and Giese, 1999 and Turazzi and Licata, 2000). Rapid cell division results in high metabolic demand, which may induce necrotic cell death in areas of the tumour exposed to unfavourable diffusion gradients for oxygen and nutrients. Consequently, clonal selection may result in the emergence of hypoxia resistant, highly malignant cells, facilitating more aggressive tumour growth (Graeber et al, 1996 and Louis, 1997). In addition, there is evidence that hypoxia is associated with the release of angiogenic factors, in particular vascular endothelial growth factor (VEGF), which results in endothelial proliferation (Louis, 1997).

Unlike the flow cytometric analysis of lysed whole blood, where lymphocytes were distributed in distinct populations of known size and granularity (Chapter 3, Figure 3.1), human glioma cell populations were less discrete. Overlap occurred between the sub-populations representing the various cell populations present in human glioma, and individual cell types could not be distinguished easily. Despite these difficulties however, the regions described by gates 2, 3, 5 and, to a lesser extent 4, were characterised by normal distributions of fluorescence intensity. This suggested

that homogeneous cell populations occurred within these regions. In comparison, the cells which possessed intermediate values of forward and side scatter in gate 1 could not be completely resolved due to population heterogeneity.

Different rates of basal reactive oxygen species generation were observed in each of the cell populations investigated, and these differences were consistent with the hypothesis that reactive oxygen intermediate generation is low and essential fatty acid sensitivity is high in tumour tissue. Analysis of the laser scatter profiles of macroscopically normal brain indicated that the cells present in G5 were most likely to represent non-malignant brain tissue, while the cells present in G4 were thought to represent malignant glioma cells. This was due to their GFAP-positivity and high side scatter, which is indicative of elevated DNA content. In comparison with the cells present in the G5 region, G4 cells possessed lower basal oxidative activity. However, both G4 and G5 cells responded with increased formation of reactive oxygen intermediates following the addition of exogenous n-6 essential fatty acids.

The morphological properties of other cell populations were investigated, and preliminary conclusions were made concerning their possible identity. The laser scatter profiles of i) a glioblastoma tumour with macroscopically visible erythrocyte contamination, ii) tumour associated necrotic tissue and iii) peripheral leukocyte preparations assisted in the identification of red blood cells, non-viable cells and reactive immune cells. It was concluded that these populations may be represented by G2, G6 and G3 respectively. The high level of basal oxidative activity observed in G3 may represent lymphocyte activation through surgery or local inflammatory changes associated with glioma pathogenesis, as similar observations were made in lymphocyte preparations acquired from patients undergoing pulmonary resection (Chapter 3). However, additional studies are required to confirm the identity of these and other tumour-associated cell populations.

5.5 Conclusions

This study has confirmed that the human glioma explants analysed for oxidative activity are characterised by cellular heterogeneity. Preliminary analysis indicated that some of the glioma cell populations indicated by flow cytometric analysis may represent red blood cells, tumour-associated lymphocytes and necrotic tissue, as the morphological and oxidative characteristics of these populations correlated with those acquired from analysis of known tissue samples.

This study also identified a group of cells which may represent malignant glioma. These cells were characterised by high side scatter, which suggests abnormal DNA content, and GFAP positivity, which is indicative of a glial phenotype. These cells were present in regions G4, G5 and, to a lesser extent G3. The cells in these regions responded with increased reactive oxygen species generation following stimulation by exogenous essential fatty acids, suggesting that at least one GFAP-positive sub-population is characterised by essential fatty acid sensitivity. Additional studies are required to confirm the identity of this and other reactive cell populations, and to examine the biological significance of reactive oxygen species generation in tumour-associated cells.

Chapter 6:

The Stimulation of Apoptotic Activity in Human Glioma and the Rat C6 Glioma Cell Line by Arachidonic Acid and Gamma-Linolenic Acid

6.1 Introduction

The purpose of this study was to provide evidence supporting the hypothesis that the stimulation of reactive oxygen species formation in human glioma tissue (which was demonstrated in Chapter 4) has biological significance. This was undertaken by analysing human glioma cell death following administration of arachidonic acid and gamma-linolenic acid at concentrations previously shown to stimulate glioma oxidative activity. Specifically, this investigation i) characterised the pro-apoptotic activities of arachidonic acid and gamma-linolenic acid in collagenase-dispersed human glioma tissue, ii) examined the kinetics of this pro-apoptotic activity, iii) investigated differences in sensitivity to the pro-apoptotic activities of n-6 essential fatty acids between tumours of different grades, and tumour-associated normal brain where available and iv) correlated endogenous endonuclease activity with cell membrane permeability.

The stimulation of apoptotic activity by arachidonic acid and gamma-linolenic acid in human glioma tissue was undertaken to provide further information on the poorly characterised mechanisms underlying essential fatty acid-mediated potentiation of glioma cell death. The study of tumour cell death is of clinical importance because although traditional cancer therapy was developed largely on attempts to limit the rate of cell proliferation, more recent evidence suggests that tumours may also have deficits in the control of cell death (Graeber et al, 1996, Malcomson et al, 1996 and Novelli et al, 1996). In particular, recent interest has been focused on the study of apoptotic cell death which is an energy requiring genetically determined process which is under the control of both environmental and autocrine regulators (Wyllie et al, 1980, Arends et al, 1990 and Bellamy et al, 1995). It has characteristic features

such as chromatin condensation and DNA cleavage which differentiate it from necrosis. The final stage of apoptosis involves segmenting cellular fragments into membrane bound apoptotic bodies which are readily engulfed by phagocytes without associated tissue inflammation (Wyllie et al, 1980 and Arends et al, 1990). Apoptosis is therefore biochemically and morphologically distinct from a pathological form of cell death termed necrosis, which may result from inflamatory or ischaemic tissue insults.

Treatments which increase apoptosis may represent a novel approach to the management of malignant glioma, and there is evidence that tumour cytotoxicity induced by essential fatty acids may be attributable to stimulation of this mode of cell death. Eicosaepentaenoic acid induced cell cycle arrest and apoptosis in pancreatic cancer cells *in vitro* (Lai et al, 1996), arachidonic acid induced a concentration and time dependant toxicity to HepG2-MV2E1-9 cells (Chen et al, 1998), arachidonic acid, gamma-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid induced apoptosis in Hep2 human larynx tumour cells (Colquhoun et al, 1998) and arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid stimulated apoptosis in various leukaemia cell lines (Finstad et al, 1998).

In vivo studies have also indicated that essential fatty acids stimulate apoptosis. Oils rich in n-3 and n-6 essential fatty acids inhibited the formation of skin papilloma induced by croton oil (Ramesh and Das, 1996 and Ramesh and Das, 1998a), hepatoma induced by diethylnitrosamine (Ramesh and Das, 1995) and ascitic tumour growth (Ramesh and Das, 1998b) and eicosapentaenoic acid and docosahexaenoic acid inhibited the growth of Morris hepatocarcinoma transplanted into ACI/T rats (Calviello et al, 1998). Additionally, immunohistochemical analysis of a series of high grade malignant gliomas suggested that apoptotic cells surround the tumour central necrotic cores (Iwaki et al, 1994). This is consistent with the stimulation of apoptosis by locally released inflammatory mediators including arachidonic acid (Iwaki et al, 1994).

Although there is evidence that essential fatty acids are associated with the stimulation of apoptotic cell death, the kinetics essential fatty acid-mediated stimulation of apoptosis are difficult to interpret. This may be due to inter-experimental variations in cellular susceptibility to pro-apoptotic stimuli. There is evidence that cells exist in at least two different states with regard to their predisposition to apoptosis (Bellamy et al, 1995). In the primed state, cells are endowed with the necessary effector proteins to enter into apoptosis if appropriately triggered. In the unprimed state, the effector mechanisms associated with apoptosis need to be synthesised before apoptosis can proceed. This susceptibility depends upon environmental considerations such as local availability of growth and survival factors, and also the activity of tumour suppressor genes and oncogenes, both of which can be upregulated prior to the initiation of programmed cell death (Collins et al, 1994). As this study was undertaken on fresh human glioma tissue obtained at biopsy, it was predicted that the phenotype of these cells, and hence inherent sensitivity to pro-apoptotic stimuli, would be more representative of malignant glioma occurring *in situ* than established cell lines and experimentally induced tumours.

During these investigations, human glioma cell apoptosis was determined in the presence and absence of 30μM arachidonic acid at 0, 15, 30 and 45 minutes, and then at hourly intervals for up to 36 hours. This concentration was selected as it is known to stimulate tumour cell peroxidation, but is not associated with changes in plasma membrane permeability. The sampling frequency of this study was designed primarily to analyse apoptotic activity over time periods when increased reactive oxygen species formation had been detected in human glioma preparations (Williams et al, 1997) (Chapter 4).

Apoptotic activity was analysed using TUNEL flow cytometry in collagenase-dispersed tumour cells. Stimulation of endogenous endonuclease was investigated following the addition of exogenous arachidonic acid and gamma-linolenic acid, and differences in the rate and extent of tumour cell apoptosis were determined in

gliomas of various grades and tumour-associated normal brain where available. Flow cytometry was also used to investigate basal apoptosis in high and low grade glioma, and results were compared with histological features of programmed cell death observed in paraffin-fixed sections of the same tumour. The effect of arachidonic acid and gamma-linolenic acid on glioma cell necrosis was determined by analysing membrane permeability to vital dyes.

Radiation-induced programmed cell death in the rat C6 cell line was used to establish experimental conditions, and to characterise the response of a single cell type undergoing early, intermediate and late apoptosis. C6 cells were also used to investigate the previously uncharacterised effect of gamma-linolenic acid on apoptotic activity in long term culture, and to determine the effect of gamma-linolenic acid on glioma cell proliferation.

6.2 Materials and Methods

(a) Standardisation of the Apoptotic Response using Radiation

Confluent C6 cells were harvested using trypsin digestion (Section 2.2c), resuspended at a density of 10^6 cells/ml in Hams F-10 medium containing 10% FCS and irradiated at 2Gy (Section 2.9b). At intervals of 0 and15 minutes and 6, 8 and 9 hours post-irradiation, 1ml aliquots were fixed in 100% ethanol.

Ethanol preserved glioma cells were used for TUNEL analysis of apoptotic activity. The cells were washed with phosphate buffered saline, incubated for 10 minutes at room temperature with cacodylate buffer and centrifuged and incubated with reaction buffer containing terminal deoxynucleotide transferase and fluorescein-12-2'-dUTP for 2 hours. The cells were resuspended in phosphate buffered saline for 15 minutes and analysed by flow cytometery. This identified apoptotic cells on the basis of fluorescein-12-dUTP-associated fluorescence (Section 2.7).

(b) Analysis of Apoptotic Activity in Human Glioma Tissue

(i) Cell preparation

Single cell suspensions of fresh human glioma tissue and, if available, tumour-associated normal brain were obtained using collagenase digestion (Section 2.2b). Cell suspensions were washed, resuspended at $1x10^5$ cells/ml in collagenase free Hams F-10 medium and incubated in the presence or absence of 30μM arachidonic acid or gamma-linolenic acid. At 0, 15, 30 and 45 minutes, and hourly intervals for periods of up to 36 hours, 1ml aliquots were fixed in 100% ethanol for TUNEL analysis of apoptotic activity (Section 2.7).

(ii) Analysis of Tumour Cell Viability

Tumour cell viability was measured immediately after ethanol fixation.100μl aliquots of collagenase-dispersed cells were incubated with 50μl trypan blue for 2 minutes at room temperature. Stained nuclei were counted in a microscopic field containing 100-200 cells at magnification x40, and expressed as a percentage of viable non-staining cells (Section 2.6).

(iii) Pathology

The pathology of each tumour analysed for apoptotic activity was determined using standard histopathological techniques (Section 2.10).

(c) Analysis of Apoptotic Activity in the Rat C6 Glioma Cell Line

Pre-confluent C6 cells were incubated with Hams F-10 medium containing 10% FCS and gamma-linolenic acid at concentrations 0μM, 1μM, 5μM, 10μM, 20μM and 40μM. Medium bathing the cells was harvested and centrifuged every 6 hours, and cell pellets were stored in 100% ethanol for TUNEL analysis of apoptotic activity. After 7 days, adherent C6 cells were harvested using trypsin digestion, and cell viability was determined by measuring propidium iodide uptake. Remaining adherent cells were stored in 100% ethanol for TUNEL analysis (Sections 2.7).

(d) Analysis of Proliferative Activity in the Rat C6 Glioma Cell Line

Proliferative activity was determined in the rat C6 glioma cell line in the presence and absence of exogenous gamma-linolenic acid using the MTT assay as a measure of the activity of intracellular dehydrogenases (Section 2.8).

6.3 Results

(a) Flow cytometric characterisation of apoptosis in the rat C6 glioma cell line

Flow cytometric analysis of irradiated cells indicated the presence of a group of cells which were distinct from those constituting the highest proportion of the total population. These cells were characterised by high dUTP/FITC-associated fluorescence, which indicated high endogenous endonuclease activity and hence the occurrence of apoptosis. In the absence of radiation, 0.038% of cells assayed for apoptotic activity exhibited high dUTP-associated fluorescence (Figure 6.1), and this increased to 0.14% at 15 minutes post-irradiation. After 6 hours a large increase in endogenous endonuclease activity was observed, and this was associated with the emergence of a population which had not been identified previously. In comparison to the population previously associated with stimulation of apoptotic activity, cells present in this new region were characterised by lower side scatter and lower dUTP-associated-fluorescence. These morphological changes are consistent with small cell size and increased membrane permeability. This is suggestive of the development of apoptotic bodies which are formed during the latter phases of apoptosis. At 8 hours the formation of these apoptotic bodies had increased by approximately 50%, although there was continued evidence of TUNEL positivity in the population thought to represent cells undergoing early apoptotic events. At 9 hours both early and late apoptotic populations were indistinguishable from the non-apoptotic population.

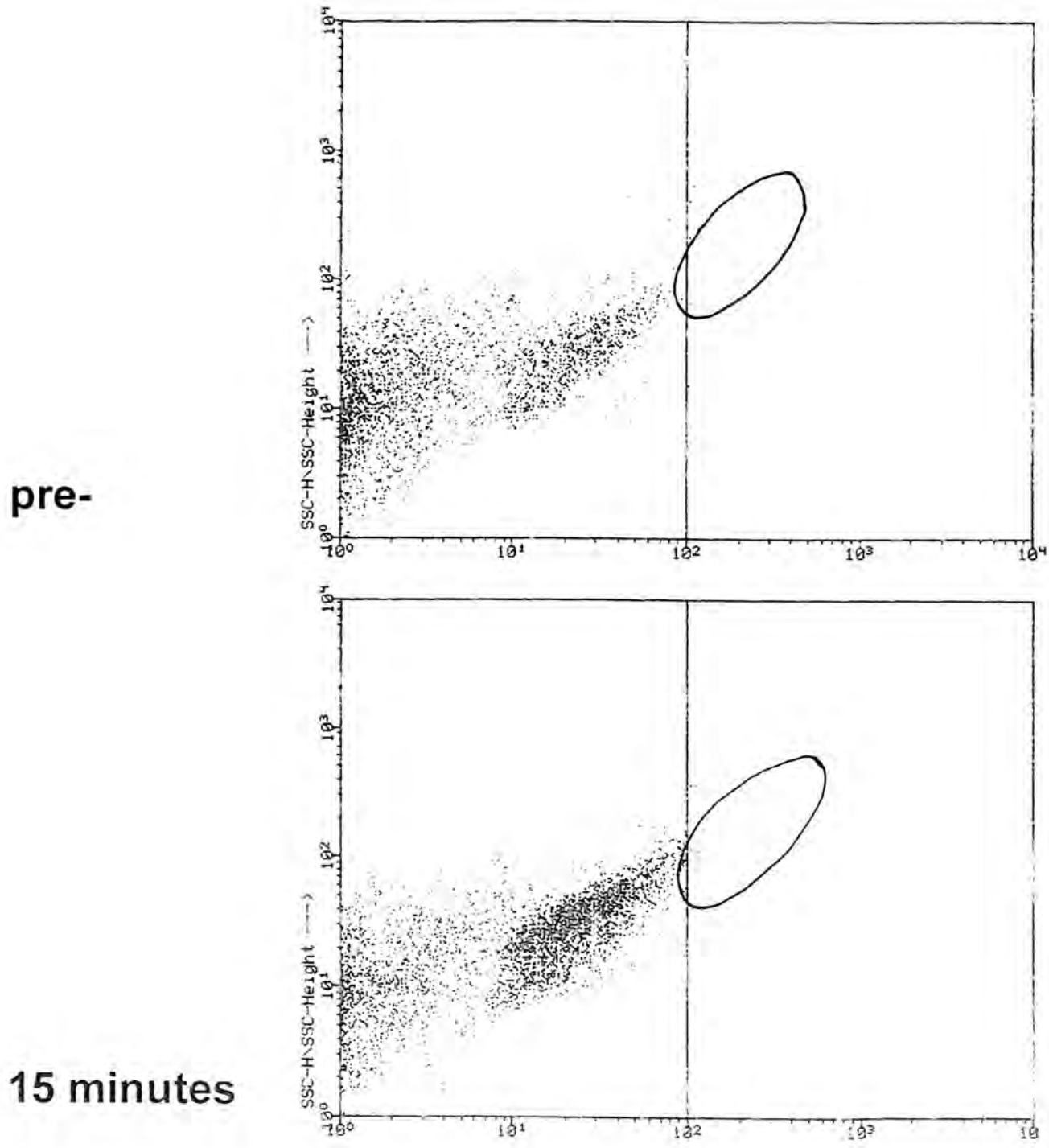
As a result of their high fluorescence intensity, cells undergoing early and intermediate apoptotic events could be defined by a single gate termed Fl-1. This method was used in preference to gating individual sub-populations because cells

undergoing apoptosis exhibited changes in morphology and fluorescence intensity, and consequently passed rapidly through smaller fixed gates. However, if these bit maps were reset during the course of an experiment, it would not have been possible to compare individual time points on a quantitative basis.

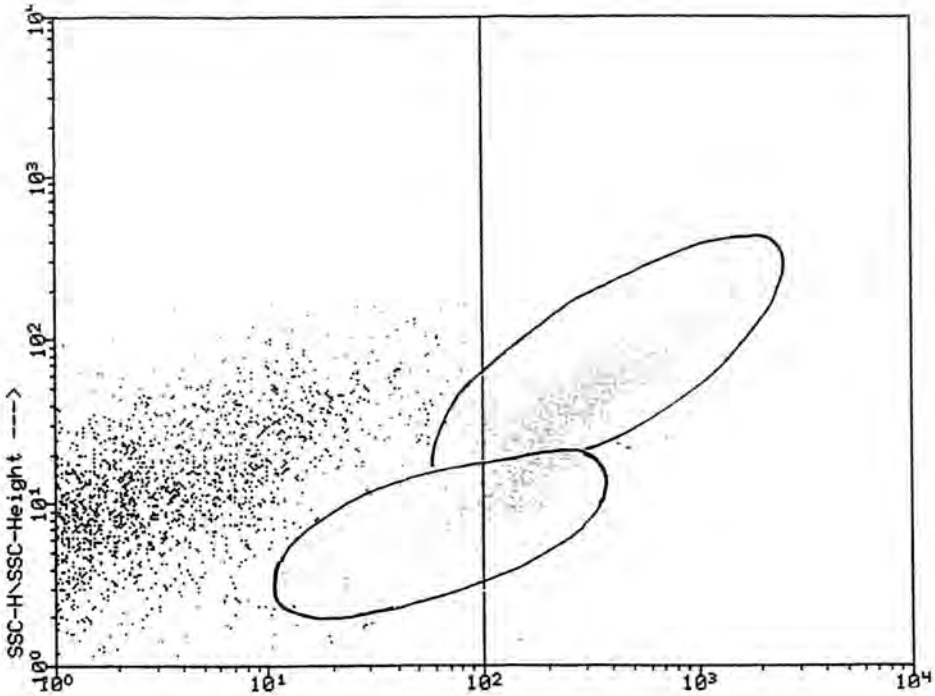
The Fl-1 gate was used to estimate apoptotic index, which is defined as the ratio of cells present in the Fl-1 gate to the number of cells in the whole population. The single gate gave lower estimates of arachidonic acid induced stimulation of apoptosis than more restricted bit maps defining individual sub-populations. This was because sub-populations in late apoptosis with intermediate to low dUTP-associated fluorescence were not included in the Fl-1 gate.

Having established the apoptotic activity of C6 cells in response to radiation, the single Fl-1 gate was used to analyse apoptotic activity in human glioma cells in the presence and absence of arachidonic acid and gamma-linolenic acid. Patterns of patterns of dUTP/FITC associated-fluorescence were similar to those observed in Figure 6.1 (Figure 6.2 and 6.3). However, in two human tumour samples (one oligoastrocytoma and one glioblastoma multiforme), it was necessary to use a horizontal gate to differentiate between populations of differing side scatters in addition to the vertical Fl-1 gate. This was because changes in endonuclease activity were detected predominantly in the small cell fraction of these tumours. Another glioblastoma multiforme showed evidence of a small cell population which was incompletely gated by the single Fl-1 gate, and was therefore underestimated.

Figure 6.1. The stimulation of apoptosis in the rat C6 glioma cell line by 2Gy irradiation. Cells were selected for TUNEL positivity using a single high fluorescence gate (Fl-1). The early apoptotic population is shown in red and the late apoptotic population in green.



hours



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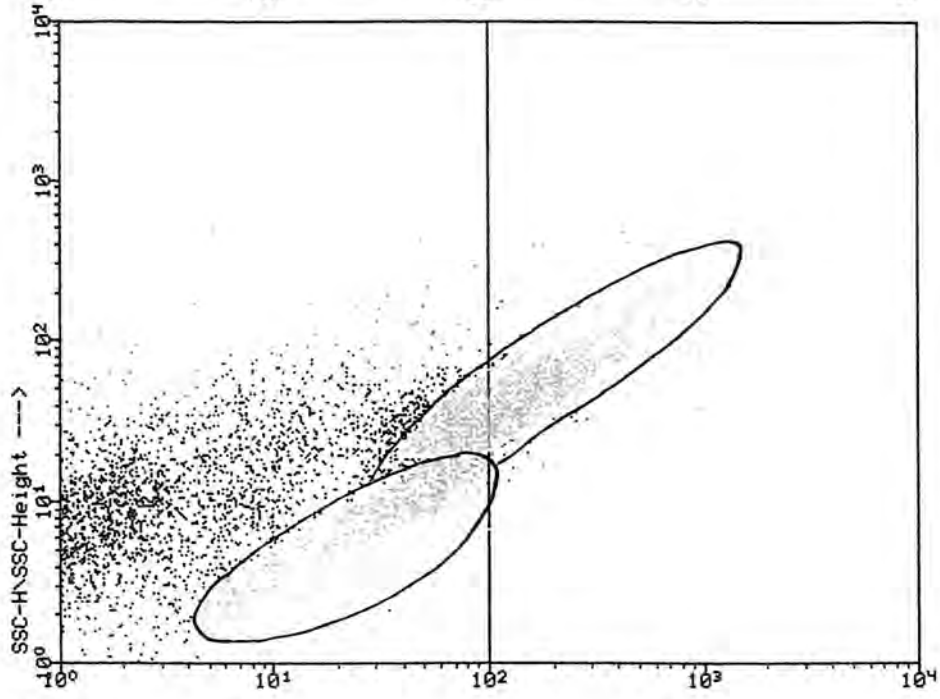

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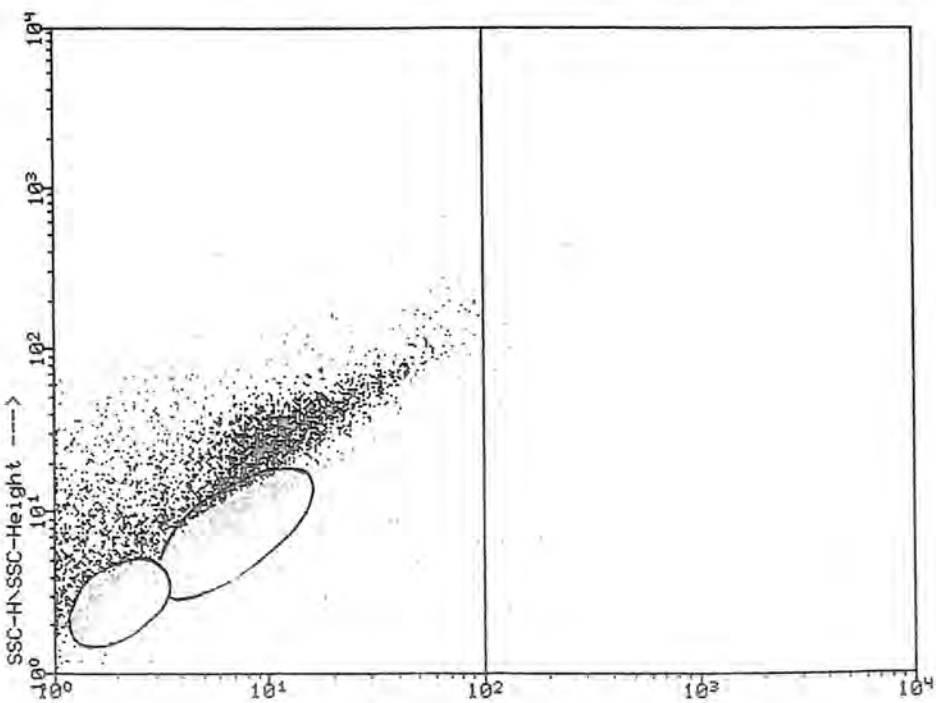

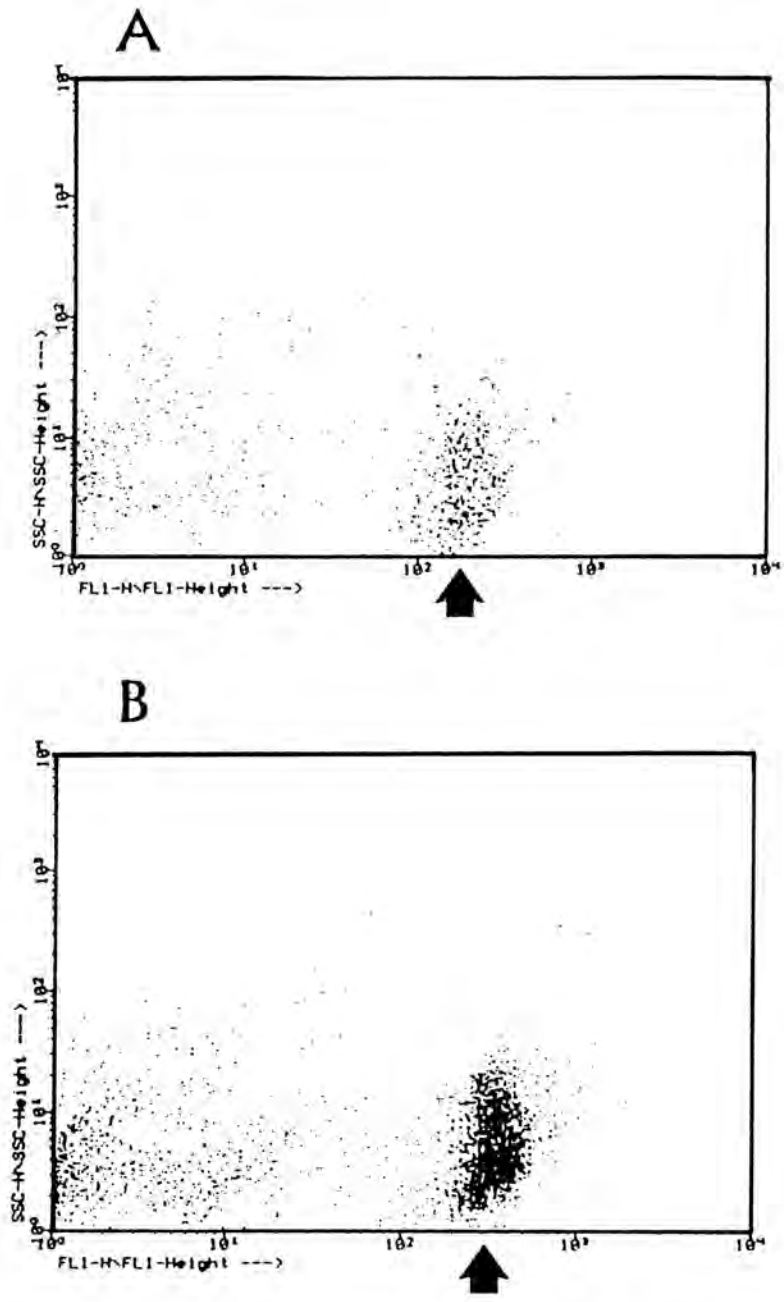

Figure 6.2. Apoptosis in cell preparations acquired from explants of human glioma using TUNEL flow cytometry. Collagenase dispersed samples of anaplastic oligodendroglioma incubated in the presence (B) or absence (A) of 30μM arachidonic acid for 15 minutes were labelled with FITC conjugated dUTP. This identified apoptotic cells (indicated by black arrow) on the basis of FITC associated fluorescence (FL-1), x axis and side scatter, y axis on a Becton Dickinson FACScan flow cytometer using LYSIS software. In each sample 5 000-10 000 cells were analysed and cells with FL-1>80 were gated. The proportion of TUNEL-positive cells in was 20.53% and 6.57% in the presence and absence of arachidonic acid respectively. The basal apoptotic rate of cells derived from this tumour is shown in Table 6.1.

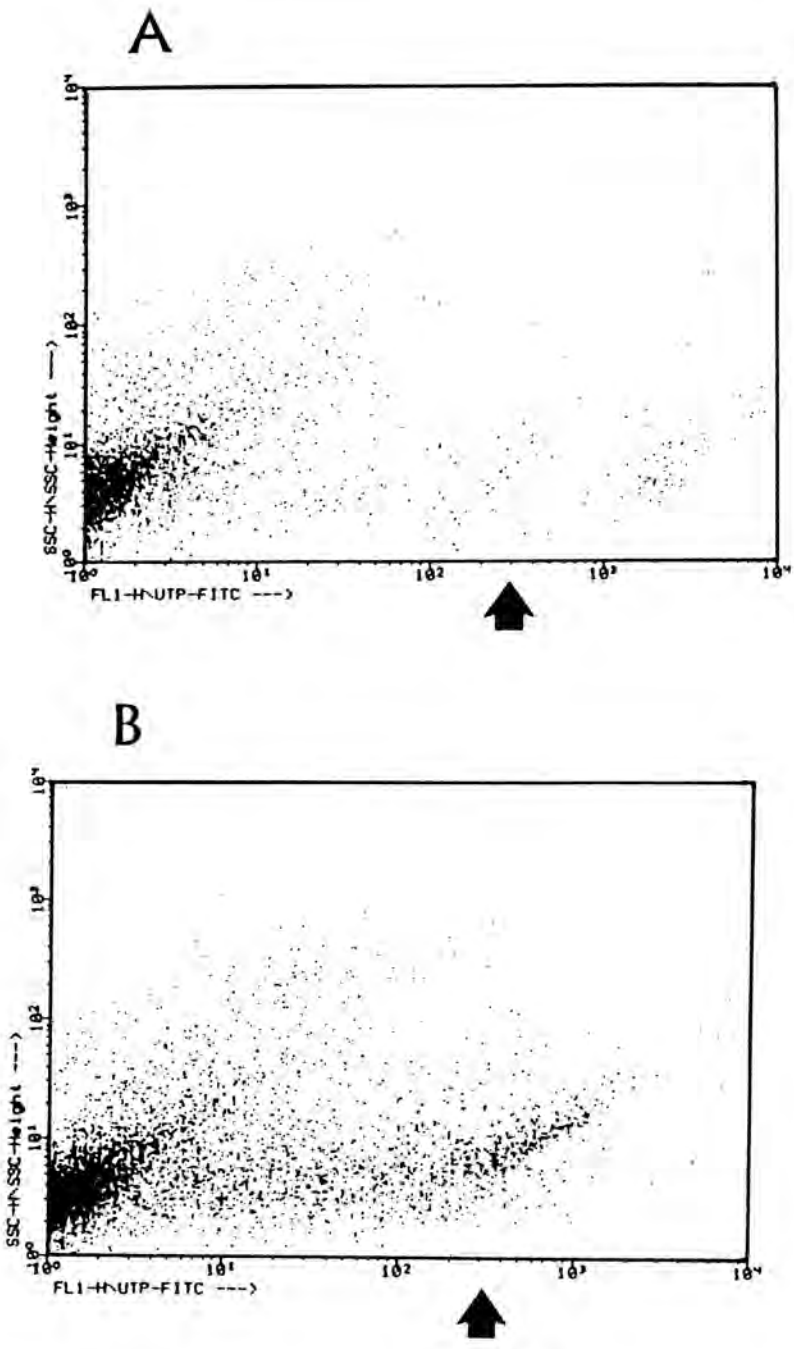

Figure 6.3. The effect of arachidonic acid on apoptosis in tumour cells prepared from a sample of cerebellar oligoastrocytoma. Isolated cells were incubated in the presence (B) or absence (A) of 30μM arachidonic acid for 15 minutes. Apoptotic activity was assessed using TUNEL flow cytometry on a Becton Dickinson FACSscan flow cytometer using LYSIS software. In each sample 10 000 cells were analysed and cells with FL-1>80 were gated. The proportion of TUNEL-positive cells was 0.76% and 6.62% in the absence and presence of arachidonic acid respectively. The basal apoptotic rate of cells derived from this tumour is shown in Table 6.1.

(b) Analysis of Basal Apoptosis in Human Glioma Cells

The basal apoptotic index of the human tumour preparations analysed was determined at each time point. Mean apoptotic index was 3.04±0.81% over the initial incubation period of 0-2 hours, and 1.64%±0.45% over the later period of 3-24 hours (Table 6.1). Although basal apoptosis varied with time and tumour type, a significant proportion of cells acquired from human tumours had a basal apoptotic index of less than 1%. This supports the finding that glial tumours have lower rates of endogenous apoptotic activity than normal brain tissue (Schiffer et al, 1995 and Kordek et al, 1996). However, a relatively high mean apoptotic rate (5.2±3.1%) was observed in one oligodendroglial tumour. Morphological features of apoptosis were also observed in a paraffin-fixed section of this tumour stained with haematoxylin and eosin.

Similarities in the basal rate of apoptosis within certain classes of tumours were noted (Figure 6.4). Early (0-2 hour) peaks of apoptotic activity followed by later peaks between 9 and 12 hours were detected in three glioblastoma multiforme tumours (Figure 6.4). However, individual variations were detected in the amplitude and duration of these periodic increases in apoptotic activity. In addition, differences between individual tumours of the same class occurred with respect to the timing and duration of these changes. As a result of this observation, the basal rate of apoptosis at each incubation time was used to determine the apoptotic index, rather than the initial or final rate of apoptosis.

grade	diagnosis	apoptosis %	(n)
II	oligoastrocytoma	0.694±0.097	(5)
		0.774±0.108	(16)
II	fibrillary astrocytoma	1.09±0.225	(10)
III	oligodendroglioma	5.2±3.1	(2)
III	anplastic oligoastrocytoma	3.08±0.777	(5)
III	anaplastic	2.19±0.176	(11)
III	astrocytoma	1.08±0.478	(14)
III	anaplastic	0.644±0.071	(18)
	oligodendroglioma	0.556±0.136	(16)
		0.308±0.047	(16)
		0.206±0.097	(14)
		2.13±0.644	(16)
IV	glioblastoma	1.32±0.300	(13)
	multiforme	1.36±0.294	(16)
		0.269±0.025	(14)
		0.23±0.050	(7)

Table 6.1. basal apoptosis in 16 human gliomas analysed at 0-12 hours. TUNEL-positive cells are indicated as a percentage total of cells analysed ±SEM of (n) 2 000-10 000 determinations from each tumour. Cells were selected for high endonuclease activity using a single high fluorescence (Fl-1) gate. This tends to underestimate cells in early apoptosis (moderate Fl-1, high side scatter), and also in late apoptosis (low to moderate Fl-1, low side scatter).

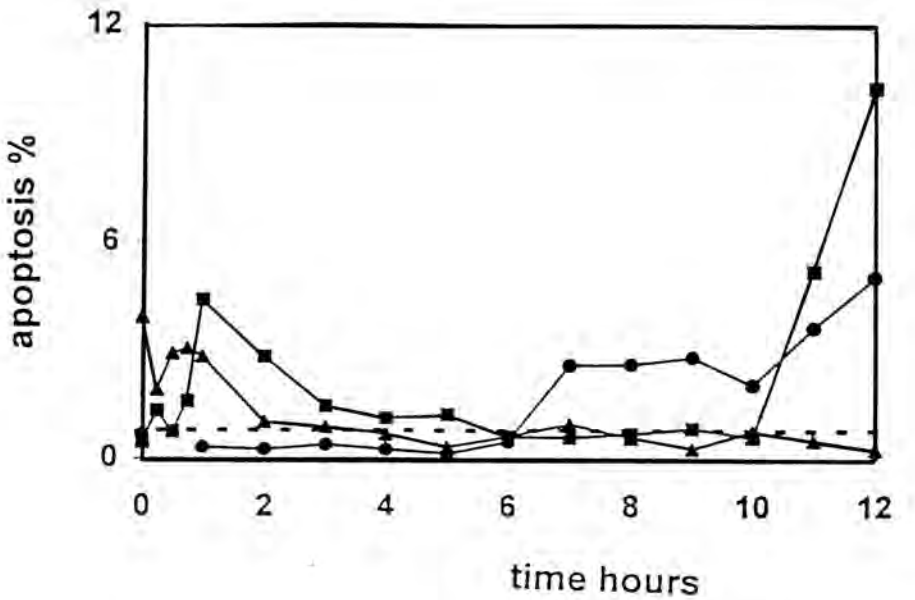

Figure 6.4. The basal rate of tumour cell apoptosis *in vitro*. Basal apoptosis was determined in three glioblastoma multiforme tumours using TUNEL flow cytometry. The mean fraction of cells exhibiting TUNEL-positivity was analysed in 45 5 000-10 000 cell samples from three different tumours. Results from individual tumours are identified using different symbols.

(c) The Effect of Arachidonic Acid on Glioma Cell Apoptosis

A statistically significant increase in human glioma cell apoptosis was detected in the presence of exogenous arachidonic acid. Increases in arachidonic acid stimulated endonuclease activity were transient, with each peak lasting for 2-3 hours. However, more than one peak in apoptotic activity was detected in most tumour preparations (Figure 6.5). Overall, 12 peaks were detected in the 7 tumour cell preparations where the proportion of essential fatty acid treated cells in the apoptotic gate was significantly greater than that of control cells ($p<0.006$, paired t-test).

Differences in the kinetics of arachidonic acid-induced stimulation of endogenous endonuclease activity were detected in tumours of different grades, with low grade tumours, particularly oligodendrogliomas, tending to respond more rapidly to the pro-apoptotic stimuli. In a collagenase dispersed explant of anaplastic oligodendroglioma, the proportion of cells characterised by high endonuclease activity increased from 6.6% to 20.53% after a 15 minutes incubation with arachidonic acid (Figure 6.2). Cells derived from an explant of anaplastic oligoastrocytoma had lower basal apoptosis before arachidonic acid stimulation, but responded with a statistically significant increase in endonuclease following a 15 minute incubation with 30μM arachidonic acid (Figure 6.3).

The stimulation of apoptosis by exogenous essential fatty acids in both high and low grade tumours was not associated with significant changes in membrane permeability during *in vitro* incubations. This suggests the induction of a cytotoxic mechanism distinct from necrosis (Table 6.2).

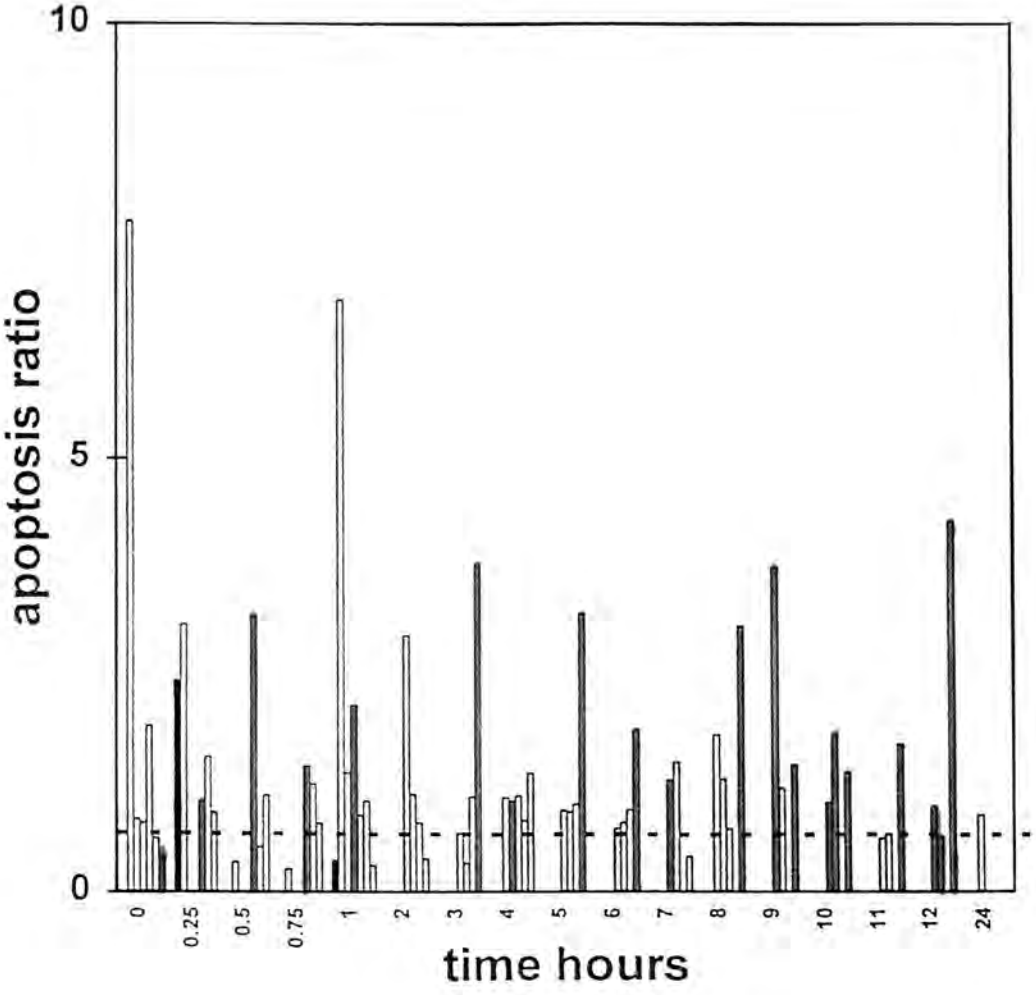

Figure 6.5. The effect of arachidonic acid on glioma apoptosis. The apoptosis ratio was determined in seven tumour preparations treated with 30mM arachidonic acid. The proportion of TUNEL-positive cells in 132 2 000-10 000 cell samples obtained from arachidonic acid treated/untreated cells between 0-12 hours is shown in 1 oligodendroglioma (black bars), three astrocytomas (white bars) and three glioblastoma multiforme tumours (red bars).

VIABILITY OF HUMAN GLIOMA CELLS: EFFECT OF ARACHIDONIC ACID

TIME (hours)	%VIABILITY CONTROL	% VIABILITY ARACHIDONIC ACID	(n)
0	91.8±3.21	92±2.9	(5)
0.5	89±4.19	89.7±4.01	(3)
0.75	84±7.07	87±3.54	(2)
1	90.8±3.55	89.4±4.53	(5)
2	84±6.36	80.5±6.01	(2)
3	88.5±3.72	82.75±1.43	(4)
4	89.25±2.63	89.5±1.87	(4)
5	88.5±3.13	84.75±2.7	(4)
6	86±3.24	83.8±3.65	(5)
8	85.4±3.31	84.2±3.1	(5)
9	78.3±3.6	78±3.68	(3)
10	77.3±4.48	74±3.68	(3)
12	78.7±2.76	85.7±4.38	(3)

Table 6.2. Viability of human glioma cells following a 0-12 hour incubation with 30μM arachidonic acid. Human glioma cell membrane integrity was measured by monitoring trypan blue permeability throughout the experiments. Results are shown as the percentage of viable cells at each time point ±SEM. The number of determinations of cell viability is shown in parentheses.

(d) The effect of Arachidonic Acid on Normal and Tumour Tissue Apoptosis

Apoptotic activity in human glioma tissue and tumour-associated normal brain was investigated over 0-12 hours in the presence and absence of exogenous arachidonic acid (Figure 6.6). In normal brain preparations, mean basal apoptotic index over the incubation period was 1.75±0.172%. In the presence of arachidonic acid, normal cells exhibited a modest increase in mean apoptotic activity. Similarly, an increase in apoptosis in the presence of arachidonic acid was detected in tumour cells, but this effect was greater, with peaks of apoptotic activity of 3.22%, 3.07% and 4.29% being detected at 5, 8 and 12 hours respectively. These increases in tumour cell apoptosis in the presence of arachidonic acid resembled those of the two other glioblastoma tumours in which possessed a similar rate of basal apoptosis. The apoptotic indices of normal and tumour cells in presence and absence of arachidonic acid is shown in Figure 6.6.

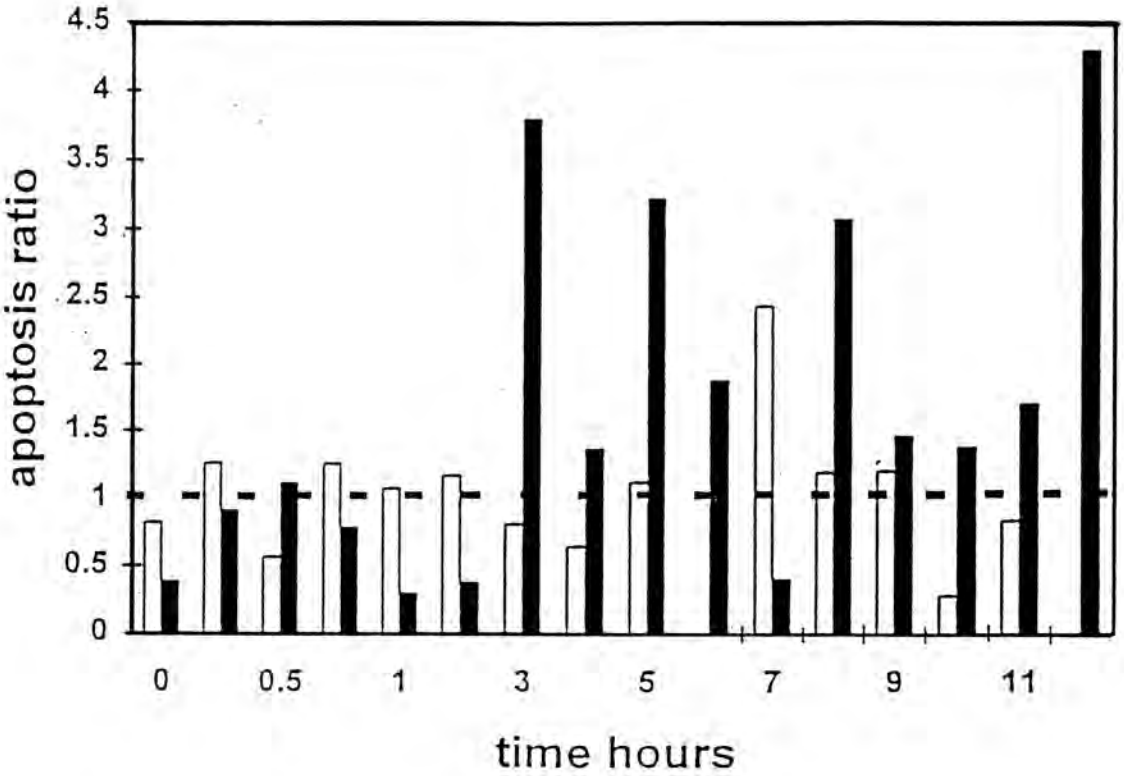

Figure 6.6. The effect of arachidonic acid on apoptosis in cells derived from normal and tumour tissue. The apoptosis ratio was determined in cell preparations acquired from an explant of glioblastoma multiforme and associated normal brain over a period of 12 hours. Cell preparations were incubated in the presence or absence of 30μM arachidonic acid. The proportion of TUNEL positive cells is shown in white bars for normal tissue and black bars for glioblastoma multiforme tissue.

(e) The effect of exogenous gamma-linolenic acid on apoptosis and proliferation in the rat C6 glioma cell line

Gamma-linolenic acid induced a concentration-dependant stimulation of apoptotic activity in rat C6 glioma cells over a period of seven days (Figure 6.7). Maximum stimulation of apoptotic activity occurred at seven days with the highest concentration (40μM) of gamma-linolenic acid. The stimulation of apoptotic activity fluctuated with time with the first peaks in endogenous endonuclease activity occurring at 1 and 3 days after the addition of gamma-linolenic acid (Figure 6.8). In the adherent C6 monolayer, gamma-linolenic acid had little effect on apoptosis in the concentration range 1-40μM. At higher concentrations of gamma-linolenic acid (50μM-1mM) no cells were available as an adherent monolayer. Stimulation of apoptosis in the rat C6 cell line by gamma-linolenic acid in the concentration range 20-40μM was associated with inhibition of cell proliferation, as measured by the MTT assay. Lower concentrations of gamma-linolenic acid (0-5μM) had little effect on apoptosis but increased cell proliferation (Figure 6.8).

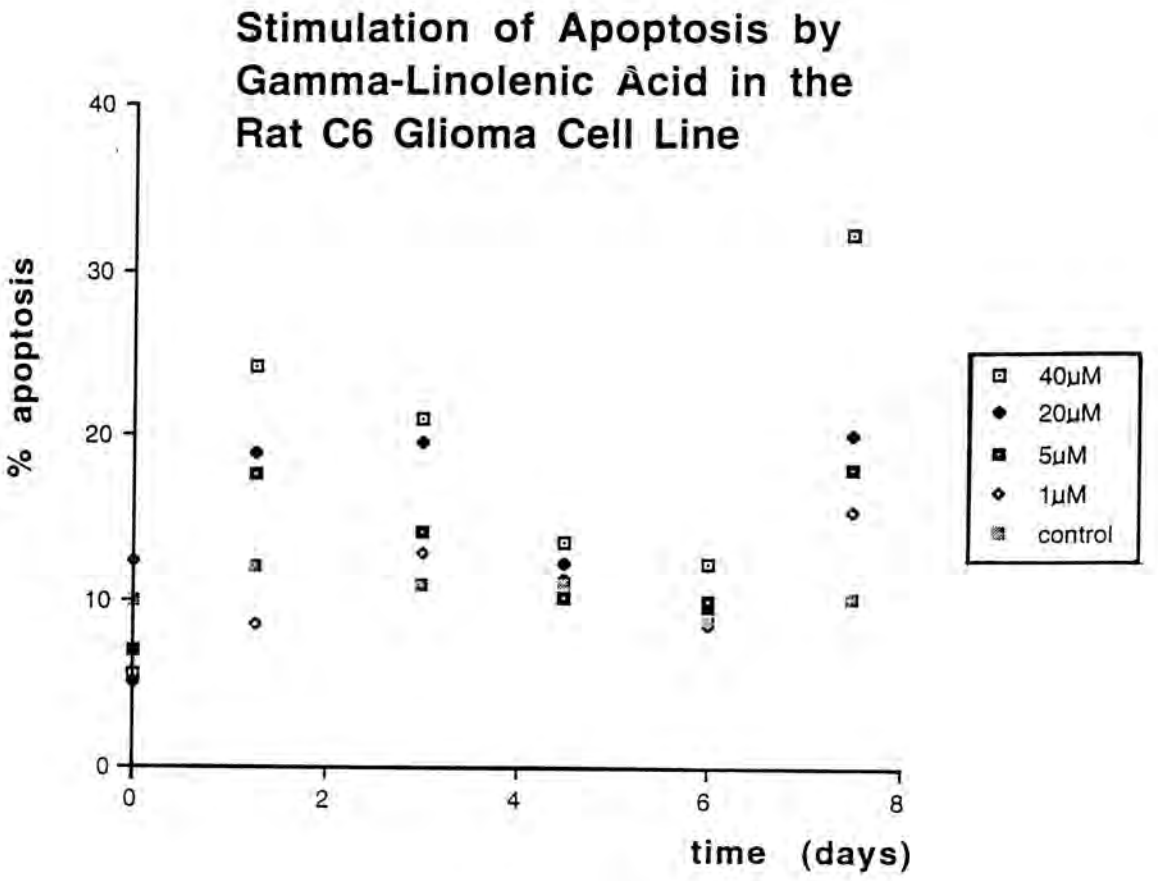

Figure 6.7. Estimation of apoptosis using the TUNEL assay in non-adherent glioma cells collected from C6 cultures incubated in the presence and absence of 0-40μM gamma-linolenic acid. Detached cells were removed every six hours for analysis.

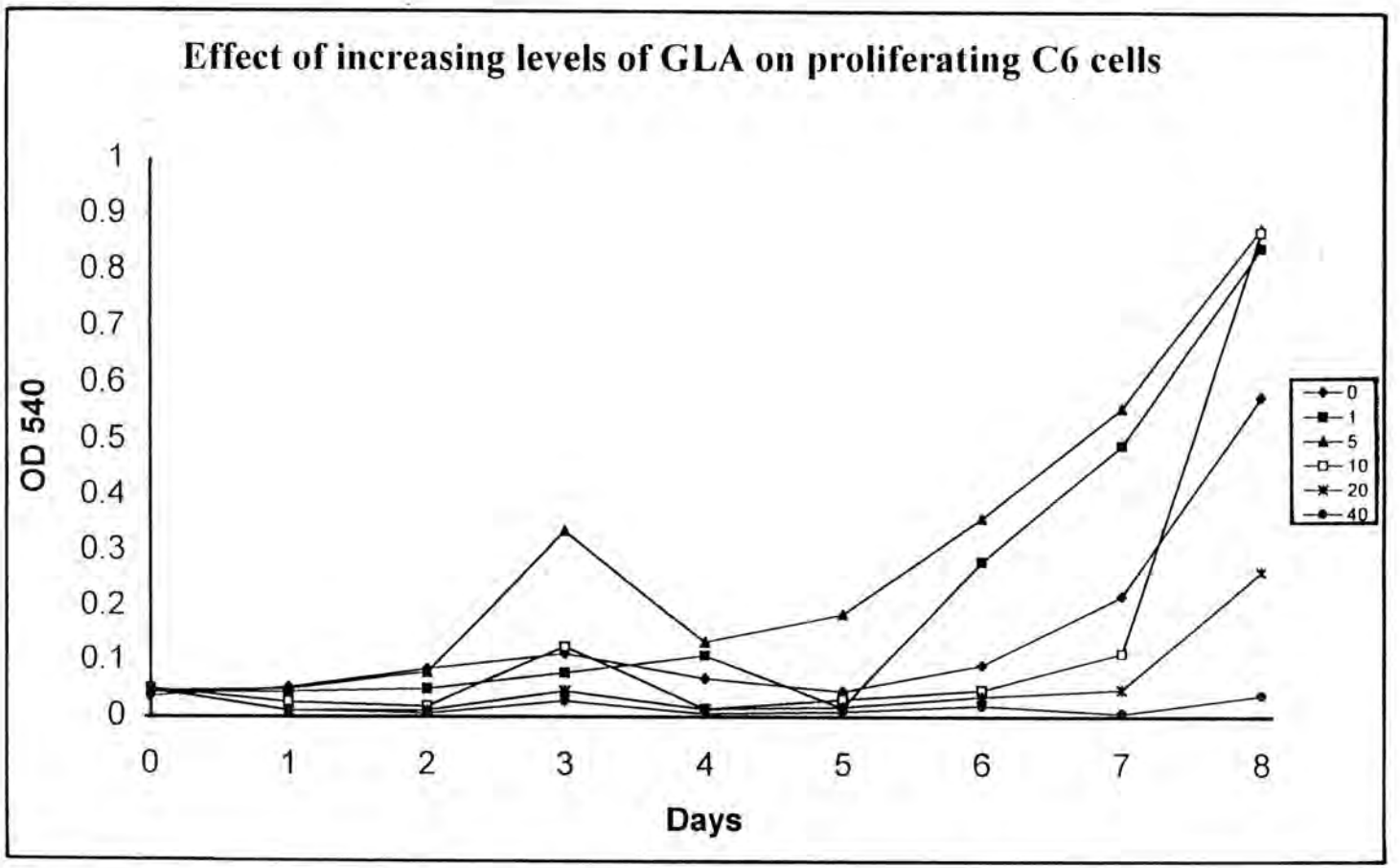

Figure 6.8. Estimation of cell proliferation in the rat C6 glioma cell line during a 7 day incubation with 0-40mM gamma-linolenic acid. The y-axis indicates the optical density (OD) at 540nm of formazan crystals produced by intracellular mitochondrial oxidases and is proportional to cellular proliferation. The x-axis indicated time in days. These results were kindly provided by Helen Bell (Department of Clinical Neurosciences).

6.4 Discussion

This study has indicated that arachidonic acid and gamma-linolenic acid stimulated apoptosis in glioma cells derived from human brain tumours and the rat C6 glioma cell line, as measured by TUNEL analysis of endogenous endonuclease activity. In human tumour preparations, the apoptotic activity of cell preparations acquired from tumours of different grades responded with different kinetics to exogenous arachidonic acid and gamma-linolenic acid. High grade tumours generally responded less rapidly, with maximum stimulation of endogenous endonuclease activity occurring between 10-12 hours. Low grade tumours typically responded within two hours. Low grade tumours may respond more rapidly to exogenous arachidonic acid and gamma-linolenic acid because constitutively expressed pro-apoptotic effector pathways sensitive to essential fatty acids and their metabolites are primed and rapidly initiate apoptosis if appropriately triggered. The relatively slow response of high grade tumours may be due to the requirement for activation of gene transcription of pro-apoptotic mediators. Development of grade IV glioblastoma multiforme involves gene mutations which can result in loss of sensitivity to pro-apoptotic stimuli (Louis, 1997). This insensitivity may be exacerbated by structural and morphological conditions. High metabolic demands resulting from rapid proliferation and competition for oxygen and nutrients can lead to selective pressures which may favour the survival of cell populations least resistant to necrosis and other forms of cell death (Louis, 1997).

A primary contribution of inflammatory processes to the initiation of apoptosis in human tumour preparations was not obvious. Morphological and histological evidence indicated limited leukocyte infiltration and astrocyte activation at the sites of apoptosis. In addition, flow cytometric analysis of tumour cell preparations sensitive to the pro-apoptotic actions of arachidonic acid did not show a predominance of high side scatter, small to medium sized cells typical of phagocyte populations. It is possible however that changes detected in tumour cells were secondary to stimulated peroxidation in adjacent cells.

The stimulation of apoptotic activity in human glioma and the rat C6 cell line fluctuated with time. In human glioma tissue there was evidence of an initial increase in endonuclease activity during the first two hours of incubation, which was followed by further peaks in the stimulation of apoptotic activity between three and 36 hours. Similarly, in the rat C6 glioma cell line there was evidence of time dependant variations in apoptotic activity, with peaks occurring at 1, 3 and 7 days. Similar kinetics have been observed in human colon carcinoma cell lines (Wyllie et al, 1992).

The molecular pathways which mediate these time-dependant episodes of increased apoptotic activity have not been well characterised. However, the initial stimulation of apoptosis may involve induction of metabolic pathways associated with pro-apoptotic activity in cells endowed with the necessary effector proteins (Bellamy, 1995). Further peaks may occur when the effector proteins associated with apoptosis have been synthesised *de novo*. There is evidence that dopamine is capable of inducing apoptosis in post-mitotic sympathetic neurones via its oxidative metabolites, which correlates with two peaks of cyclin B and proliferating cell nuclear antigen expression (Shirvan et al, 1997). The essential fatty acid-sensitive inositol trisphosphate/Ca^{2+} signalling pathway may also be involved in generating fluctuations in apoptotic activity (Berridge, 1997), and it has been reported that up-regulation of apoptosis during embryogenesis involves several time dependant changes in gene expression (Furlow et al, 1997).

This study indicated that individual human tumours exhibited variable basal rates of apoptosis. This finding concurs with that of Schiffer et al (1995) and Kordeck et al (1996), who described varying levels of apoptosis in different brain tumour types. Despite these variations, high grade tumours were generally characterised by low basal rates of apoptosis in this study. An inverse association between tumour proliferative activity and apoptosis has also been established in glial tumours of embryonal orogin, where a trend towards low apoptotic activity was observed in tumours with high mitotic indices (Schiffer et al, 1995).

6.5 Conclusions

This study has demonstrated that arachidonic acid and gamma-linolenic acid stimulate apoptotic activity in human glioma tissue and the rat C6 glioma cell line. This stimulation fluctuated with time, with peaks of apoptotic activity generally being followed by a reduction in dUTP-associated fluorescence one hour later. The kinetics of n-6 essential fatty acid-mediated stimulation of apopotosis were grade dependant. Tumour tissue responded with high sensitivity to exogenous arachidonic acid in comparison with tumour-associated normal brain, and high grade GBM tumours generally took longer to respond to the pro-apoptotic stimulus than low grade astrocytomas. It was also observed that high grade tumours were characterised by low basal apoptotic activity. At the concentrations of arachidonic acid and gamma-linolenic acid used, there was no evidence of statistically significant changes in cell membrane integrity, further supporting the hypothesis that the induction of apoptosis is the principal cause of essential fatty acid-mediated cytotoxicity. These observations provide additional evidence supporting a clinical role for n-6 essential fatty acids in the treatment of malignant glioma.

Chapter 7:

The Interaction of Irradiation with Arachidonic Acid and Gamma-Linolenic Acid

7.1 Introduction

The purpose of this study was to investigate the potentiation of tumour reactive oxygen species formation and apoptosis in rat C6 glioma cells in the presence and absence of arachidonic acid, gamma-linolenic acid and radiation. These activities were investigated at concentrations of arachidonic acid and gamma-linolenic acid previously shown to stimulate glioma oxidative activity and programmed cell death, and over time periods where peaks in apoptotic activity had been observed.

These investigations were undertaken to investigate a potential clinical role for n-6 essential fatty acids as a therapeutic adjuvant in glioma radiotherapy. These considerations are important because of the poor clinical response of malignant glioma to standard fractionated radiotherapy. Despite the recent introduction of stereotactic radiosurgery and gamma knife radiotherapy, which increase the precision of positioning of small treatment beams, the prognosis for patients suffering from malignant glioma is not significantly better now than it was thirty years ago (Taphoorn et al, 1994, Forsyth and Cairncross, 1995 and Sichez, 1996). In addition, ultra high dose rates (Cygler et al, 1994), intraoperative radiotherapy (Shimbamoto et al, 1994 and Hara et al, 1995), accelerated hyperfractionation (Glinski, 1993, Sugawara et al, 1994 and Freeman et al, 1996) and concurrent radiotherapy and standard and multiagent chemotherapy (Eyre et al, 1993, Heideman et al, 1993, Jeremic et al, 1994, Dillman et al, 1995, Hui et al, 1995 and Buchsbaum and Robertson, 1996) have not been associated with significant clinical benefit.

It has been proposed that essential fatty acid-mediated potentiation of cellular oxidative activity may increase the sensitivity of malignant glioma to radiotherapy because of the pivotal role of reactive oxygen intermediates in radiation induced cytotoxicity is well established (Halliwell and Gutteridge, 1985 and Hagen, 1989). Radiation-induced stimulation of reactive oxygen intermediate production may,

under appropriate conditions, result in self-propagating chain reactions leading to DNA double strand breaks and locally denatured regions (Halliwell and Gutteridge, 1985, Hagen, 1989 and Wardman and Ross, 1990). This induces p53 dependant G_1 phase cell cycle arrest which allows time for DNA repair or elimination of lethally damaged cells by apoptosis (Gupta et al, 1996).

Results presented in this thesis and elsewhere indicate that human glioma cells are characterised by low basal reactive oxygen species formation (Levchenko and Demchik, 1991 and Williams et al, 1997). This may be partially due to deficiency of n-6 essential fatty acids including arachidonic acid and gamma-linolenic acid (Martin et al, 1996). These observations suggest that addition of exogenous essential fatty acids may be associated with potentiation of radiation-induced cytotoxicity by providing pro-oxidative substrate upon which radiation can act.

In these investigations, the kinetics of cellular peroxidation, apoptotic activity and cell membrane integrity were investigated in rat C6 glioma cells in the presence and absence of radiation and exogenous n-6 essential fatty acid. The cells were irradiated at 2Gy because this is a therapeutically relevant dose (Chang et al, 1995) associated with tumour cytotoxicity in human glioma cell lines (Ross et al, 1994). In addition, 2Gy is close to the mid-point used in irradiation experiments using the C6 cell line and sensitising agents (Zhang et al, 1993, Stapper et al, 1995 and Bergenheim et al, 1995).

In addition, the interaction of arachidonic acid and sodium nitroprusside was investigated. Sodium nitroprusside is a NO donor which may have a role as a therapeutic adjuvant in the management of human glioma due to its action as a vasodilator (Whittle 1996). This may increase the delivery of cytotoxic drugs to tumour tissue. In addition, there is evidence that NO donors potentiate free radical activity (Ioannidis and deGroot, 1993 and Hata et al, 1996), which may contribute to tumour cell cytotoxicity *in vivo* (Krinsky et al, 1992). Essential fatty acid-mediated potentiation of this action may be associated with increased glioma cell death.

7.2 Materials and Methods

(a) Cell Preparation

(i) Cell Irradiation

Rat C6 glioma cells were harvested and resuspended at a density of 10^6 cells/ml in Hams-F10 medium. This cell suspension was used to completely fill 50ml cell culture flasks, taking care to exclude all air gaps. The flasks were irradiated at 2Gy with a 6MV X-ray beam in the presence and absence of 20μM arachidonic acid or gamma-linolenic acid (Section 2.9b). At intervals of 1, 2, 3, 4, 6, 8 and 10 hours post-irradiation, 1ml aliquots were analysed for peroxidative and apoptotic activity, and cell membrane integrity (Sections 2.3, 2.6 and 2.7).

(ii) The Interaction of n-6 Essential Fatty Acids with Sodium Nitroprusside

C6 cells were harvested, resuspended at a density of 10^6 cells/ml in Hams F-10 medium and incubated in the presence and absence of 0.1mM sodium nitroprusside and/or 20μM arachidonic acid (Section 2.9a). Glioma cell peroxidation, viability and apoptosis was analysed as described previously (Section 7.2ai).

(b) Analysis of Peroxidative Activity

C6 cells were centrifuged and incubated with 5μM 2',7'-dichlorofluorescin diacetate for 10 minutes at 37°C. The cells were washed and resuspended in Hams F-10 medium containing 10% FCS. The rate of reactive oxygen intermediate production was determined using flow cytometric quantitation of 2',7'-dichlorofluorescein production (Section 2.3).

(c) Analysis of Apoptotic Activity

C6 cells were fixed by exposure to 100% ethanol and stored at -4°C for 1-3 weeks. Apoptotic activity was determined using TUNEL flow cytometery (Section 2.7).

(d) Analysis of Cell Viability

Cell viability was determined immediately after analysis of peroxidative activity using flow cytometric analysis of propidium iodide permeability (Section 2.6).

7.3 Results

(a) Effect of Irradiation, Arachidonic Acid and Gamma-Linolenic Acid on Oxidative Activity in the Rat C6 Glioma Cell Line

Oxidative activity in the rat C6 glioma cell line was increased in response to irradiation and exogenous n-6 essential fatty acid stimulus (Figure 7.1). Potentiation of glioma cell reactive oxygen species formation following addition of arachidonic acid and gamma-linolenic acid typically occurred within 3 hours of stimulation, with peaks of up to 700% of the untreated control being observed after 2 hours. Subsequent smaller increases of up to 200% occurred within the next 7 hours. The maximum stimulation of oxidative activity in response to radiation was less than that observed in response to arachidonic acid or gamma-linolenic acid, with peaks of up to 400% of the untreated control occurring. This stimulation occurred throughout the incubation period with fewer fluctuations in reactive oxygen intermediate generation with time. In one experiment the maximum stimulation of oxidative activity occurred between 4 and 8 hours. These results suggest different kinetic mechanisms for the stimulation of reactive oxygen intermediate production by essential fatty acids and radiation. No statistically significant differences were observed between the kinetic profiles of arachidonic acid or gamma-linolenic acid either alone or in conjunction with radiation.

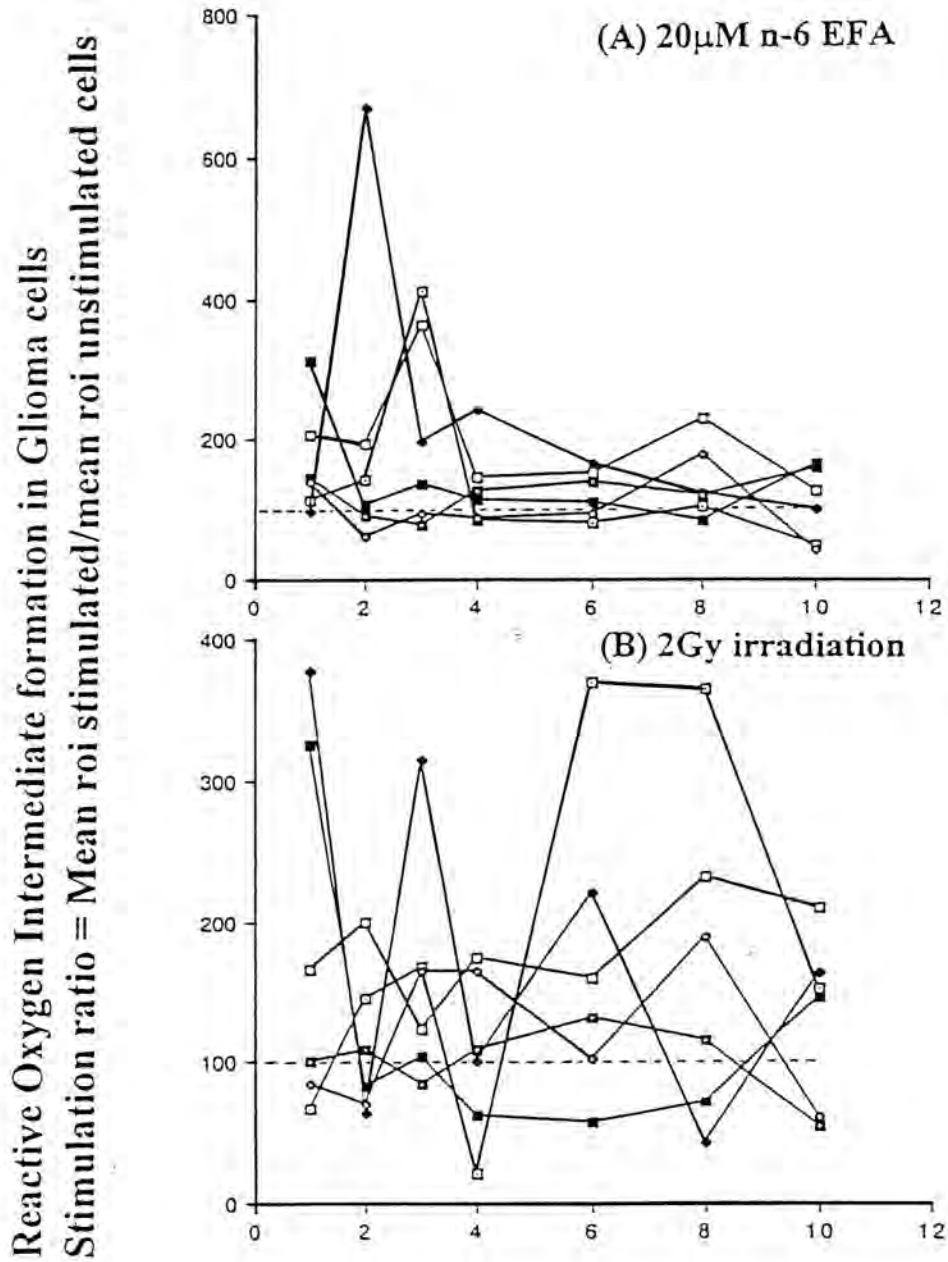

Figure 7.1. The stimulation of cellular peroxidation in 6 single cell suspensions of the rat C6 glioma cell line by (A) 20μM exogenous arachidonic acid or gamma-linolenic acid and (B) 2Gy irradiation. The y-axis indicates reactive oxygen intermediate formation which is expressed as percent stimulation of the untreated control and the x-axis indicates time in hours.

(b) Analysis of Apoptotic Activity in the Rat C6 Glioma Cell Line

Irradiation and exogenous n-6 essential fatty acid stimulus potentiated apoptotic activity in the rat C6 glioma cells (Figure 7.2 and Figure 7.3). There was evidence of an early stimulation of endogenous endonuclease activity, although in certain cases additional peaks of smaller magnitude were observed after 4 and 6 hours. The kinetics of the stimulation of apoptotic activity were complex, and peaks in apoptotic activity were often followed by a reduction in TUNEL positivity one hour later. The kinetics of these episodic increases in apoptotic activity were similar to those observed in human astrocytoma (Chapter 6).

Radiation induced stimulation of endogenous endonuclease activity also occurred within one hour, although the magnitude of this early response was generally less than that observed in response to exogenous arachidonic acid and gamma-linolenic acid (Figure 7.2b). This stimulation was maintained for up to 10 hours post-irradiation. In certain cases, peaks of apoptotic activity occurred at 5 and 6 hours post-irradiation, with maximum stimulation being approximately 600%. In general however, fewer episodic fluctuations of endogenous endonuclease activity occurring with time in comparison with the effect of 20μM exogenous arachidonic acid or gamma-linolenic acid alone.

The maximum stimulation of apoptotic activity induced by exogenous essential fatty acid stimulus occurred within the first two hours of incubation. This is in contrast to the later response observed following exposure to radiation. However, the magnitude of the maximum stimulation was similar in both cases, with increases of up to 600% of the unstimulated control being observed. These results suggest the induction of different kinetic mechanisms for the stimulation of apoptosis by radiation and arachidonic acid and gamma-linolenic acid.

Simultaneous exposure to radiation and exogenous n-6 essential fatty acids was associated with greater than additive potentiation of apoptotic activity. In most cases this synergistic stimulation was maintained over the 10 hour incubation period,

however maximum peaks were observed at 4, 7 and 8 hours post-irradiation. Again, the kinetics of this stimulation were complex and there was evidence that peaks in apoptotic activity were followed by a reduction in endogenous endonuclease activity 1 hour later. The maximum stimulation of apoptosis was approximately 4 000% of the untreated control. No statistically significant differences were observed between the stimulation of apoptosis by arachidonic acid or gamma-linolenic acid.

During the analysis of apoptotic activity, contemporary sampling was used to calculate changes in endogenous endonuclease activity. This was due to the observation that the apoptotic index (AI) in untreated tumour samples changed with time. There was evidence of variable basal apoptotic indices in human tumours (Chapter 6). There is also evidence that variation of AI occurred with time in single cell suspensions of the rat C6 cell line. This may be partly related to cell damage caused by trypsinisation and resuspension, and subsequent recovery from mechanical damage sustained during cell preparation. In addition, membrane damage due to essential fatty acid effects was studied. Pre-stimulation analysis of apoptotic activity was also carried out initially but this was not routinely calculated. This was due principally to subsequent negative values in certain tumour preparations which were difficult to analyse statistically and the initial low viability of cell preparations following trypsinisation and resuspension.

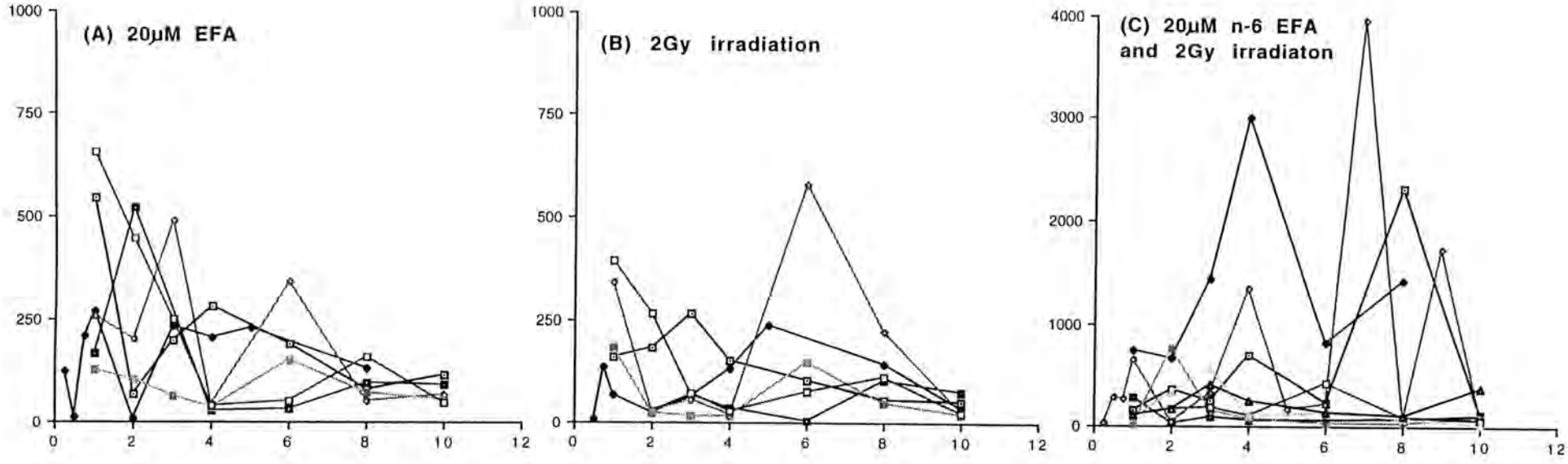

Figure 7.2. The stimulation of apoptosis in the rat C6 glioma cell line in response to (A) 20μM exogenous arachidonic acid or gamma-linolenic acid, (B) 2Gy irradiation and (C) exogenous arachidonic acid or gamma-linolenic acid and radiation combined. The stimulation of apoptotic activity is expressed as the percent stimulation of TUNEL positivity in stimulated compared with unstimulated cells (y-axis). The x-axis indicates time in hours.

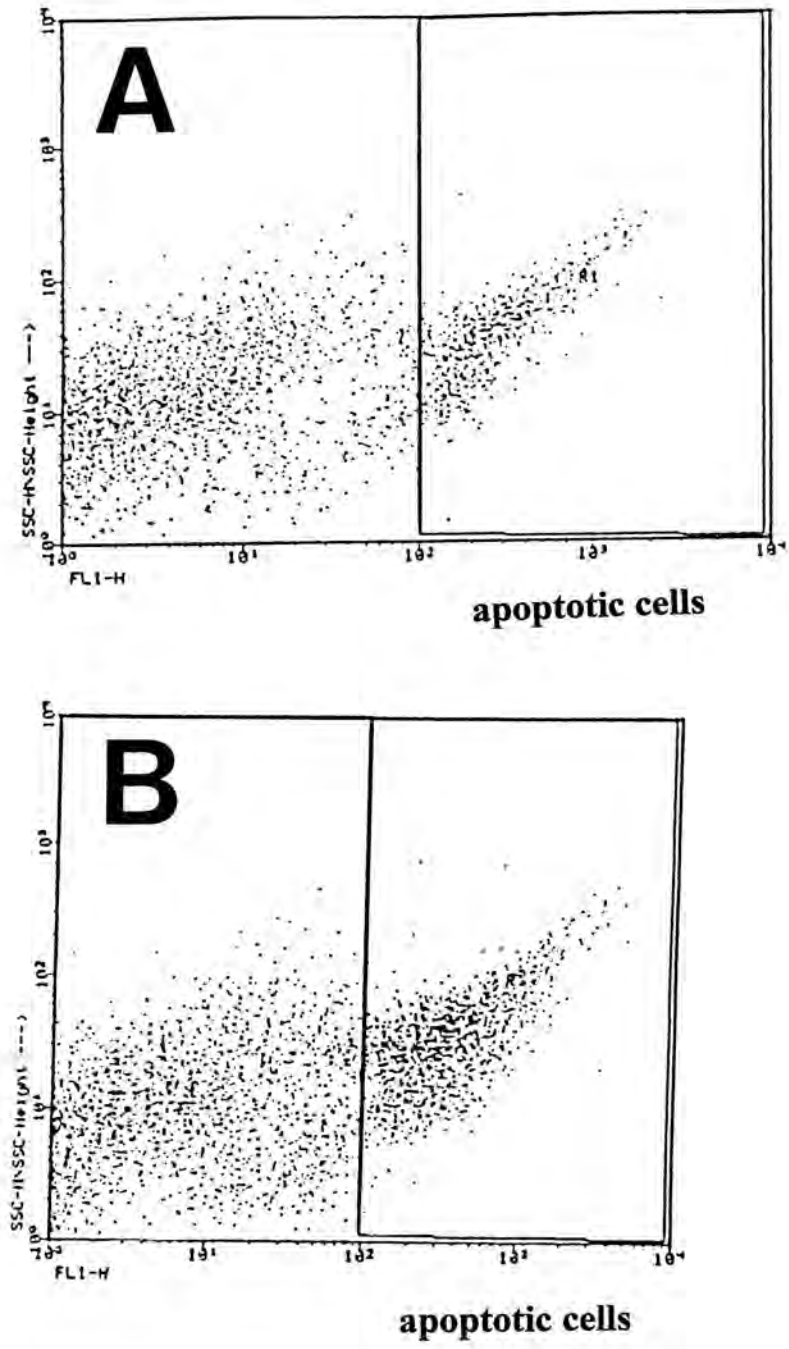

Figure 7.3. The stimulation of apoptosis in rat C6 glioma cells irradiated at 2Gy in the presence (B) and absence (A) of 20μM exogenous arachidonic acid or gamma-linolenic acid. Cells were selected for TUNEL positivity by using a high fluorescence (Fl-1) gate.

(c) The Effect of Sodium Nitroprusside on Oxidative Activity in the Rat C6 Glioma Cell Line

Sodium nitroprusside stimulated reactive oxygen intermediate formation in rat C6 glioma cells (Figure 7.4). This stimulation occurred within one hour and was maintained throughout the 10 hour duration of the investigation. The magnitude and kinetics of sodium nitroprusside-mediated potentiation of glioma reactive oxygen species formation was similar to that observed in response to addition of exogenous arachidonic acid, and although the stimulation of reactive oxygen intermediate generation was generally higher in response to sodium nitroprusside, this difference was not statistically significant.

Simultaneous exposure to arachidonic acid and sodium nitroprusside resulted in potentiation of oxidative activity. This potentiation was maximal during the first hour of incubation but was maintained throughout the 10 hour exposure.

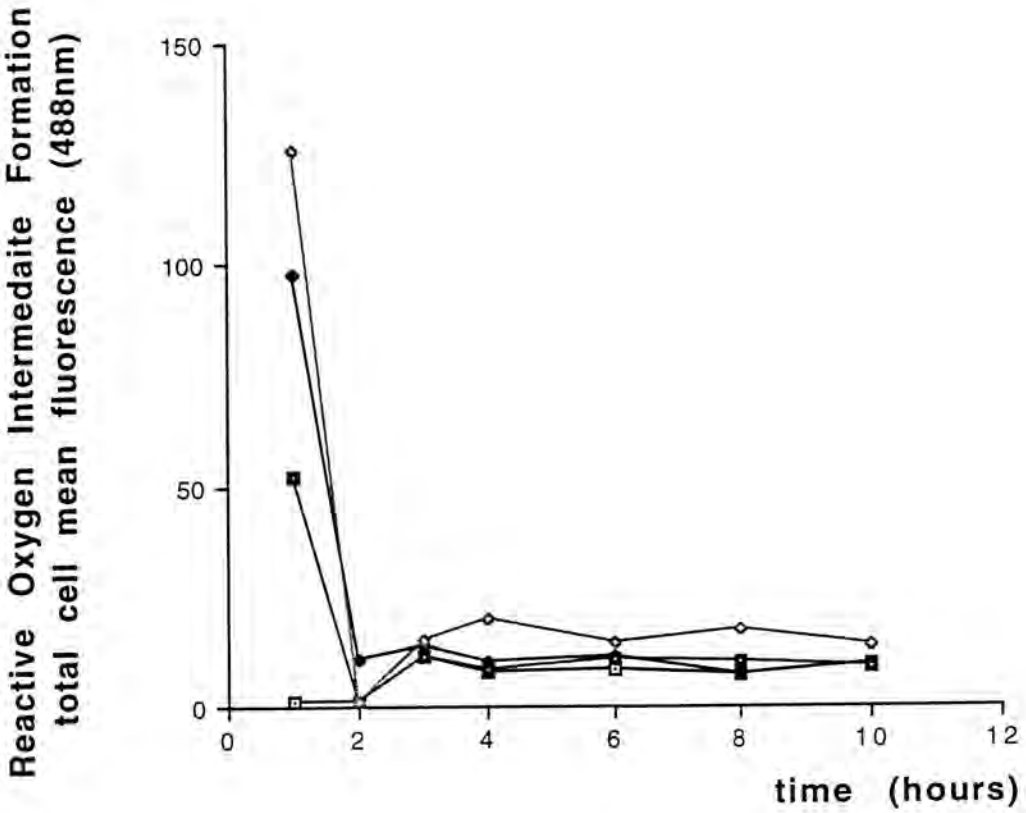

Figure 7.4. The stimulation of reactive oxygen intermediate formation in rat C6 glioma cells by the nitric oxide donor sodium nitroprusside. Results are expressed as mean reactive oxygen intermediate formation in cells incubated with 20μM arachidonic acid (closed diamonds), 0.1mM sodium nitroprusside (closed squares), 20μM arachidonic acid and sodium nitroprussude combined (open diamonds) and vehicle alone (open squares).

7.4 Discussion

This study has provided preliminary evidence that arachidonic acid, gamma-linolenic acid and radiation stimulated apoptotic and peroxidative activity in single cell suspensions of the rat C6 glioma cell line. However, differences were observed between the kinetic profiles of these stimulatory effects. Arachidonic acid and gamma-linolenic acid stimulated apoptosis and reactive oxygen intermediate formation within the first three hours of exposure. This early oxidative response observed in glioma cells following arachidonic acid and gamma-linolenic acid exposure was similar to that described for the short term stimulation of peroxidative activity in Chapter 4. In contrast, radiation induced stimulation of oxidative activity occurred throughout the duration of the experiment, although the maximum stimulation was lower. These results suggest different kinetic mechanisms for the stimulation of reactive oxygen intermediate production by radiation and essential fatty acids.

Preliminary investigations also indicated that sodium nitroprusside potentiated peroxidative activity in the rat C6 glioma cell line. The kinetics of this stimulation were similar to those induced by arachidonic acid. There was also evidence that reactive oxygen intermediate generation was potentiated when C6 cells were simultaneously exposed to both sodium nitroprusside and arachidonic acid. This potentiation was greatest during the first two hours of exposure.

The stimulation of apoptosis in response to exogenous n-6 essential fatty acids also occurred relatively rapidly, with maximum increases in endogenous endonuclease activity typically occurring within the first three hours of incubation. Although radiation-induced stimulation of apoptosis also occurred within one hour, maximum stimulation was typically observed towards the end of the incubation period. The stimulation of apoptosis induced by exogenous arachidonic acid or gamma-linolenic acid was generally greater than that induced by radiation.

There was evidence of potentiation of apoptotic activity following simultaneous exposure to arachidonic acid or gamma-linolenic acid and radiation. This stimulation occurred throughout the ten hour incubation period, but was typically maximised at 6-8 hours post-irradiation. In one case, dUTP-associated fluorescence in C6 cells exposed to gamma-linolenic acid and radiation was up to 4 000% of the non-irradiated control.

These results are consistent with the hypothesis that essential fatty acids potentiate glioma cell response to radiotherapy. Although the molecular pathways responsible for this activity have not been well characterised, it has been proposed that essential fatty acids may reduce radioresistance by modulating PIP_3 activity (Woloschak et al, 1990 and Uckun et al, 1993). Inhibition of protein kinase C is associated with sensitisation of human tumours to ionising radiation (Hallahan et al, 1992), and there is evidence that essential fatty acids reduce the activity of activated protein kinase C (Chen and Murakami, 1992, Hilian and Nelson, 1992 and may et al, 1993). This inhibition may be associated with reduced expression of activated ras, an oncogene which confers radiation resistance and is associated with protein kinase C activity (Sklar, 1988 and Borner and Weinstein, 1990). There is also evidence that pre-exposure of glioma cell lines to the cytokines IFNγ and TNFα, whose activity and/or secretion can be modified by linoleic acid, arachidonic acid, and gamma-linolenic acid (Ku et al, 1991 and Baldie et al, 1993), can inhibit bcl_2 mediated rescue from apoptosis following irradiation by sensitising for FAS/APO-1 dependent killing (Weller et al, 1995).

In addition to increasing efficacy, essential fatty acids may reduce the occurrence of pathological side effects associated with radiotherapy (Hopewell et al, 1993). It has been proposed that damage to peri-tumoural tissue which occurs following exposure to radiation may result from loss of essential fatty acids rather than the accumulation of toxic metabolites. This implies that increasing the availability of essential fatty acids will limit and/or reverse radiation-induced damage to normal tissue (Horrobin, 1990). These radio-protective actions may result, in part at least, from modification

of eicosanoid metabolism. Radiation causes an imbalance in the ratio of mono:dienoic eicosanoids (Schneidkraut et al, 1984, Weshler et al, 1987 and Ward, 1990) however gamma-linolenic acid increases the production of monoenoic PGE_1 rather than dienoic prostaglandins such as PGE_2 and TXA_2 (Hopewell et al, 1993). In addition, gamma-linolenic acid inhibits leukotriene formation (Horrobin and Manku, 1990).

7.5 Conclusions

This study has provided evidence that arachidonic acid and gamma-linolenic acid interact with radiation to potentiate oxidative and apoptotic activity *in vitro*. The increase in reactive oxygen intermediate formation occurred within the first three hours of exposure to exogenous essential fatty acid stimulus post-irradiation, suggesting that stimulation of reactive oxygen intermediate production is an early event. This result concurs with the observation that oxidative activity increased within 10 seconds of exogenous essential fatty acid stimulation in collagenase dispersed explants of human malignant glioma, and that this increase can be maintained for up to 3 hours (Chapter 4).

In conclusion, this study has provided evidence that exogenous arachidonic acid, gamma-linolenic acid and therapeutically relevant doses of irradiation stimulated reactive oxygen intermediate generation and apoptotic activity in the rat C6 glioma cell line. There was also preliminary evidence that arachidonic acid interacted with the nitric oxide donor sodium nitroprusside to potentiate reactive oxygen intermediate formation. These results are consistent with a possible role for arachidonic acid and gamma-linolenic acid as a therapeutic adjunct in the treatment of human primary glioma.

Chapter 8:

Discussion

8.1 Aims and Hypotheses

Despite the considerable progress which has been made in understanding the molecular biology of malignant gliomas, the prognosis for patients suffering from these tumours has not improved substantially in recent years (Roth and Weller, 1999). Furthermore, cytoreductive surgery and radiotherapy remain the preferred treatments for malignant glioma, although it is thought that novel cytotoxic drugs may represent the most promising therapeutic strategies for the future (Darling, 1990 and Roth and Weller, 1999). The evaluation of alternative anti-tumour agents is therefore an important aim of neurooncology research.

One promising strategy may be modulation of the oxidative activity of these tumours through administration of essential fatty acids. These highly unsaturated lipids act as substrates for the production of lipid peroxides, which may be implicated in glioma cytotoxicity and radiosensitisation (Cornwell and Morisaki, 1984 and Gonzalez, 1992). *Cis*-unsaturated fatty acids inhibited the growth of glioma cell lines *in vivo* and *in vitro* (Das et al, 1990a), PUFA administration was associated with the rejection of transformed microglial tumour cells *in vivo* (Frei et al, 1994), peroxidised low density lipoprotein was cytotoxic to human glioma cell lines (Kikuvhi et al, 1997) and gamma-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid were cytotoxic to 36B10 rat astrocytoma cell lines *in vitro* (Vartak et al, 1997 and 1998). Additionally, oral administration of gamma-linolenic acid induced tumour regression and improved patient survival in a small clinical trial (Das, 1995). However, only fifteen patients were assessed in this study, and adequate control groups were not included.

Although these previous studies provided evidence supporting a clinical role for n-6 essential fatty acids in malignant glioma therapy, limited information was available on the activity of essential fatty acids in fresh human glioma tissue. Most

investigations had been carried out on established cell lines, and the limitations of these experiments were concerned predominantly with loss of tumour phenotype associated with cell culture, and the lack of comparative analysis of the oxidative activities of tumours of different grades. Additionally, the mode of cell death resulting from essential fatty acid administration had not been well characterised. There was also limited information on the interactive effects of essential fatty acids and radiation in malignant glioma cells, particularly with respect to the kinetics of reactive oxygen species formation and the mode of cell death induced. The purpose of this study was to provide additional information on the role of essential fatty acid metabolism in glioma cytotoxicity and radiosensitisation by investigating the hypotheses that:-

1) Oxidative activity is impaired in human glioma tissue in comparison with tumour-associated normal brain

2) Addition of exogenous essential fatty acids stimulates tumour reactive oxygen species generation

3) Potentiation of tumour reactive oxygen species generation is associated with tumour cytotoxicity, in part at least through stimulation of apoptosis

4) Stimulation of tumour oxidative and apoptotic activity by arachidonic acid and gamma-linolenic acid is potentiated by simultaneous exposure to therapeutic doses of radiation

8.2 Investigations Undertaken

(a) General practical considerations

Investigation of these hypotheses lead to a number of important practical considerations. These related predominantly to tissue availability, and the complexity of experimental and data analysis which arose from the cellular heterogeneity which is characteristic of this tumour type. Preliminary investigation

of the initial hypotheses suggested that analysis of essential fatty acid-mediated stimulation of glioma oxidative and apoptotic activity was likely to be complex. This was due to variations in basal oxidative activity, cell composition, viability and tumour grade between tumours acquired from different patients, and also the cellular heterogeneity of tumour preparations derived from individual tumours. As a result of these confounding factors, it was initially necessary to use relatively homogeneous cell preparations to characterise the experimental conditions required to analyse the both the pro-oxidative and the pro-apoptotic activities of arachidonic acid and gamma-linolenic acid *in vitro*.

These preliminary investigations were undertaken using fresh leukocyte preparations, which were used to define the conditions most appropriate for analysis of tumour oxidative activity. In addition to characterising experimental conditions, analysis of the oxidative response of these cells may be relevant to the overall cytotoxic activities of essential fatty acids, as infiltrating lymphocytes are sometimes present in human gliomas (Roszman et al, 1991 and Tada and Tribolet, 1993). Results of this study also assisted the identification of the characteristics a human tumour sub-population thought to represent infiltrating phagocytes.

Cellular heterogeneity was also responsible for the complexity of analysis of apoptotic activity in human glioma. Consequently, the terminal desoxynucleotidyl transferase binding characteristics of the rat C6 cell line stimulated into apoptosis by a therapeutically relevant dose of radiation was used to identify human tumour cell populations undergoing programmed cell death. As insufficient quantities of human glioma tissue were available for analysis, and cellular heterogeneity would have made tumour-specific responses difficult to delineate, the rat C6 cell line was also used for investigation of essential fatty acid-mediated radiosensitisation. Although further investigations are required to analyse essential fatty acid-mediated radiosensitisation in human glioma tissue, the results of this study provided preliminary information on the previously uncharacterised association between the

kinetics of reactive oxygen species formation and apoptotic cell death in glioma cells.

(b) Human glioma oxidation

It has been postulated that impaired reactive oxygen species generation is associated with increased proliferative activity (Barber and Wilbur, 1959, Bernheim, 1963, Mead and Fulco, 1976 and Masotti et al, 1988, Cheeseman et al, 1984, Levchenko and Demchik, 1991 Bartoli and Galeotti, 1979, Barber and Wilbur, 1959, Bernheim, 1963, Mead and Fulco, 1976 and Masotti et al, 1988), and that tissues with low basal oxidative activity respond to exogenous essential fatty acid stimulus with increased production of potentially cytotoxic lipid peroxides. This study demonstrated for the first time that these hypotheses are pertinent to human malignant glioma. Reactive oxygen intermediate formation was an inverse function of proliferative activity in collagenase-dispersed glioma tissue, the basal rate of tumour oxidation being significantly lower than that of associated normal brain ($p<0.0000017$). Additionally, the kinetics of essential fatty acid-mediated stimulation of glioma oxidative activity were grade dependent. Although the kinetics of essential fatty acid-stimulated reactive oxygen intermediate formation followed an exponential function in both normal and tumour cell preparations, tumour cells showed significantly higher sensitivity than normal tissue ($p<0.0000017$).

Sub-population analysis also provided preliminary evidence supporting the hypothesis that tumour cells have low basal oxidative activity and high sensitivity to exogenous essential fatty acid stimulation. Preliminary investigations revealed a GFAP-positive population which was characterised by high side scatter (which is indicative of high granularity and hence DNA content). These morphological and immunohistochemical features are characteristic of the known properties of human glioma cells. The cell population identified in this way possessed low basal oxidative activity and reacted with high sensitivity to exogenous arachidonic acid and gamma-linolenic acid.

(c) Glioma apoptosis and proliferation

The biological significance of arachidonic acid and gamma-linolenic acid-mediated stimulation of cellular oxidation was investigated through analysis of glioma cell proliferation and apoptosis. It was confirmed that at concentrations previously shown to stimulate glioma reactive oxygen species formation, arachidonic acid and gamma-linolenic acid inhibited cell proliferation and stimulated apoptosis. The effects of gamma-linolenic acid on C6 glioma cell proliferation were investigated using the MTT assay, and preliminary results indicated that gamma-linolenic acid inhibited tumour cell proliferation over 7 days. These results concur with previous investigations which demonstrated that gamma-linolenic acid inhibited cell proliferation in glioma cell lines (Das, 1990), and that inhibition of cell proliferation in the GHP-212 neuroblastoma cell line was associated with stimulation of cellular oxidative activity (Hrelia et al, 1996).

The stimulation of apoptotic activity by arachidonic acid and gamma-linolenic acid was investigated in collagenase-dispersed explants of fresh human primary glioma obtained at biopsy. Both arachidonic acid and gamma-linolenic acid stimulated apoptosis for up to 36 hours, with arachidonic acid being more active. The stimulation of apoptotic activity by exogenous arachidonic acid and gamma-linolenic acid varied with time: episodes of increased apoptotic activity were generally followed by lower levels of apoptosis one hour later.

The apoptotic activity of cell preparations acquired from tumours of different grades responded with different kinetics to exogenous arachidonic acid and gamma-linolenic acid. High grade tumours generally responded less rapidly, with maximum stimulation of endogenous endonuclease activity occurring between 10-12 hours. Low grade tumours typically responded within two hours. Low grade tumours may respond more rapidly to exogenous arachidonic acid and gamma-linolenic acid because constitutively expressed pro-apoptotic effector pathways sensitive to essential fatty acids and their metabolites are primed and rapidly initiate apoptosis if appropriately triggered. The relatively slow response of high grade tumours may be

due to the requirement for activation of gene transcription of pro-apoptotic mediators. Development of grade IV glioblastoma multiforme involves gene mutations which can result in loss of sensitivity to pro-apoptotic stimuli (Louis, 1997). This insensitivity may be exacerbated by structural and morphological conditions. High metabolic demands resulting from rapid proliferation and competition for oxygen and nutrients can lead to selective pressures which may favour the survival of cell populations least resistant to necrosis and other forms of cell death (Louis, 1997).

Although the down-stream pro-apoptotic targets of reactive oxygen intermediates have not been fully characterised, a number of candidate molecular pathways have been proposed. There is evidence that hydrogen peroxide induces apoptosis in hepatoma cells by modulating intracellular Ca^{2+} and Mg^{2+}, resulting in DNA fragmentation and activation of the pro-apoptotic enzyme poly(ADP-ribose) polymerase (Li et al, 2000). Other putative targets include Bcl_2, cytochrome P450 and intracellular anti-oxidants. Recent studies indicate that essential fatty acid-mediated stimulation of apoptosis is associated with depletion of glutathione and inhibition of carnitine palmitoyl transferase I in cells over expressing cytochrome P450 (Das, 1999). These studies also suggested that essential fatty acids reduce expression of anti-apoptotic bcl_2, possibly through P450 phosphorylation. Each of these activities was associated with increased lipid peroxidation (Das, 1999).

Additionally, there is evidence that modification of the activities of certain growth factors sensitive to essential fatty acids and their metabolites may be implicated in the initiation of programmed cell death. Deprivation of PDGF initiates apoptosis in human glioma cells (Collins et al, 1994 and Westermark et al, 1995) possibly by modulating the Ras.Raf pathway (Westermark et al, 1995). PDGF is secreted by transformed glial cells and has been identified in neurones and their processes where it stimulates astrocyte migration and proliferation (Westermark et al, 1995, Engebraaten et al, 1993, Whelan et al, 1993 and Pedersen et al, 1994). PDGF and PDGF receptors are frequently over-expressed in human glioma and specific and

non-specific PDGF antagonists block the growth of some glioma cell lines *in vivo* and *in vitro* (Kuratsu et al, 1995, Kurimoto et al, 1994, Kurimoto et al, 1995, and Westermark et al, 1995). Additionally, it is known that the v-sis oncogene of simian sarcoma virus, which is a retroviral homologue of the B-chain of PDGF, induces malignant glioma in experimental animals (Westermark et al, 1995). It is known that eicosapentaenoic acid inhibits certain well characterised actions of PDGF including inhibition of PDGF-mediated elevation of intracellular calcium (Locher et al, 1991).

There is also evidence that NGF inhibits growth and stimulates apoptosis in a human anaplastic glioma cell line (Marushige et al, 1992), possibly by reducing the expression of bcl_2 (Kamada et al, 1996) and augmenting FAS-mediated killing (Kamada et al, 1996 and Yashima et al, 1996). Arachidonic acid lipoxygenation may stimulate nerve growth factor secretion in astroglial cultures (Carman-Krzan and Wise, 1993). NGF may also inhibit tumour growth by stimulating lipid peroxidation by stimulating the production of hydrogen peroxide (Weese et al, 1993). NGF-mediated stimulation of hydrogen peroxide may further increase NGF synthesis (Pechan et al, 1992).

Additional research is also required to characterise which oxidative products of lipid metabolism mediate tumour cytotoxicity, either directly or through modulation of downstream molecular pathways. Although the stimulation of apoptosis in glioma cell lines following addition of selenium has been attributed to increased reactive oxygen intermediate generation (Nakatsu et al, 1996 and Zhu et al, 1996), relatively little is known about the identity of the individual oxygen based free radicals which are implicated in cell death pathways. Preliminary evidence suggests that increased formation of conjugated dienes and/or hydroperoxyl groups in polyunsaturated fatty acid molecules is pertinent to gamma-linolenic acid-induced cytotoxicity (Takeda et al, 1993). Studies on human arterial smooth muscle cells indicated that moderately oxidised low density lipoprotein, which contained the highest concentration of lipid hydroperoxides, induced smooth muscle cell apoptosis within six hours, whereas other forms of low density lipoprotein had no effect (Siow et al, 1999). It has been

proposed that lipid hydroperoxides may stimulate cell death by inhibiting bcl_2-mediated rescue from apoptosis (Sandstrom, 1995 and Siow et al, 1999).

(d) Glioma radiosensitisation

The inherent radioresistance of human malignant glioma is a barrier to cure (Mansur et al, 2000). Research is therefore required to characterise therapeutic interventions which selectively increase the radiosensitivity of tumour cells while minimising toxicity to surrounding normal brain. A number of studies have suggested that essential fatty acids may fill both these criteria. Both radiation and essential fatty acids stimulate tumour reactive oxygen intermediate generation (Barber and Wilbur, 1959, Dennis and Shimbamoto, 1990 and Yamaguchi et al, 1994), and it has been demonstrated that potentiation of tumour oxidative activity is associated with tumour cytotoxicity (Karmali et al, 1984, Das et al, 1987 and Begin et al, 1988). The relevance of these studies to glioma radiosensitisation has been demonstrated *in vitro*. Gamma-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid decreased the clonogenic capacity of the 36B10 rat astrocytoma cell line following exposure to therapeutically relevant (0-10Gy) doses of radiation (Vartak et al, 1997), and a later study demonstrated that gamma-linolenic acid-mediated potentiation of radiosensitivity was associated with tumour reactive oxygen species formation (Vartak et al, 1998). However, these investigations did not evaluate the mode of cell death resulting from combined essential fatty acid and radiation exposure, and the kinetics of reactive oxygen species formation in these cells was not reported.

This study addressed these issues by demonstrating that arachidonic acid and gamma-linolenic acid synergistically stimulated oxidative and apoptotic activity in rat C6 glioma cells in conjunction with a therapeutically relevant dose of radiation (Chapter 7). Initially, the individual effects of n-6 essential fatty acids and radiation on glioma reactive oxygen species generation and apoptosis were investigated. Arachidonic acid and gamma-linolenic acid administration was associated with an early potentiation of oxidative activity (typically within the first three hours of the investigation). Conversely, radiation-induced stimulation of tumour reactive oxygen

species production typically occurred 6-10 hours post-irradiation. This suggested that radiation stimulated the induction of a reactive oxygen intermediate producing mechanism different from that induced by arachidonic acid and gamma-linolenic acid.

An early apoptotic response was also observed in C6 glioma cells exposed to arachidonic acid or gamma-linolenic acid alone. In comparison, radiation-induced stimulation of apoptosis occurred over a period of 12 hours and was maximal between 6 and 8 hours post-irradiation. This increase was observed when the stimulation of apoptosis induced by arachidonic acid or gamma-linolenic acid alone was low. These preliminary experiments suggested that arachidonic acid and gamma-linolenic acid and radiation may interact to potentiate reactive oxygen intermediate generation and apoptotic events sensitive to lipid peroxides and their products.

These results provide additional information on the controversy concerning the physiological and pathological roles of oxidative metabolism. A pathological role for oxygen based free radicals has been proposed following exposure to ionising radiation and cytotoxic drugs (Halliwell and Gutteridge, 1985). Ultraviolet light, X-rays and gamma-radiation stimulate the formation of reactive oxygen intermediates including the hydroxyl radical, which has been implicated in the formation of DNA lesions known to increase genomic instability (Boon et al, 1984 and Drecher and Junod, 1996). It has also been proposed that free radicals formed *in vivo* attack biological molecules such as lipids, carbohydrates, proteins and DNA to induce membrane damage, denaturation of protein, inactivation of enzymes, DNA strand breakage and modification of DNA bases, which may eventually cause a variety of pathological events including cancer and ageing (Niki et al, 1991, Davies, 1991, Sies, 1991 and Halliwell and Gutteridge, 1989).

The hypothesis that reactive oxygen intermediates are implicated in the initiation of malignant transformation would predict that provision of essential fatty acids would

potentiate cell damage and tumour proliferation by increasing the availability of substrate upon which free radicals can act. However, *in vivo* and *in vitro* evidence has indicated that essential fatty acids decrease tumour proliferation (Gonzalez, 1992) and stimulate apoptosis (Lai et al, 1996 and deKock et al, 1996). Additionally, it has been shown that gamma-linolenic acid reduced radiation-induced damage to porcine epidermis (Hopewell et al, 1993). An alternative hypothesis has been proposed which states that while reactive oxygen intermediates may mediate some degree of cell damage, in many situations it is free radical-induced loss of membrane essential fatty acids which produces much of the pathology (Horrobin, 1991).

The present study supports the hypothesis which suggests a therapeutic role for essential fatty acid-mediated stimulation of glioma oxidative activity. However, additional studies are required to evaluate the combined effects of radiation and essential fatty acid administration in untransformed human brain tissue. Although an *in vitro* study has indicated that the radiosensitising properties of gamma-linolenic acid are not associated with normal cell toxicity (Vartak et al, 1997), and results presented in this thesis indicate that tumour-associated normal brain reacts to essential fatty acid stimulation with lower reactive oxygen species production in comparison with human glioma tissue (Williams et al, 1997), additional research is required to further clarify the role of oxidative metabolism in human brain *in vivo*.

8.4 Summary

The following findings and conclusions have been made:-

1) Analysis of the peroxidative activity of cell preparations derived from fresh human glioma samples and tumour-associated normal brain indicated that basal cellular oxidation was significantly lower in tumour cells ($p<0.0004$-0.00001),

2) Arachidonic acid and gamma-linolenic acid stimulated glioma oxidation. Statistical analysis of the kinetics of the essential fatty acid induced response suggested that more aggressive tumours, for example glioblastoma multiforme, responded in a more rapid and sustained manner than less malignant tumours e.g. astrocytomas.

3) Preliminary immunohistochemical and flow cytometric analysis indicated a group of cells which was identified as the tumour sub-population. These cells were characterised by low oxidative activity but high sensitivity to exogenous arachidonic acid and gamma-linolenic acid.

4) Arachidonic acid and gamma-linolenic acid were not associated with significant loss of cell membrane integrity, as measured by the uptake of vital dyes, suggesting that forms of cell death other than necrosis are responsible for mediating the cytotoxic activities of arachidonic acid and gamma-linolenic acid.

5) FlowTUNEL analysis of human primary brain tumour preparations indicated that arachidonic acid and gamma-linolenic acid stimulated apoptosis over 24 hours. Long term cell culture experiments with the rat C6 glioma cell line demonstrated dose-dependent episodes of increased apoptotic activity over a period of 7 days.

6) There was evidence of a synergistic stimulation cellular oxidation and apoptotic activity by radiation and exogenous arachidonic acid and gamma-linolenic acid.

Peaks in oxidative activity may correlate with transient reductions in cell viability, as measured by the uptake of vital dyes.

8.3 Conclusions

This study has provided evidence that glioma tissue has low basal oxidative activity in comparison with tumour-associated normal brain. These observations support the findings of Cheeseman et al (1984) and Bartoli and Galeotti (1979) who described low levels of basal oxidative activity in breast and hepatic tumour tissue. Exogenous arachidonic acid and gamma-linolenic acid stimulated peroxidative activity and apoptosis in a grade dependant manner (Chapter 4). This finding is in agreement with previous reports that essential fatty acids stimulate cellular oxidative activity (Yamamoto, 1985, Porter, 1986, Esterbauer et al, 1987 and Meydani et al, 1990).

Studies on the cellular heterogeneity of human glioma samples have indicated that the stimulation of reactive oxygen intermediate by exogenous arachidonic acid and gamma-linolenic acid occurred in GFAP-positive cells (Chapter 5). This finding provided further evidence supporting the hypothesis that human glioma cells possess high sensitivity to exogenous arachidonic acid and gamma-linolenic acid. Additionally, cells possessing high side angle scatter, which was indicative of GFAP positivity, rapidly underwent apoptosis when stimulated with exogenous arachidonic acid and gamma-linolenic acid. These findings are consistent with the hypothesis that sensitivity to exogenous essential fatty acids is inversely related to cellular proliferative activity (Horrobin, 1990).

Phagocyte populations from lung cancer and malignant glioma patients have also been analysed, and there is evidence that they responded with increased reactive oxygen intermediate production to arachidonic acid and gamma-linolenic acid (Chapter 3). The magnitude of this increase was generally less than that observed for human glioma tissue (Chapter 5).

Concentrations of arachidonic acid and gamma-linolenic acid shown to stimulate oxidative activity induced apoptosis in human glioma tissue and the rat C6 cell line. This finding concurs with the observation that gamma-linolenic acid induced apoptosis in human cervical carcinoma (deKock et al, 1984). Preliminary evidence suggested that this activity was also associated with inhibition of glioma proliferation (Chapter 6). Gamma-linolenic acid had previously been shown to inhibit the proliferation of a glioma cell line *in vitro* (Das et al, 1990). In addition, potentiation of the oxidative and apoptotic response of the rat C6 cell line to exogenous arachidonic acid and gamma-linolenic acid has been demonstrated in the presence of a therapeutically relevant dose of radiation (Chapter 7). Analysis of the kinetics of this stimulation indicated that radiation and exogenous arachidonic acid and gamma-linolenic acid stimulated oxidation and apoptosis at different phases of the 12 hour incubation period. However, radiation and arachidonic acid and gamma-linolenic acid may interact to potentiate the stimulation of peroxidative and apoptotic activity. These results concur with those of Vartak et al (1997 and 1998) who demonstrated that gamma-linolenic acid inhibited the clonogenic capacity of a rat astrocytoma cell line in conjunction with radiation.

These results are consistent with a potential therapeutic role for arachidonic acid and gamma-linolenic acid in the management of malignant glioma. Additional research is necessary to further characterise the pathways which mediate these cytotoxic activities. Additionally, the response of malignant glioma to exogenous essential fatty acid administration *in vivo* is not well characterised. Additional research is necessary to investigate behavioural and toxic side effects of essential fatty acid administration *in vivo*, in addition to biochemical and neuropathological responses.

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Publications

The following publications are a result of work presented in this thesis.

1) Arachidonic Acid in the Reticuloendothelial Network. H.A. Leaver, J.R. Williams, I.M. Dawson and P.L. Yap. Biochemical Society Transactions Symposium: Lipids in Immune Function and Inflammatory Disorders 297-302, 1995.

2) Role of Calcium in Arachidonic Acid Stimulated Cellular Cytotoxicity and Superoxide Generation. H.A. Leaver, P.L. Yap, S. Janah, A. Gregor, J. R. Williams and J. Ironside. European Journal of Clinical Investigation, 25: Supplement 2; 117, 1995.

3) Arachidonic Acid and Gamma Linolenic Acid Stimulate Peroxidation in Human Primary Brain Tumours. J.R. Williams, H.A. Leaver, J.W. Ironside, G. Malcolm and A. Gregor. Abstract for the British Neuro-oncology Meeting, University of Sheffield, 22-23 June, 1995. Neuropathology and Applied Neurobiology, 1995.

4) Stimulation of lipid peroxidation in glioma cells by essential fatty acids: A Novel Cytotoxic Strategy? J.R. Williams, H.A Leaver, G. Malcolm, A. Gregor and J.W. Ironside. Abstract for the British Neuropathological society, Royal College of Physicians, London, 10-12 January, 1996. Neuropathology and Applied Neurobiology, 22, 145, 1996.

5) Anti-proliferative effects of Arachidonic Acid on the C6 cell line and human glioma tissue. J.R. Williams, H.A. Leaver, J.W. Ironside, E.P. Millar, G.P. Malcolm, I.R. Whittle and A. Gregor. Journal of Neuro-Oncology, 30, 107, 1996.

6) Arachidonic Acid pathophysiology in human phagocytes and primary brain tumours. H.A. Leaver, J.R. Williams, S..R. Craig, A. Gregor, J.W. Ironside, I.R. Whittle and P.L Yap. European Journal of Haematology, 57, 76, 1996.

7) Arachidonic acid metabolism and activation of monocyte and neutrophil reactive oxygen in lung cancer patients undergoing pulmonary resection. H.A. Leaver, S.R. Craig, P.L Yap, J.R. Williams and W.S. Walker. Biologicals, 24, 319-324, 1997.

8) Apoptosis in human primary brain tumours: Effects of n-6 essential fatty acids. J.R. Williams, H.A. Leaver, J.W. Ironside, A. Gregor and I.R. Whittle. Journal of Neuro-Oncology, 35, 177, 1997.

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10) Analysis of arachidonic acid pathophysiology in human phagocytes and primary brain tumours. Leaver A, Williams J.R., Craig S.R., Gregor A., Ironside J.W. and Whittle I.R. Annals of the New York Academy of Science, 823, 200-214, 1997.

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12) Apoptosis in human gliomas: actions of arachidonic acid. Williams, J.W., Leaver, H.A., Ironside, J.R., Gregor, A., Miller, E.P. & Whittle, I.R. Prostaglandins Leucotrienes and Essential Fatty Acids, 58, 193-200, 1998.

13) Dynamics of reactive oxygen intermediate production in human glioma: n-6 essential fatty acid effects. Leaver, H.A., Williams, J.R., Ironside, J.W., Miller, E.P. Gregor, A., Su, B., Prescott, R.J. and Whittle, I.R. European Journal of Clinical Investigation, 29, 220-231, 1999.