CHAPTER 1
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1.1 BACKGROUND

In the last decade of the 20th century most developed countries have an aging population and as a consequence, the numbers of people at high risk from cancer are increasing rapidly. After cardiovascular disorders, cancer is now the second major cause of death in the western world, and the most feared disease. Despite significant progress in the area of cancer treatment, there is also increasing awareness of the importance of early detection and diagnosis of malignant disease. Predicting the clinical course of the individual patient is necessary to provide the basis for rational treatment. The traditional gold standards for such assessment, namely histopathological typing and grading of the malignancy and clinicopathological staging, are subjective and often inaccurate, therefore a new and objective technique of tumor detection and prognosis needs to be developed.

Tumor typing and grading are based on a combination of microscopic observations of cytologic and architectural deviation from the assumed normal tissue of origin, nuclear pleomorphism and mitotic activity of cells within a tumor. Staging systems provide precise anatomical descriptions of the spread of tumors through local tissues and their more distant spread at a particular time, as well as details regarding involvement of local and distant lymph nodes and presence of metastases. Both grading and staging are important as the latter does not distinguish between older, more slowly growing tumors and young aggressive ones. Both assessments are associated with inaccuracies of sampling errors (particularly when examining lymph nodes, since a large volume of tissue is required) and both may be influenced by subjective decision of the histopathologist examining a biopsy specimen.

Because of many inadequacies involved in the classical approach to tumor diagnosis, some new methods are being developed. These are based on monitoring changes in cell biology and biochemistry which occur during the process of tumor development and progression. Immunological markers, detecting either the absence of tissue-specific or the presence of tumor-associated antigens, have been developed for breast and colorectal cancers (Trojani and deMascarel, 1987; Yuan, 1990). Flow cytometry shows percentage of cells in the proliferative phase of the cell cycle, which is usually high in tumor tissue (Fielding *et al.*, 1992). A particularly accurate technique for detecting gene mutation, polymerase chain reaction (PCR), has also been used in cancer diagnosis (Fielding *et al.*, 1992), and its variant, mutant-allele amplification can aid conventional histological staining for colorectal micrometastasis detection in lymph nodes (Hayashi *et al.*, 1995).

Another method of investigating chemical changes involved with tumor development and progression is magnetic resonance spectroscopy (MRS). It has been demonstrated that MRS can distinguish preinvasive from invasive malignant lesions, identified by histopathology in many organs, including colon (Lean *et al.*, 1993; Ende *et al.*, 1996), cervix (Delikatny *et al.*, 1993), thyroid (Mackinnon *et al.*, 1996), ovary (Mountford *et al.*,1987), breast (Singer *et al.*, 1995; Mountford *et al.*, 1997), prostate (Kurhanewicz *et al.*, 1993; Hahn *et al.*, 1997; Swindle *et al.*, 1998), brain (Yoshino *et al.*, 1996) and lymph nodes (Mountford *et al.*, 1993). MRS can also be applied to detect some pathological changes that were not detectable by conventional histopathological assessment (Lean *et al.*, 1993; Delikatny *et al.*, 1993; Mountford *et al.*, 1993; Mackinnon *et al.*, 1996; Yoshino *et al.*, 1996).

Colorectal cancer (CRC) in Australia is the most common internal malignancy in men and women combined. It has been estimated that 4% of the population will develop this cancer in their lifetime. On a world wide basis CRC has been ranked as the third or fourth most common cancer (15% of all cancers). While the genesis of some colonic cancers is still unknown, many are preceded by benign polyps (Hill, 1991). This fact has led to a widespread policy of colonoscopic polypectomy for the prevention of CRC. There is also increasing experimental, histological, epidemiological and genetic evidence supporting the concept of an adenoma-carcinoma sequence in the progression and development of colon tumors (Carrel *et al.*, 1976; Leibovitz *et al.*, 1976; Brattain *et al.*, 1983; McBain *et al.*, 1984; Whitehead *et al.*, 1985; Fearon and Vogelstein, 1990; Paraskeva *et al.*, 1990; Williams *et al.*, 1990; Hill, 1991; Mackinnon *et al.*, 1994).

It is now possible to simulate colorectal tumor development and progression *in vitro* using cultured human colorectal cell lines with known growth characteristics and varying degrees of differentiation, tumorigenicity, metastatic potential and genetic abnormalities (Leibovitz *et al.*, 1976; Paraskeva *et al.*, 1990; Goyette *et al.*, 1992). The well documented evidence on confirmation of MRS data taken from excised colorectal tissues (where conventional grading and clinical staging is recorded) by studies on *in vitro* cultured colorectal cell lines (Lean *et al.*, 1992, 1993; Singer *et al.*, 1993; Mackinnon *et al.*, 1994; Ende *et al.*, 1996) shows a major advantage of using cell models to investigate the adenoma-carcinoma sequence. It also allows multiple studies to be carried out on well defined samples in controlled and/or experimentally modified conditions.

To provide evidence for the adenoma-carcinoma sequence in colorectal cancer progression using MRS, a model consisting of six human colorectal cell lines: PC/AA, PC/AAC1, PC/JW, SW1222, SW480, SW620, ranging from non-tumorigenic, well-differentiated adenoma to highly malignant, poorly differentiated carcinoma (Mackinnon *et al.*, 1994) was examined. These studies demonstrated that changes in lipid, carbohydrates and other metabolites correlate with the degree of malignancy and differentiation within the cell line sequence. In particular MRS showed that reduced differentiation in the cell model correlated with an increase in the levels of lipid, choline metabolites, the glycosylation intermediate UDP-GlcNAc and cell-surface fucosylation. However most of the cell lines used in this model had different origins (were not interrelated) and varied in their tumorigenic potential. Therefore factors other than the degree of cellular differentiation, like the expression of their genotypic variations and differences in tumorigenicity, could have possibly effected the MR-visible changes. To address this problem, a study involving a comparison of MRS data obtained from one cell line at different stages of cellular differentiation was designed for the purpose of this thesis.

1.2 CHEMICAL CHANGES IN MALIGNANT CELLS

1.2.1 Changes in plasma membrane properties and glycosylation

The plasma membrane, being the barrier separating intra- and extracellular environments**,** plays a significant role in the behaviour of cells. In particular, it is involved in regulating physiological processes and features like cell shape, growth rate, cellular recognition, communication, adhesiveness, migration, drug resistance, metastatic

spread and immunological competence. Generally, the cell membrane is composed of three chemically different classes of molecules: lipids (organized in phospholipid bilayers or incorporated in the membrane as distinct structures); proteins (attached to the lipid fraction with hydrophobic bonds formed between non-polar amino acid residues and acyl chains); and carbohydrates (attached covalently either to lipid or protein molecules and exposed to the aqueous environment).

The liquid-crystalline mosaic model of biological membranes, summarized by Singer and Nicholson (1972), considers the membrane as a "fluid" ***lipid*** bilayer into which proteins are inserted. Mobility of molecules within the membrane is restrained by mutual interactions. There are many reports on the increase in membrane fluidity that results from tumor progression (Nicolau *et al.*, 1974; Shinitzky and Inbar, 1976; Kier, 1990). The spectroscopic methods used to demonstrate and measure plasma membrane "fluidity" include magnetic resonance and fluorescence techniques. However, great caution needs to be taken when interpreting and comparing different measurements because of the variety of parameters contributing to the term "fluidity" of a given molecule or chemical group. Orientational order (static, time-averaged quantities) and microviscosity (dynamic quantities determinable by correlation and relaxation times) are two concepts which, in particular, should be kept apart. By not taking into account that rotational diffusion is hindered to some extent, and that the equilibrium orientational distribution function is anisotropic, some published biological membrane 'fluidity' values measured by fluorescence depolarization are incorrectly elevated. Heyn (1979), however, states that after appropriate corrections these data agree well with those determined by MR techniques.

Plasma membrane composition, especially phospholipid:cholesterol and polyunsaturated:monounsaturated phospholipid ratios as well as the presence of lipid domains also contribute to alterations in the molecular mobility in tumor cell membrane components. Tumor and activated cells are usually characterized by a lower proportion of polyunsaturated:monounsaturated fatty acids (Schroeder and Gardiner, 1984; Reynier *et al.*, 1991; Mc Donagh *et al.*, 1992). There is no evidence for a consistent change in cholesterol concentration in malignant cells, however an increased level of cholesterol and other lipid metabolites (choline, phosphoryl choline, ethanolamine and glycerophosphocholine) indicates fast lipid turnover associated with intense proliferation.

An increase in ***cholesterol, cholesteryl esters and triacylglycerols*** in tumor tissue is often observed (Lin *et al.*, 1978; Madesh *et al.*, 1996). Triglycerides and cholesteryl esters were not originally associated with plasma membranes. However, in a few cases of proliferating cells these neutral lipids were recognized as membrane components, *e.g.* in mitochondrial membrane of rat hepatocytes, leukemic lymphoblasts, melanoma, neuroblastoma, chinese hamster ovary cells (Mountford *et al.*, 1984; Schroeder and Gardiner, 1984; Harsas *et al.*, 1985; Chakravarthy *et al.*, 1985; Mackinnon *et al.*, 1992). Neutral lipids, di- and triacylglycerols are non-bilayer forming (Gorrissen *et al.*, 1982). Within physiological temperature ranges, multiple broad transitions accompanied by non-ideal mixing behaviour indicate that biological membranes consist of domains of gel-phase and liquid crystalline lipids. It was postulated that domains composed of isotropically tumbling neutral lipids may cause a local increase in membrane lipid mobility found in activated lymphocytes and leukemic lymphoblasts (Mountford and Wright, 1988).

Carbohydrate chains, being most extended from the cell surface, play a very important role in the processes of neoplastic development and progression. Consequently they are the most widely investigated species in relation to malignant disease. Glycosylated species on the cell surface are expressed mainly as glycoconjugates (glycolipids, glycoproteins and glycosaminoglycans). These molecules consist of a backbone (lipid or hydrophobic protein) and a carbohydrate chain of varying length and branching structure. One form of a lipid anchor involves the covalent attachment of a glycosyl-phosphatidylinositol (GPI) glycolipid moiety to the C-terminus of protein. Some plasma membrane-associated enzymes (alkaline phosphatase, aminopeptidase P) belong to this class of proteins (often referred to as ‘glypiated’ or PIG-tailed proteins). It is hypothesized that PIG-tailed proteins may have a role to play in cell signalling by being released from cell surface after PLC-cleavage of the GPI anchor (Turner, 1994).

The lipid backbone and usually protein molecules are incorporated into the plasma membrane, while the glycosylated parts are exposed to the environment on the extracellular (rarely on the intracellular) sides of the membrane. Here they play the roles of receptors aiding in signal or substance transmission across the cell membrane, and/or antigens expressing or masking cell identity. The big carbohydrate parts of glycoconjugates consist of a core region, a backbone and a peripheral region.

Sialic acid (*N*-acetyl neuraminic acid, NANA) was early associated with the surface glycosylation of malignant cells. It was first attributed to membrane glycoprotein, and later also to glycolipid determinants (Van Beek *et al.*, 1973; Warren *et al.*, 1975; Krishnaraj *et al.*, 1982). The unique structural features of the sialic acid molecule (negative charge due to a carboxyl group) enable it to play a role in cellular functions

such as the transport of positively charged compounds, cell-to-cell repulsion, influence on the conformation of glycoproteins of cell membranes, and even masking antigenic determinants on receptor molecules (Narayanan, 1994).

Except on the cell surface, increased levels of sialic acid-containing glycolipids were found in the serum of tumor-bearing humans and animals (Kloppel *et al.*, 1977; Tewarson *et al.*, 1993). The shedding of tumor-associated antigens with plasma membrane fragments from the cell surface has been demonstrated in different cell systems, including sialic acid-rich gangliosides (Chatterjee and Kim, 1977; Ladisch *et al.*, 1992). This process is correlated with the metastatic capacity of cells (Schirrmacher and Barz, 1986). It is theorised that sialic acid has a role in blocking the host's immune reaction against a tumor, and therefore in promoting metastasis. This theory is strongly supported by the presence of sialylated glycoconjugates in the blood and by the fact that the sialic acid residues hide the expression of tissue-specific antigen determinants (Ladisch *et al.*, 1992). Moreover, studies *in vivo* and *in vitro* showed the importance of sialic acid in the adhesion of cells to matrix (Yamada *et al.*, 1995; Fang *et al.*, 1997).

The amount and type of sialylation of tumor cell membranes depend on the activity of a number of different sialyltransferases. Abnormally high levels of these enzymes were found in cells and serum of tumor-bearing patients (Papadopoulou-Boutis *et al.*, 1985). Increased activity of specific sialyltransferases depends on the stage of tumor progression. N-glycan- specific α(2-6)-sialyltransferase and Gal(β2-6)GlcNAc - specific α2,6-sialyltransferase were significantly increased in metastasizing colon and brain carcinoma, but a Gal(β1-3)GalNAc-specific sialyltransferase was increased in nonmalignant tumors (Kemmner *et al.*, 1994; Kaneko *et al.*, 1996). Increased sialylation

of the cell surface is not malignancy-specific. It does not distinguish between growth processes such as the regeneration of liver or breast during pregnancy and lactation and neoplastic growth of these organs (Khadapkar *et al.*, 1975). Increased sialylation is also present in inflammatory diseases (Narayanan, 1994). Sialic acid measurements, however, have value in comparing tumors at different stages of development, *e.g.* monitoring cancer patients during treatment.

Fucose has been known for a long time to be a component of the glycocalyx of malignant cells. Fucose-containing glycolipids were found in adenocarcinoma cells and their immunological homology with blood-group substances was demonstrated by Hakomori *et al.* (1967). Fucosylated glycoproteins were also found on the surface of HeLa cells (Bosmann *et al.*, 1968). Changes in surface fucose-labeled glycoproteins were associated with the tumorigenic potential of transformed cells (Smets *et al.*, 1976). Studies involving labelling of membrane glycoproteins with radioactive fucose showed a relationship between structural and qualitative changes in these molecules and the process of cellular differentiation (Muramatsu *et al.*, 1978; Herscovics *et al.*, 1980).

The role of fucose in the process of metastasis is more obscure than that of sialic acid. Increased cell surface fucosylation or incorporation of labelled fucose was found in metastatic variants of many tumors or transformed cells (Guy *et al.*, 1980; Schwartz *et al.*, 1984; Wright *et al.*, 1988; Bruyneel *et al.*, 1990; Shirahama *et al.*, 1993). However, some studies involving the binding of fucose-specific lectins, *Lotus tetragonolobus* and *Ulex europeus I*, indicate the opposite tendency (Walker, 1984; Walker *et al.*, 1986; Finne *et al.*, 1989).

Fucose occupies terminal positions on carbohydrate structures and is enzymatically incorporated by specific fucosyltransferases (Bosmann *et al.*, 1968). There are specific receptors for these enzymes on plasma membranes determining the exact patterns of fucosylation of cell surface antigens. Differences in the activities of these enzymes correlate with the degree of cellular differentiation in hepatomas with varying growth rates and are accompanied by changes in the levels of fucosidases, GDP-fucose and CMP-N-acetylneuraminic acid (Bauer *et al.*, 1977). Decreased activities of fucosyltransferases accompany the process of differentiation of many malignant cell systems (Muramatsu and Muramatsu, 1983; Ronquist and Nou, 1983; Ambros and Kurman, 1993).

1.2.2 Changes in cellular metabolism

A difference in ***carbohydrate metabolism*** between normal and tumor cells due to increased glycolytic rate after transformation has been known since Warburg's works in the 1920s (Warburg, 1930). An increased rate of aerobic and anaerobic glycolysis was observed as characteristic of progressively malignant cells. In the control of aerobic glycolysis certain enzymes (phosphofructokinase, hexokinase, ATP-ase) are involved, whose expression is altered in tumor cells (for review see Wenner and Tomei, 1972). Fast-growing neoplastic cells need to adjust their glycolysis rate to cover their elevated energy requirements. Increased glucose uptake by virally transformed cells was demonstrated by kinetic studies to be due to an increase in the number of transport sites on the cell membrane, rather than to a conformational modification of these sites (Kletzien and Perdue, 1975). The higher rate of glycolysis in malignant cells prevents full oxidation of the glycolysis product, pyruvate in the citric acid cycle. It is, therefore, converted into lactic acid by lactate dehydrogenase, an enzyme which sometimes

increases in activity with tumor progression (Beck *et al.*, 1979; Dobrossy *et al.*, 1980).

Lactate accumulating in malignant tissues also modifies activities of other enzymes. In tumors, however, pyruvate kinase, the rate limiting enzyme for the last steps of glycolysis, has a high tolerance of lactic acid and is adapted to producing energy from either glucose or amino acids, depending on substrate and oxygen supply (Eigenbrodt and Reinacher, 1986).

Cancer cells rely to a greater degree than normal cells on the energy-generating pentose phosphate pathway (PPP), as an alternative to glycolysis, as was shown by elevated activity of the enzymes of the PPP (transaldolase and glucose-6-phosphate dehydrogenase) in the liver (Heinrich *et al.*, 1976; Hacker *et al.*, 1982).

Another modification of neoplastic metabolism is the high rate of ***glutamine*** utilization (Ardawi and Newsholme, 1983; Brand *et al.*, 1986; Galons *et al.*, 1989) which, after conversion to glutamate in glutamate-glutamine cycle, can be transformed into glucose and glycogen and used as an additional source of energy. Glutamine, together with fructose, is a substrate in the synthesis of *N*-acetyl neuraminic acid.

Hexose nucleotides are intimately involved in the glycosylation of membrane proteins and lipids. *N*-acetylated UDP hexoses, UDP-GlcNAc and UDP-GalNAc were found to accumulate in HT-29 human colon cancer cells in culture, but only in those cells that did not differentiate (Wice *et al.*, 1985). Other sugars, namely GDP-Man, CMP-Neu5Ac and UDP-GlcUA were less affected by differentiating conditions (glucose withdrawal). Even CMP-Neu5Ac, which is synthesized from glucose, did not change in

concert with rapid glucose changes. This suggests a unique role of those two glycosylation precursor UDP-hexoses in the process of enterocytic differentiation.

UDP-hexoses are also involved in carbohydrate metabolism, particularly, in human tissues UDP-Glc is an intermediate in the hydrolysis and synthesis of glycogen. The conversion of UDP-GalNAc into UDP-GlcNAc is catalysed by an enzyme epimerase.

Many of the chemical changes described above are related to changes in MR spectra of tumor as distinct from normal colorectal cells and tissues, and are described in section 1.5.3 of this introduction.

1.3 CELLULAR DIFFERENTIATION

1.3.1 Biological significance of differentiation

The human organism consists of about one hundred different types of cells, all of which derive from one cell - the zygote. The process of specialization of cells into different types which express phenotypic properties of functionally mature cells is called cellular differentiation. This does not imply that the process is complete or that it is irreversible (Freshney, 1987 p.187). At least some stages of the differentiation processes in ontogenesis are irreversible. Differentiation usually leads to the cessation of proliferation and decrease in the developmental potential of cells, limiting the number of specialized encoded functions to a few or even one. From the point of view of the developmental potential of cells undergoing differentiation one can distinguish three

types of differentiation: primary differentiation taking place in the embryonal phase, intermediate differentiation in the foetal phase and terminal differentiation which can take place in post-natal life. Terminal differentiation implies that a cell has progressed down a particular lineage to a point at which the mature phenotype is fully expressed and beyond which the cell cannot progress (Freshney, 1987 p.187).

Phenotypical changes resulting from differentiation are associated with certain phases of the mitotic cycle, during which some specific modifications in the genome may occur. These changes include modification of receptors on the cell surface, alterations to chromatin conformation, or exchange of chromosomal proteins. Cells usually undergo differentiation when entering the G_0 through G_1 cell cycle phase (nuclei containing 2C DNA), or, less often, when entering the G_0 through G_2 cell cycle phase (nuclei containing 4C DNA) (Dell' Orco *et al.*, 1975; Tobey and Hildebrand, 1977).

Differentiation processes in adult human organism follow two main pathways:

1. A small population of multipotent or pluripotent undifferentiated stem cells gives rise to committed precursor cells which progress towards terminal differentiation and lose their capacity to divide. This process is typical where continual renewal is required, such as in the hemopoietic system and epithelial tissues (skin, gastric mucosa).
2. Re-differentiation following dedifferentiation is a process, which occurs after response to a trauma, such as wound repair or liver regeneration. It requires re-induced proliferation of previously differentiated cells.

The functioning of such populations is very dynamic. Therefore any, even small, error in regulating these proliferation processes in renewing or regenerating cell systems

causes pathological states, such as neoplastic transformation.

Dedifferentiation is the loss of the specific phenotypic properties associated with mature cells, which occurs when a tissue becomes malignant or when it is grown in culture. Dedifferentiation *in vivo* also occurs as a normal process when cells (like fibroblasts) lose some of their differentiated properties and re-enter the cell cycle responding to a local reduction of cell density and/or the presence of growth factors.

After the tissue has regained an appropriate cell density, differentiation is reinduced. This is not the case, however, in neoplastic tissues (at least in more advanced stages of tumor development) where, after losing the differentiated state, cells usually stop responding to physiological differentiation controlling factors. Tumor cells may often retain the ability to respond in some conditions to inducers of differentiation, though not always those active on normal cells (Cole and Paul, 1966; Gross and Goldwasser, 1971).

1.3.2 Regulation of differentiation

There are four groups of factors controlling differentiation of cells:

1. Cell-cell interactions between homologous (same origin) or heterologous cells occur optimally at high cell density. Following gastrulation, the mutual communication between cells originating in different layers initiates and promotes differentiation (Cooper, 1965; Taderera, 1967). Communication by exchange of metabolites, second messengers (cAMP), or electrical charge via gap junctions harmonizes the differentiation within a population of similar cells (Finbow and Pitts, 1981).

2. Matrix interactions - the contact of a complex mixture of glycoproteins and proteoglycans surrounding the surface of most cells with the extracellular tissue-specific matrix regulates differentiation *in vivo*. Construction of different artificial matrices and surfaces *in vitro* can regulate gene expression (Reid, 1990) and cellular differentiation (Yang *et al.*, 1979; Folkman and Haudenschild, 1980).
3. Cell polarity and shape also have an effect on the access of specific nutrients and signals to different parts of the cell. Therefore they may act in maintaining differentiated state *in vitro* (Sattler *et al.*, 1978; Chambard *et al.*, 1983).
4. Soluble chemical factors
    - Physiological differentiation inducers vary for different cell types and may be active in some *in vivo* systems as well as *in vitro*. They include established endocrine hormones *e.g.* hydrocortisone for glioma (McLean *et al.*, 1986), hepatocytes (Granner *et al.*, 1968), mammary epithelium (Stockdale and Topper, 1966), myeloid leukemia cells (Sachs, 1978); second messenger cyclic AMP for neuroblastoma (Prasad *et al.*, 1972); paracrine factors released by adjacent cells *e.g.* TGF-β for bronchial epithelium (Lechner *et al.*, 1981), prostaglandins for myelocytes (Fuller and Meyskens, 1981); vitamins such as vitamin D_3 for myeloma cells (Murao *et al.*, 1983), retinoic acid for bronchial (Wu and Wu, 1986) and tracheal epithelium (Klann and Marchok, 1982), melanoma (Meyskens and Fuller, 1980) and myeloid leukemia (Breitman *et al.*, 1980) or vitamin E for neuroblastoma (Prasad *et al.*, 1980); inorganic ions, particularly Ca^{2+} promoting keratinocyte differentiation (Boyce and Ham, 1983); and other substances like butyric acid coming from bacterial fermentation of fibre in gut and inducing differentiation of erythroleukemia (Sato *et al.*, 1979) and colorectal carcinoma (Augeron and Laboisse, 1984; Chung *et al.*, 1985).

- Non-physiological inducers are widely used in cell culture techniques. The first nonphysiological compound discovered to trigger differentiation was dimethylsulfoxide used for murine erythroleukemia (Rossi and Friend, 1967), and subsequently for other cell systems, like myeloma (Tarella *et al.*, 1982), neuroblastoma (Kimhi *et al.*, 1976) and mammary epithelium (Rudland *et al.*, 1982). Other differentiation inducers belonging to this class include *N*-methyl acetamide and *N*-methyl formamide both of which are effective in glioma cells (McLean *et al.*, 1986), or dimethyl formamide in colon cancer (Dexter *et al.*, 1979), tumor promoters such as TPA used for squamous cell differentiation (Willey *et al.*, 1984), and many other substances like cytotoxic drugs, benzodiazepines, or synthetic derivatives of physiological inducers.

Not all the mechanisms of action of the soluble differentiation inducers are known. It has been established that differentiation inducers act through regulation of gene expression, in many different ways, *e.g.* histone hyperacetylation (butyrate: Miyashita *et al.*, 1994), changes in DNA methylation (polar compounds like DMSO: Rifkind *et al.*, 1983), and this causes other changes in cell phenotype. These changes are often referred to as markers of differentiation. They include physiological markers (slower metabolism, blocked proliferation), morphological markers (altered composition and structure of cytoskeleton accounting for cell shape and size, as well as appearance of tissue-specific membrane structures and properties), and biochemical markers of differentiation (expression of specific cell products or enzymes, growth factor receptors, loss of tumor-specific antigens and appearance of other antigens specific to a given tissue).

The colorectal tissue-specific markers of a differentiated phenotype are adressed in the

following section (1.4) and will be investigated on the SW620 cell line in chapters 3 and 6.

1.4 COLON NEOPLASM AND DIFFERENTIATION

1.4.1 Cytology of the colon

The colon wall consists of three layers: mucosa (a single layer of epithelium lining the vertical crypts, containing lymphoid follicles), submucosa (loose connective tissue, lymphatic channels, blood vessels, neural network and a variety of cells, mainly lymphocytes) and the muscularis propria (made up of inner circular and outer longitudinal layers).

The normal large intestinal epithelium is made up of three differentiated cell types: goblet cells, tall columnar cells and endocrine cells. Mucus secreting goblet cells have relatively large, vesiculate nuclei and mucin vacuoles in their cytoplasm. They by volume account for the largest proportion of the crypt epithelium, however numerically, they are outnumbered by tall columnar cells by a ratio of 4:1 (Neutra and Padykula, 1984). After discharging mucin, goblet cells take on the appearance of tall columnar cells (Shamsuddin and Trump, 1981). Tall columnar cells are slender in shape, have brush border or microvilli on their apical membrane, lack mucin vacuoles, yet are also active as glycoprotein secretors. Vesicles are seen by electron microscopy in the apex of each columnar cell (Shamsuddin *et al.*, 1982). Moreover, these cells are believed to have a role in absorption of water, lipid and other soluble compounds from the lumen. Endocrine cells are small and few in number, pyramidal in shape with a narrow apex which presents a small luminal surface covered with microvilli (Neutra and Padykula,

1984). They have argyrophil granules, basally situated beneath the nucleus, containing such substances as serotonin, polypeptides, glucagon and somatostatin.

Apart from differentiated cells, the crypts also contain stem cells (located at the crypt base, low cuboidal in shape with no specialized ultrastructural features) which make the proliferative compartment for the epithelial population of the whole crypt (Shamsuddin *et al.*, 1982).

1.4.2 Brush border-associated enzymes

The brush border is a common characteristic of differentiated columnar epithelial cells formed by numerous cylindrical extensions of the cell enclosed by plasmalemma exposed to a luminal space. Its primary function is the absorption of nutrients and ions from the intestine. Many digestive enzymes are associated with human intestinal brush border membrane: phosphatases, carbohydrases and peptidases.

A nonspecific ***alkaline phosphatase,*** active in neutral pH, was demonstrated histochemically in human intestinal epithelia more than 50 years ago (Gomori, 1941) and is widely used as a marker for brush border membranes. Its role is the digestion of phosphorylated compounds (phosphomonoesters) present in the diet. This enzyme may be a transporter for inorganic phosphate, since its inhibition caused decrease, and stimulation resulted in increase in phosphate uptake (Shiriazi *et al.*, 1978). Humphreys and Chou (1979) proposed that brush border HCO_3^--ATPase and alkaline phosphatase could be properties of the same enzyme, implicating a role for phosphatase in ion transport.

All dietary carbohydrates of nutritional importance have to be converted to their monomeric constituents before they can enter absorptive cells. In the gut, small oligomers produced by salivary and pancreatic amylases together with sucrose, lactose and trehalose are hydrolysed by the brush border-associated ***carbohydrases*:** maltase, sucrase, isomaltase, trehalase, lactase and β-glucosidase (Maestracci *et al.*, 1973). The most intensively investigated brush-border proteins, sucrase and isomaltase occur together as a glycoprotein hybrid of two polypeptide chains with separate active sites (Conklin *et al.*, 1975). They appear to act together because of their specificity: isomaltase is the only gastrointestinal enzyme capable of cleaving (α1-6) glycosidic linkages and sucrase complements this action by acting on (α1-4) glycosidic linkages of glucose containing disaccharides. The resultant free glucose is able to penetrate the brush border membrane.

The ***peptidases*** of the intestinal mucosa are the final step of the proteolytic pathway breaking down undigested peptides into amino acids which can be utilized by the host organism. Intestinal peptidases are located both in the brush border and the cytosol of columnar cells. Cytosol peptidases have very limited activity against molecules larger than tripeptides, while brush border enzymes can digest peptides containing up to six amino acid residues (Kim *et al.*, 1974). The identified intestinal brush border peptidases include aminopeptidases, a neutral endopeptidase, a dipeptidyl peptidase, and enterokinase.

γ-glutamyl transferase (γ-glutamyl transpeptidase) is found in many tissues including intestinal mucosa, but its highest abundance is in kidney cortex. This enzyme has a transferase activity and catalyses the transfer of the glutathione across the

plasma membrane. The γ-glutamyl moiety of glutathione when bound to several amino acids forms the γ-glutamyl dipeptide which can enter so-called γ-glutamyl cycle. Extracellular amino acid acts as the γ-glutamyl acceptor and the glutamylated peptide is transferred into the cell. The amino acid is released inside the cell by the action of a cyclotransferase and, through an enzymatic cycle requiring ATP, glutathione is regenerated in the cell (Meister *et al.*, 1979). γ-glutamyl transpeptidase, however, may not be a useful marker of cellular differentiation in colonic systems. Aviram *et al.* (1988), measuring the activity of this enzyme, found no significant difference between tumor and uninvolved tissue, while the alkaline phosphatase level was reduced in tumor specimens.

Three marker enzymes: alkaline phosphatase, sucrase and amino-oligo peptidase were, therefore, chosen as appropriate markers of differentiation in the colorectal cell model studied.

1.4.3 Short chain fatty acids in colon cancer prevention

Colorectal cancer is a major health problem in industrialized countries and its high incidence has been linked to diet. Epidemiological studies suggest that a low-fat and high-fibre diet is protective both for colorectal cancer and precursor adenomas (Burkitt, 1971; Howe *et al.*, 1992; Sandler *et al.*, 1993). Fibre can protect against colorectal cancer in many ways: increases the transit rate and bulk therefore decreasing exposure to carcinogens present in the diet; absorbs carcinogens; modifies intestinal microflora altering bile salt and carcinogen metabolism. Fibre fermented by intestinal bacteria increases the concentration of short-chain fatty acids (acetate, propionate and butyrate) in the large bowel (McIntyre *et al.*, 1993).

Butyric acid has two major roles within colonic epithelium (Hague *et al.*, 1996): it is an energy source, preferred over glucose and glutamine (Roediger, 1982; Rodriguez *et al.*, 1995); but it is also able to induce cellular differentiation (Augeron and Laboisse, 1984; Whitehead *et al.*, 1986; Gamet *et al.*, 1992) and apoptosis (Hague *et al.,* 1993; Heerdt *et al.*, 1994) of colonocytes. Jass (1985) proposed a hypothesis linking metabolism of the cell with its differentiation state, in which the initial deficiency in the butyrate level in colon is coupled with increased energy requirements of the neoplastic tissue. This promotes a switch to anaerobic metabolism, and also explains why malignant cells are sensitive to differentiating effects of butyrate. Apoptosis, being a physiological process by which cells effectively commit suicide, also regulates the number of epithelial cells in healthy colonic mucosa, therefore protecting from pathological neoplastic growth.

Contrary to the epidemiologic studies indicating protective role of fibre against colorectal cancer, some studies on colorectal tissues *in vivo* and *ex vivo* revealed that most fibre types and sodium butyrate have stimulatory effects on colonocyte proliferation (for review see Lupton, 1995). However, in these studies, nonmalignant tissues were taken for experiments, so their responsiveness can be expected to differ from even early neoplastic specimens. Also, results obtained from a rodent model may not be conclusive, taking into account that the natural diet of these animals, in particular the amount and type of fibre it contains, as well as physiology of their digestive processes are quite different from that of humans.

1.4.4 Colonic tumor development and progression

Any localized lesion projecting from the mucosa may be regarded as polyp. Polyps may arise from mucosal glands or from connective tissue. They can be neoplastic, hamartomatous*, inflammatory or of other origin. Neoplastic (adenomatous) polyps consist of dysplastic epithelium, but they do not necessarily exhibit malignant changes. They can be subdivided into tubular adenoma, tubulo-villous adenoma or villous adenoma (Talbot and Price, 1987).

The relationship between adenomas and carcinomas in the large intestine is yet to be established conclusively. Some authors support the view that the development of all colorectal cancers occurs from flat mucosa, the so called “de novo hypothesis” (Castleman and Krickstein, 1962). Yet the contrary view also exists, the so-called “polyp-only cancer hypothesis” of colorectal carcinogenesis which claims that all cancers of the large bowel arise from pre-existing polyps (Muto *et al.*, 1975; Fenoglio and Lane, 1974). A sub-hypothesis of the “polyp-only cancer hypothesis” suggests that only a minority of adenomas (which reach sufficient size and extent of dysplasia) become malignant (Williams and Bodenne, 1990), and therefore, there is no need for surgical intervention for most adenomatous polyps. There is now good evidence that many polyps become malignant if left untreated, this process being dependent on polyp size, villous content and degree of dysplasia as well as their numbers and growth rate (Muto *et al.*, 1975; O’Brien *et al.*, 1990; Sugarbaker, 1991; Hill, 1991). Muto *et al.* (1975) discounted any evidence for de novo colorectal carcinogenesis, while Hill (1991) pointed out the possibility of malignant transformation in microscopic adenomatous lesions, normally

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* Hamartoma - a focal malformation that resembles a neoplasm, grossly and even microscopically, but results from faulty development in an organ.

overlooked by routine diagnosis. Based on the “polyp-only cancer hypothesis” many *in vitro* models of colorectal cancer development via the adenoma-carcinoma sequence have been constructed (Carrel *et al.*, 1976; Leibovitz *et al.*, 1976; Brattain *et al.*, 1983; McBain *et al.*, 1984; Whitehead *et al.*, 1985; Paraskeva *et al.*, 1990; Mackinnon *et al.*, 1994).

1.4.5 Cell surface antigens associated with colon tumors

The fundamental assumption underlying the whole tumor immunology is that tumor cells carry antigenic specificities not found on corresponding normal cells. Existence of these tumor-specific antigens was convincingly demonstrated on human systems both *in vivo* and *in vitro.* The involvement of cellular glycosylated antigens in various malignant diseases is, however, incompletely understood. Aberrant glycosylation may be the basis of inappropriate cell/cell and cell/matrix interactions leading in tumor cells to antisocial behaviour such as uncontrolled proliferation, immunosuppression, invasiveness, metastatic potential and adhesion. It is now evidenced that specific cell surface glycoconjugates play a role in cell motility and metastasis (Stanford *et al.*, 1986; Miyake and Hakomori, 1991; Hakomori, 1991).

There are two major classes of glycosylated antigens expressed on the surface of colorectal cells (Hakomori, 1989):

! ***Glycolipid antigens*** have their oligosaccharide epitopes (β1-)attached to the ceramide via the hydroxyl group of sphingosine. In adult gastrointestinal and colorectal epithelia there are two types of lacto-series chain antigens:

- Type 1 chain: Gal(β1-3)GlcNAc(β1-3)Gal predominates over the type 2 chain

[Gal(β1-4)GlcNAc(β1-3)Gal] (McKibbin *et al.*, 1982). During their synthesis, type 1 chain undergoes fucosylation: either α1→4 at the penultimate GlcNAc to form Le^a or α1→2 fucosylation at the terminal Gal (Le^d), or co-occurrence of both processes to form Le^b. Both antigens Le^a and Le^b accumulate in various tumors in a higher quantity than can be found in normal colon epithelium, as their synthesis is enhanced in malignant cells. In tumors, the type 1 chain undergoes various enhanced reactions: α2→3 sialylation at terminal Gal and α2→6 sialylation at GlcNAc leading to two tumor-specific antigens which accumulate in various adenocarcinomas and epitheliomas (Fukushi *et al.*, 1986), which after subsequent α1→4 fucosylation, form sialyl-Le^a and disialosyl-Le^a , respectively (Figure 1.3.), also expressed specifically on colorectal cancer cells (Itzkowitz *et al.*, 1988 ; Hansson and Zopf, 1995).

- Lacto-series type 2 chain: Gal(β1-4)GlcNAc(β1-3)Gal is the main carrier for blood group ABH determinants of human erythrocytes and a relatively minor component in epithelial cells [virtually absent in normal intestinal mucosae (McKibbin *et al.*, 1982)]. Alterations to the type 2 chain constitute the major change in glycosylation in most human cancers, and are of central importance. The oncofetal pathway involving α1→3 fucosylation of type 2 chain on GlcNAc gives Le^x which, after subsequent α1→2 fucosylation on terminal Gal, forms Le^y. α2→3 sialylation followed by α1→3 fucosylation of the type 2 chain gives sialyl Le^x. Finally, the product of type 2 chain elongation, can be subsequently poly-fucosylated at position 3 of GlcNAc and/or position 2 of terminal galactose and/or sialylated at terminal Gal. This gives rise to di- or trimeric Le^x, trifucosyl Le^y and sialyl dimeric Le^x. (Hakomori *et al.*, 1984). The structures of some type 2 chain antigens are shown in Figure 1.3.

! ***Glycoprotein carbohydrate antigens*** are most often formed by O-glycosylation of the terminal serine or threonine residues of protein chains.

- Some of the lacto-series (glycolipid antigen) epitopes can be carried by mucin-type and other glycoproteins. Sialyl Le^a and sialyl difucosyl Le^x were found associated with high and low molecular weight glycoproteins, respectively, in the sera of patients with gastrointestinal cancer (Magnani *et al.*, 1983; Kannagi *et al.*, 1984 - cited after Hakomori, 1989). Le^x and Le^y antigens were found in carcinoembryonic antigen (CEA) purified from several cases of colonic cancer (Nichols *et al.*, 1985; Yamashita *et al.*, 1987).
- Tn antigen (GalNAc(α1)-O-Ser/Thr), and its derivatives, namely T (Thomsen-Friedenreich) antigen (Gal(β1-3)GalNAc(α1)-O-Ser/Thr) and sialyl/sialosyl Tn antigen (Neu5Ac(α2-6)GalNAc(α1)-O-Ser/Thr) are also expressed on normal gastric epithelial and, in high concentration, on malignant cells (Hakomori, 1991), however their cellular distribution varies with degree of differentiation (Jass *et al.*, 1995).

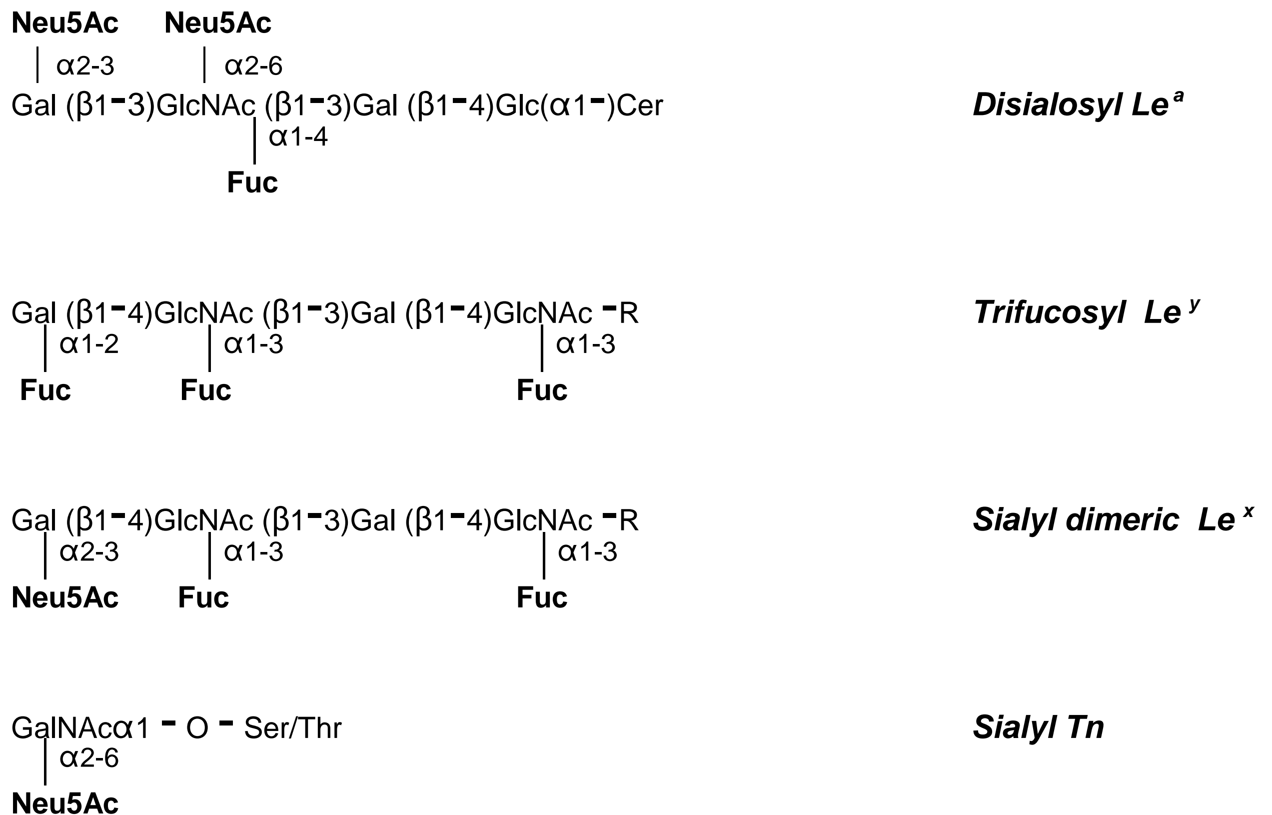

FIGURE 1.3. Chemical structures of some glycosylated antigens associated with colorectal cancers.

The above description indicates that the enhanced terminal fucosylation and sialylation of oligosaccharide structures plays a major role in the synthesis of colorectal cancer-associated antigens. α1→3 in particular, and to a lesser extent α1→4 and α1→2 fucosylation of GalNAc, GlcNAc or Gal, as well as α2→3 and α2→6 sialylation of GlcNAc residues, are significant markers of malignancy in colorectal cells. The involvement of changes in Fuc and GlcNAc carbohydrate residues with the process of cellular differentiation will be investigated in this thesis.

1.5 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

1.5.1 History and applications

Nuclear magnetic resonance was observed for the first time by Rabi in 1939 (Rabi *et al.*, 1939). Then, in 1945, two independent groups: Purcell, Torrey and Pound at Harvard University (Purcell *et al.*, 1946) and Bloch, Hansen and Packard at Stanford University (Bloch *et al.*, 1946) used this phenomenon in their studies on the exchange of energy between nuclei, and between nuclei and their surroundings.

Since that time, this technique has undergone a rapid development and has found numerous applications within many scientific disciplines. In 1948 NMR was applied to determine the distance between hydrogen atoms in crystals and detect molecular motions in other solids. In the years 1949-50 ‘chemical shift’ was described and this opened the countless possibilities of using high resolution magnetic resonance spectroscopy (MRS) in organic chemistry. The initial problem of the low sensitivity of MRS compared to other spectroscopic methods was overcome in the 1970s by the introduction of pulse Fourier spectroscopy. Together with the development of multidimensional spectra by Ernst’s group (Aue *et al.*, 1976), this paved the way to modern MR spectroscopy and to an unprecedented expansion of its applications in chemistry, biochemistry, biology and medicine. MRS techniques in the biological sciences include studies of the structural and dynamic properties of proteins (first observed with ribonuclease by Saunders *et al.*, 1957), nucleic acids, lipids (Cerbon *et al.*, 1965) and other macromolecules, and of a multitude of low molecular weight

compounds of biological, pharmacological and medical interest, membranes and other multimolecular structures, as well as intact cells and tissues. Introduction of high field and superconducting magnets allowed the increased resolution of MR spectra.

The success of MRS compared with other spectroscopic methods was based on both its ability to determine native conformations of macromolecules in physiological solutions and its being a non-invasive method useful for studies of living organisms or tissues without doing any damage to the sample or patient. High resolution MRS of protons has a particular importance in biomedical research, due to the great abundance of hydrogen in organic material and its highest gyromagnetic ratio of all stable isotopes (see Appendix).

1.5.2 ^{1}H MRS and cancer

The first application of proton magnetic resonance spectroscopy in cancer research was based on Damadian's discovery (1971; *et al.*,1973) that the water contained in many tumors (breast, skin, gastrointestinal tract, muscle, brain, blood plasma in leukemias and other neoplastic diseases of the blood and bone marrow) had longer longitudinal relaxation times than the water in healthy tissues. Initially the relaxation time values were measured in the plasma of patients with various tumors, then in solid tissues. Thus, also the first attempts of applying MR to the diagnosis of gastrointestinal cancer made by Goldsmith *et al.* (1978) involved the measurement of 'malignancy index' based on T_1 and T_2 of water in the tissue. This allowed to distinguish between normal and malignant specimens and raised the claim that this MR technique added a new dimension to the pathologist's ability to discern malignancy-associated abnormalities. These phenomena are due to the fact that tumors have higher water

content than normal tissues (Mellors *et al.*, 1953), however some types of tumors are exempt. Moreover, great caution needs to be involved in measuring the malignancy index in tissues, especially when analysing the three-exponential character of signal decay (due to localization of water molecules in free state, but also as external hydration of proteins and polysaccharides, and chemically bound with different molecules).

One of the most important early discoveries of ^{31}P-MRS of tumors was that, contrary to the acidic extracellular fluids, the intracellular pH of many human tumors was more alkaline than in normal tissue (Gillies *et al.*, 1982; Oberhaensli *et al.*, 1986; Cadoux-Hudson *et al.*, 1989; Gillies *et al.*, 1994)

It was originally supposed that chemical differences in tissue, relating to specific pathologies, could be detected from cellular fractions or perchloric acid (PCA) extracts. Many significant findings were obtained from MRS of cellular extracts, e.g. cell-type specific metabolites are conserved through across species and transformation as indicated by the similarity of the spectra from related cell types from brain and nervous system tumors, both human and rat's (Florian *et al.*, 1995, 1996). However, for a tight correlation between the pathology and the cellular chemistry, tissues or cells must be examined intact and viable (May *et al.*, 1986, 1988; Mackinnon *et al.*, 1992). Most MR-visible diagnostic molecules are uniquely distributed within cellular compartments or are associated with the plasma membrane. This distribution is destroyed when cells undergo disruption during PCA extraction. As a result, the spectra of cellular fractions and extracts report on the entire concentration of cellular components and the diagnostic specificity is much reduced. This was demonstrated by comparison of whole leukaemic lymphocytes and CHO cells with highly purified plasma membrane from each cell line (May *et al.*, 1988; Mackinnon *et al.*, 1992).

Historically, spectroscopists have used resonance integrals to compare the concentrations of chemical species in 1D MR spectra. This is the most precise method for quantification of well resolved resonances. However, in the 1D MR spectra from intact biological cells and tissues almost each resonance is a composite of signals from many different chemical species. As a result, an integral of a particular resonance for two different samples may not be reporting on the same set of chemical species.
It has however been demonstrated in a number of human organs, including cervix, colon, breast, prostate, ovary and lymph nodes (Delikatny *et al.*, 1993; Mountford *et al.*, 1993; Russell *et al.*, 1994; Lean *et al.*, 1995; Rutter *et al.*, 1996; Mackinnon *et al.*, 1997; Barry *et al.*, 1998) that comparison of the resonance intensities at discrete frequencies correlates with established biological and pathological criteria. Even in cases of different linewidth for different samples, this procedure allows MRS to provide an alternative modality to histopathology for detecting and diagnosing diseases.

Colorectal cancer was first investigated by high resolution proton MRS when Hakomori *et al.* (1984) distinguished two fucolipid structures in extracts of human adenocarcinoma tissues. Later, narrow lipid resonances were detected in malignant colorectal cells and attributed to mobile phospholipids (Guidoni *et al.*, 1987). Rosi *et al.* (1987) detected a correlation between the differentiation-inducing effect of all-trans-retinol on colon adenocarcinoma cells and MRS characteristics. In the same year, following studies of lipid and metabolite resonances in histopathologically staged colorectal tumors by proton MRS, Chen *et al.* (1987) proposed that this technique could aid the traditional diagnosis of this disease. The glycolipid antigen profile of colon cancer metastasized to liver was investigated by Taki *et al.* (1988) using HPLC and ^{1}H MRS. Another study on novel glycolipid from colon cancer was performed by Thurin *et al.*

(1989). Galons *et al.* (1989) showed changes in undifferentiated and differentiated (after glucose-depletion) HT29 colorectal cells in relation to their metabolite levels (choline, phosphorylcholine, phosphocreatine) by ^{31}P and ^{1}H MRS. Vesicles shed from colorectal cells were also characterized by proton MRS (Masella *et al.*, 1989; Lean *et al.*, 1991, 1992).

Structural determination of mucin oligosaccharides secreted by the human colonic cancer cells was performed by ^{1}H MRS by Capon *et al.* (1990). Lean *et al.* (1992) reported that the cell surface fucosylation pattern detectable by MR could distinguish between lowly and highly tumorigenic colorectal cell lines, and subsequently (1993) confirmed this on colorectal biopsies. Further support for the diagnostic use of proton MRS in colorectal cancer was provided by research of Moreno *et al.* (1993), Bezabeh *et al.* (1996), Moreno and Arrus (1996). A correlation between the degree of differentiation, ^{1}H MRS profile and the chemical analysis of several colorectal cell lines was demonstrated by Mackinnon *et al.* (1994). Recently Ende *et al.* (1996) used chemical shift imaging for the spatial location of MR visible lipid in the wall of the normal human colon and in carcinomatous colonic tissue.

1.5.3 MR-visible species in colorectal cancer.

Some colorectal tumor-associated chemical changes include alterations to cell surface glycosylation, lipid turnover and plasma membrane organization and alterations to carbohydrate metabolism.

Cell surface glycosylation plays an important role in the behaviour of cells, being involved in neoplastic development and progression as well as metastatic spread,

invasiveness and dedifferentiation. The carbohydrate species expressed on the cell surface consist of a core region, a backbone and a peripheral region. Because of higher mobility of peripheral sugar residues, these are the most likely to be observed by ^{1}H MRS, and therefore alterations in their structure and composition are good candidates for MR-visible indicators of the state of cellular differentiation.

Fucose was assigned in ^{1}H MR spectra of malignant leukemic lymphoblasts, after introducing a T_2-filtered COSY technique, a modification of the COSY pulse sequence that filters out resonances on the basis of their T_2 relaxation time, therefore enhancing the slowly relaxing fucose signal (Williams *et al.*, 1988). Correlation of these data with previous findings based on the investigation of fucosidase-treated rat mammary adenocarcinoma cells (Wright *et al.*, 1988) additionally showed that the metastatic potential of cells was inhibited by fucosidase, and implied a role for fucose in the process of cancer spread. Fucose H5 and H6 (methyl) couplings in 2D spectra of malignant cells result in three or four cross-peaks. The largest, denoted Fuc I - 4.27,1.33 ppm, is superimposed on β,γ cross-peak of threonine as shown by acid hydrolysis of plasma membrane fragments shed from colorectal cell surface (Lean *et al.*, 1991,2); the other cross-peaks are Fuc II at 4.28,1.25 ppm, which sometimes splits into IIa and IIb, and Fuc III - 4.30,1.41 ppm. It was evidenced that fucose resonances were absent or reduced in the spectra of normal, better differentiated and less tumorigenic cells and tissues in comparison to more malignant or tumorigenic and less differentiated specimens (Lean *et al.*, 1991-1993; Mountford *et al.*, 1993; Mackinnon *et al.*, 1994). However, the precise position of observed fucose residues in carbohydrate chains of the cell surface is yet unknown.

Structural ^{1}H MR studies of fucose-containing antigens and blood group determinants were undertaken on type 1 chain (Bechtel *et al.*, 1990), type 2 chain (Levery *et al.*, 1986), and on Le^x in a CHO mutant (Stanley and Atkinson, 1988) with different degrees of sialylation. Considering the pattern of fucosylation described above for colorectal cells, it seems unlikely that the observed species belong to any typical lacto-series type 1 or 2 determinants. This is because their chemical shifts differ from those measured in structural studies, even when the same solvent and temperature were used. For example, it has been shown that in the Le^a determinant, H5 of fucose has an unusually high chemical shift (4.9 ppm) due to electrophilicity induced by the neighbouring oxygens of Gal and GlcNAc (Bechtel *et al.*, 1990). It can be suggested that the fucosylation pattern observed for malignant colorectal material is due to α-1,6 (rather than -1,3 or -1,4) bound fucose being separated by no more than 2 sugar residues from sialic acid. On the other hand, the chemical shift values for H6 obtained in the studies of Bechtel *et al.* (1990) or Stanley and Atkinson (1988) are significantly lower than those from many studies on cancer (Wright *et al.*, 1988; Williams *et al.*, 1988; Lean *et al.*, 1991-1993; Mountford *et al.*, 1993; Mackinnon *et al.*, 1994). This was most likely caused by a difference in the models studied - the structural studies were performed on solutions of purified fucosylated antigens, not the whole-cell samples.

High resolution spectra with motionally narrowed ***lipid*** resonances are obtained from fast proliferating cells like fibroblasts, activated lymphocytes, macrophages and neutrophils, as well as malignant cells. Controversy however arose about the cellular structure that contains mobile MR-visible lipids. Cytoplasmic lipid droplets are the most noticeable compartments containing triacylglycerols which are stored as an energy reserve in the cell. Many authors therefore postulated that lipid droplets are the main

cause for the narrow lipid resonances in proton MR spectra (Callies *et al.*, 1993; Rémy *et al.*, 1997). Since it has been proven that the plasma membranes of malignant cells contain neutral lipids, it was hypothesized (Mountford *et al.*, 1982) that neutral lipids of plasma membrane contribute, at least in part, to the narrow lipid resonance at 1.3 ppm. Mackinnon *et al.* (1989, 1992) showed that triglyceride-containing lipid droplets in CHO cells are not the MR-visible mobile lipid pool and that cholesteryl esters and triacylglycerols are present in the plasma membrane of this cell line. Similar evidence was delivered by Freitas *et al.* (1990), who detected mobile lipids in the absence of lipid droplets in tumor cells. Based on these data, a new model of tumor cell membrane was designed by Mountford and Wright in 1989, in which unilamellar spherical vesicles 25-28 nm in diameter containing neutral lipids are incorporated in the phospholipid bilayer.

The introduction of the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence allowed the observance of the narrow resonance components of the methylene resonance at 1.3 ppm and the selective measurement of the relaxation time selectively for lipid signals. These experiments showed that the metastatic potential and tumorigenicity of cells are correlated with long T_2 (Mountford *et al.*, 1984; Mountford *et al.*, 1986; Lean *et al.*, 1993). The two dimensional correlation spectroscopy method (COSY) allowed further separation of signals which overlapped in one dimension. By providing more specific information on intramolecular couplings between chemical groups, precise assignment of the resonances of triglycerides and fucose was possible (May *et al.*, 1986; Mountford and Wright 1988; Holmes and Mountford 1991). Up to 10 different scalar couplings (denoted A-L on Figure 1.4.) are present in ether-linked triglyceride and result in the same number of cross-peaks in the COSY spectrum (coordinates listed in table 5.2).

Changes in lipid signals in the ^{1}H MR spectra of tumors can also be correlated with the degree of necrotic changes that were shown to account for an increase in the methylene resonance in astrocytomas (Kuesel *et al.*, 1994). The same effect was observed for apoptotic death induced in a number of different cell lines (Blankenberg *et al.*, 1996). Elevation of lipid signals was also found to be associated with the response of tumor cells to treatment with different drugs (Van Haaften-Day *et al.*, 1988; Delikatny *et al.*, 1996a), also present in drug-resistant variants (May *et al.*, 1988; Le Moyec *et al.*, 1996).

A study on cellular lipid changes in the process of enterocytic differentiation undertaken by Mackinnon *et al.* (1994) showed that, although there were no consistent changes in the total neutral lipid content, the narrowing of the methylene signal clearly correlated with the degree of cellular differentiation within a series of cell lines. The same MR study found a decrease in the overall number of saturated bonds in fatty acyl chains in more differentiated cell lines. Furthermore, changes in mobile lipid signal in one line of transformed murine fibroblasts was shown to depend on cell culture conditions, such as extracellular pH, cell density and cell cycle phase profile (Delikatny *et al.*, 1996).

MRS also detected tumor-associated changes in ***phospholipid metabolites***, such as choline, phosphorylcholine, glycerophosphocholine and inositol, which are markers of fast membrane turnover characteristic of proliferating cells. Among the most often claimed markers of malignancy are the changes to the *N*-trimethyl group of total cholines at 3.2 ppm. The increase in this signal was larger in malignant compared with normal tissue (Lean *et al.*, 1993) and was parallel to the increase in tumorigenicity of

colorectal cell lines (Lean *et al.*, 1992), the degree of dedifferentiation of colorectal tumor cell lines (Mackinnon *et al.*, 1994), and the metastatic potential of rat mammary carcinoma (Mountford *et al.*, 1993). The signal at 3.2 ppm was also higher in stimulated rat pancreas cells (Esclassan *et al.*, 1990).

MRS studies involving the induction of cellular differentiation in cultured cell lines showed inconsistent alterations in phosphorylcholine (Cho-P) and glycerophosphocholine (Gro-P-Cho) signals. DMSO caused an increase in Cho-P with a simultaneous decrease in Gro-P-Cho resonances (Carpinelli *et al.*, 1984) in Friend leukemia cells. In glucose-free conditions both signals, together with free choline, were elevated (Galons *et al.*, 1989) in HT29 colon carcinoma cell line. In contrast, low glucose conditions in CX-1 colorectal cell line, derived from HT29, caused an increase in Cho-P and a slight decrease in Gro-P-Cho resonances (Shedd *et al.*, 1993). Using ^{13}C-MRS for comparing three colorectal cell lines varying in the degree of differentiation, Künnecke *et al.* (1994) found that increased loss of cellular differentiation correlated with a large increase in the Gro-P-Cho signal. Choline metabolites may also be involved in the process of immortalization and transformation. Bhakoo *et al.* (1996) found distinct changes to Cho-P and Gro-P-Cho to be associated of the expression of *ts Tag* or H-*ras* oncogenes in transformed Schwann cells.

Carbohydrate metabolism in neoplastic cells was also investigated by MRS of protons and other nuclei. Many changes in glycosylation precursors have been associated with the degree of cellular differentiation. Künnecke *et al.* (1994), tracing ^{13}C-labelled glucose pathways, discovered a pronounced accumulation of glycosylation precursors, UDP-hexoses, and the anaerobic glycolysis product, lactate, in poorly

differentiated, as distinct from moderately and well differentiated, cell lines. The 2D cross-peak between H1 and H2 protons of ribose in UDP-hexoses was also measured in a series of six colorectal cell lines and its intensity was found to well correlated with the degree of cell dedifferentiation (Mackinnon *et al.*, 1994). A decrease in glucose concentration in cell culture medium of CX-1 cell line significantly lowered UDP-hexose levels (Shedd *et al.*, 1993). However basic cell morphology did not depend on UDP-hexose levels. When the CX-1 line was grown to confluence, 25 mM glucose caused accumulation of UDP-hexoses. This effect was eliminated by increasing the Cho or Etn in the medium (Shedd *et al.*, 1993; Franks *et al.*, 1996). Also, high levels of UDP-hexoses have been associated with the more differentiated subtype of small cell lung carcinoma, and lower levels with its less differentiated subtype (Pederson *et al.*, 1992). However, indirect involvement of these molecules in the process of cellular differentiation is not yet conclusive; their levels may rather be dependent on changes in metabolism that result from alterations in cell phenotype.

Other small-molecular metabolites reported to change with the degree of cellular differentiation in MR spectra include an increase in ***glutamate and glutamine*** (amino acids utilized by tumor cells as an energy source) and a decrease in (myo)inositol and taurine levels (Galons *et al.*, 1989).

α - L - fucose

N - acetyl group

UDP

Ether-linked triglyceride

Phosphocholine

FIGURE 1.4. Some molecules and chemical groups observed to change in colorectal cancer progression

1.6 OBJECTIVE AND HYPOTHESES

Objective:

To use the differentiation-inducing cell model to clarify which of ^{1}H MR-visible changes, previously recorded (Lean *et al.*, 1993; Mackinnon *et al.*, 1994), are related specifically to the process of cellular differentiation.

Hypotheses:

- Sodium butyrate is a successful reversible differentiation inducing agent for the poorly differentiated SW620 colorectal cell line.
- Changes in ^{1}H MR spectra of SW620 cells caused by sodium butyrate can be related to the process of cellular differentiation.
- The concept of MR-visible markers of cellular differentiation can be substantiated by investigation of other differentiation inducers' effects on the same SW620 cell line.