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Growth Factors and the Redox State as New Therapeutic Targets for Colorectal Cancer

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1. Introduction

Colorectal cancer (CRC) is an important health problem in many western countries due to its significant morbidity/mortality. Despite advances in its diagnosis and treatment, survival associated with this cancer when it has extended to adjacent organs, lymphatic nodules or distal organs is drastically reduced. The liver is the most common site of CRC metastasis, since it represents a unique microenvironment for the formation of metastases, not only due to its sinusoidal endothelium (Barberá-Guillén et al., 1989), but also due to its abundant expression of growth factors (GFs) (Stoeltzing et al., 2003).

At present, curative treatment of localized metastases is possible via partial liver resection. However, this surgical procedure is only potentially curative, since 65% of patients subjected to resection of liver metastases experience relapse within 5 years (Sun & Tang, 2003; Allendorf et al., 2004). In the light of this frequent recurrence, it is essential to develop new preventive therapeutic strategies, which require a detailed knowledge of the biological events that occur following hepatectomy. In this sense, we have previously demonstrated the tumor-enhancing effect associated with liver resection in a mouse tumor model; in addition, we showed that hepatectomized rat serum increased cell proliferation in vitro, when compared with laparotomized rat serum or fetal calf serum (García-Alonso et al., 2003; García-Alonso et al., 2008a, 2008b). These findings indicated that GFs produced by the liver promote the development of metastases.

At present, CRC treatment includes various active drugs, either as individual agents or in combination: 5-fluorouracil (5-FU), capecitabine, irinotecan and oxaliplatin, among others. Despite this wide array of anti-tumor agents, relapse often occurs in CRC patients, due in large part to the resistance of the tumor cells to these anti-neoplastic agents. Various different mechanisms have been reported as being responsible for the development of chemoresistance and, though each may be important in itself, they take on an even greater significance if we consider how they may be interrelated.

One of these mechanisms of resistance to anti-neoplastic agents is the presence of GFs, which may be able to protect certain tumor cells against cytotoxic cell death. For this reason, one of the most promising cell targets nowadays are these GFs and their receptors. Thus, since 2004, three new agents have been approved which in combination with cytotoxic
agents are administered in cases of advanced and metastatic CRC: bevacizumab, a monoclonal antibody to vascular endothelial growth factor (VEGF) (Hurwitz et al., 2004), and cetuximab and panitumumab, which are monoclonal antibodies to the epidermal growth factor receptor (EGFR) (Cunningham et al., 2004; Odom et al., 2011).

An increasing amount of evidence indicates that the intracellular redox state plays an essential role in the mechanisms underlying the actions of GFs. In particular, GFs have been reported to generate reactive oxygen species (ROS) which can function as second messengers, mediating important cellular functions, such as proliferation and programmed cell death. Intracellular redox homeostasis is sustained primarily by glutathione (GSH), which has long been known to be an important factor in cancer chemoresistance.

In the present chapter, we analyze three important concerns in relation to CRC chemoresistance:

- The influence of GFs in CRC biology and in the response to current cytotoxic therapies.
- The involvement of the redox state in the mechanisms of action of GFs in CRC cells.
- The exogenous modulation of the redox state as a new pharmacological strategy to improve the response to chemotherapeutic agents.

2. Growth factors and colorectal cancer

GFs play a fundamental role in CRC biology, mediating critical functions in cancerous cells, such as proliferation, angiogenesis and the inhibition of cell death. The recurrence of cancer after excision surgery is still a major clinical problem. Accumulating clinical and experimental evidence has indicated that specific factors involved in liver regeneration may influence the growth patterns of residual or dormant micrometastases after resection, suggesting that the process of hepatic regeneration has a significant proliferative effect on tumor cells. In this regard, GFs appear to be involved in tumor recurrence and in metastasis formation. Thus, after partial resection of liver metastases, various types of GFs, which are responsible for liver regeneration, are locally released. However, these may also stimulate the proliferation of undetected tumor cells in the remaining liver, i.e. highly metastatic colon cancer cells can respond to liver regeneration associated mitogens, whose expression is induced after hepatectomy. GFs such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β), basic-fibroblastic growth factor (b-FGF), insulin growth factor-I (IGFI) and vascular endothelial growth factor (VEGF) have been reported to be associated with tumor progression and metastasis (Christophi et al., 2008).

**Hepatocyte Growth Factor (HGF)** is essential for the process of hepatic regeneration. It is a potent mitogenic agent produced by stellate, endothelial and Kupffer sinusoidal cells, which binds to a receptor of the tyrosine kinase (TK) family. This family of genes is encoded by the proto-oncogene c-Met which is expressed in hepatocytes, as well as in other cell types, including tumor cells (Di Renzo et al., 1991). It has a pro-angiogenic effect and stimulates cell motility as well as the secretion of matrix metalloproteinases (MMPs) by pericytes, suggesting an important role in tumor invasion. In the case of CRC, the co-expression of HGF and its receptor is correlated with tumor pathogenesis and with the metastatic phenotype, and for this reason, it has been proposed as a possible molecular marker to be incorporated into CRC staging procedures (Kammula et al., 2007). Moreover, it is known that epithelial tumor metastases undergo an epithelial to mesenchymal transition (EMT) before becoming invasive. The stimuli which promote this transition include HGF and other
GFs such as b-FGF, EGF, TGF-β, as well as extracellular matrix (ECM) constituents including MMPs (Kalluri & Zeisberg, 2006; Christophi et al., 2008). For these reasons, HGF is considered to be a potentially valuable new therapeutic target for different tumors. Studies using NK4, a HGF antagonist, have shown an inhibitory effect on proliferation, invasion and angiogenesis in cell lines of gastric and pancreatic carcinoma, and of CRC (Hirao et al., 2002; Wen et al., 2007). In addition, anti-HGF monoclonal antibodies have been developed, thereby blocking binding to its receptor (Cao et al., 2001). Other developments include anti-c-Met antibodies (Jin et al., 2008), and strategies aimed at silencing the expression of c-Met or HGF via antisense oligonucleotides (Stabile et al., 2004), or RNA (Shinomiya et al., 2004).

Epidermal Growth Factor Receptor (EGFR) ligands, the most physiologically relevant of which include EGF, TGF-α, and Amphiregulin (AR). All of these bind to the extracellular domain of EGFR, which is a member of the ErbB transmembrane TK receptor family (Hynes & Lane, 2005). Binding of these ligands to the receptor activates the Ras/Raf/MAPK and PI3K-AKT signaling pathways which are involved in tumor cell proliferation, inhibition of apoptosis, invasion, migration and angiogenesis (Le Golvan & Resnick, 2010; Wanebo & Berz, 2010). Abnormal expression of these ligands has been demonstrated in many advanced tumors, including breast cancers, gliomas, and lung cancer. In the case of CRC, EGFR overexpression has been detected in 60-80% of cases (Le Golvan & Resnick, 2010) and a correlation has been reported with early tumor recurrence and extra-hepatic metastasis (Christophi et al., 2008). However, its exact role in the CRC metastatic cascade has not yet been characterized due to controversial results obtained with anti-EGFR antibody therapy. In this regard, the therapeutic use of two monoclonal antibody agents (cetuximab and panitumumab) has been authorized in patients with metastatic CRC; although they have a modest effect when used as single agents, they have been found to be beneficial in some patients when used in combination with conventional chemotherapeutic agents (Wanebo & Berz, 2010; Tol & Punt, 2010). In fact, it has been shown that the response to this therapy is independent of EGFR expression in tumor tissue (Chung et al., 2005). Thus, some studies suggest that EGFR expression in the primary tumor does not necessarily correspond with the same level of expression in metastatic tissue, while other studies have reported 78-100% concordance in EGFR expression in both tissue compartments (Tol & Punt, 2010). These discrepancies may partially be due to differences in the detection techniques employed. Nevertheless, recent studies have demonstrated that the therapeutic efficacy of the anti-EGFR antibody is limited to patients in whom the K-Ras oncogene is not mutated, since mutation of this oncogene can induce constitutive activation of the Ras/Raf/MAPK signaling pathway, which is independent of the activation of EGFR via ligand binding (Benvenuti et al., 2007; Tol & Punt, 2010).

Transforming Growth Factor β (TGF-β) acts as a tumor suppressor due to its inhibition of growth and its activation of apoptosis. However, in CRC, this suppressor activity is lost due to the existence of mutations in the genes which encode TGF-β, the type II receptor (TGF-β 2), or SMAD proteins, in such a way that the antiproliferative signal associated with this factor is interrupted (Markowitz & Bertagnolli, 2009). On the other hand, TGF-β has a protumor effect due to its effect on the stroma, promoting angiogenesis, and on the tumor cells themselves, stimulating their motility and their invasive capacity (Blobe & Gordon, 2000). Thus, TGF-β, whose serum values are correlated with a poor CRC prognosis, acts as a tumor promoter, inducing the development of hepatic metastasis (Shim et al., 1999).

Insulin Growth Factor I (IGF-I) and its TK receptor are implicated in the development and progression of CRC due to their induction of proliferation. A correlation has been found
between serum levels of IGF-I, high levels of IGF-IR expression in tumor cells and the development of hepatic metastasis. This pro-tumor effect is due to the fact that the signal induced by the binding of IGF-I to its receptor promotes the migration of endothelial cells, invasion and the formation of new blood vessels following the stimulation of VEGF production by endothelial cells (Wu et al., 2002), suggesting that IGF-I is an important contributor to tumor growth and hepatic metastatic development after hepatectomy (Christophi et al., 2008).

Vascular Endothelial Growth Factor (VEGF) is an endothelial cell mitogen which induces cell migration, proliferation, invasion and increased vascular permeability and has a potent pro-angiogenic activity. It has been shown that a large percentage of tumors which produce high levels of VEGF are associated with a high density of vessels in the tumor, metastasis, chemoresistance and poor prognosis (Sullivan & Brekken, 2010).

The VEGF family is made up of six growth factors. These exert their effects via binding to one of the three VEGFRs which belong to the tyrosine kinase receptor (TKR) family. These are localized predominantly on endothelial cells and angioblasts (Tol & Punt, 2010). In addition, in solid tumors, it is postulated that the production of VEGF is increased following liberation of hypoxia-inducible factor 1α (HIF-1α) (Kaur et al., 2005), EGF (Niu et al., 2002) and HGF (Dong et al., 2001). In turn, VEGF induces the synthesis of other factors related to tumor development, such as stroma-derived factor 1 (SDF-1) which induces an increase in the population of cancer-associated fibroblasts (CAFs) (Kalluri & Zeisberg, 2006, Christophi et al., 2008).

The risk of developing hepatic metastasis associated with CRC may be related to the expression of different VEGF isoforms which bind to the different VEGFRs. Thus, it has been shown that in 50% of CRCs, VEGFR-2 is expressed on the surface of the tumor cells (Duff et al., 2006). This extensive expression, which reflects the dependence of some solid tumors on neoangiogenesis, has led to the proposal that VEGF and VEGFR may be therapeutic targets in the treatment of CRC. Bevacizumab is a humanized monoclonal antibody which binds to VEGFA blocking the binding of this GF to VEGFR, thereby avoiding the corresponding intracellular signal transduction. Although parameters which allow a prediction of the efficacy of this monoclonal antibody have not been reported, bevacizumab has been approved as a first and second-line therapy for the treatment of metastatic CRC, enhancing survival, stabilizing the disease and achieving partial regression when used with chemotherapy. Two recent and complete reviews by Tol & Punt (2010) and Wanebo & Berz, (2010) analyze randomized and non-randomized trials of neo-adjuvant therapy using bevacizumab in metastatic CRC.

3. The role of the redox state in the mechanism of action of growth factors

The redox state is a key characteristic which influences important cell biological processes including enzymatic reactions, cell signaling, cell proliferation and apoptosis. The term redox signaling refers to a regulatory process in which the signal is transmitted through redox reactions. The intra- and extracellular redox levels allow the carrying out of different extra and intracellular signaling (intra-cytoplasmic and nuclear), which subsequently give rise to the cascade of effector signals that regulate diverse cellular activities such as cell proliferation.

GF signals are transmitted from the cell surface by means of the activation of TK-type transmembrane receptors and the induction of the corresponding intracellular effects.
Among these signal transduction pathways, protein phosphorylation plays a fundamental role. This process is reversible and dynamic, being controlled by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). As a consequence of the binding of GF to its specific receptor, dimerization occurs followed by the autophosphorylation of tyrosine residues in the intracellular domain of the receptor (Cadena & Gill, 1992). These residues are key sites of interaction with cytoplasmic proteins which contain SH2 (Src homology type 2) domains; these mediate the signal transduction of GFs, such as PLC-\(\gamma\), GAP-ras (GTP-ase-activating protein of ras), PIK3 and Grb2 (Johnson & Vaillancourt, 1994). The action of all these proteins, via different mechanisms, converges to activate the Ras protein, which in its turn, activates the Raf tyrosine. Subsequently, a phosphorylation cascade is produced in such a manner that Raf phosphorylates another kinase, the MAPK kinase which phosphorylates members of a family of serine/threonine kinases, the MAP kinases. Finally, MAP kinases phosphorylate the transcription factors which promote the transcription of genes necessary for the final cellular response (Davis, 1993).

Many studies have demonstrated that the cellular redox status plays a key role in GF-mediated signaling systems (Thannickal & Fanburg, 2000). Although there is evidence that GFs generate ROS, it is not yet clear how ROS activate these cell signaling pathways. One plausible mechanism is that ROS could act as second messengers which participate in phosphorylation/dephosphorylation processes (Storz, 2005). ROS, such as hydrogen peroxidase (H\(_2\)O\(_2\)), induce the phosphorylation and activation of some PTKs, such as the kinases implicated in the MAP kinase cascade (Rao, 1996). In contrast, PTPs have a cysteine residue in their catalytic domain, which must be in its reduced form for total activity of the receptor. It has been shown that in cell signaling phenomena, ROS may induce the inactivation of PTPs (Rhee et al., 2000). Interestingly, ROS play a crucial role in vascular angiogenesis, not only due to their induction of VEGF (Sen et al., 2002), but also to their implication in the VEGF signaling pathway. Thus, VEGF stimulates ROS production via the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is essential for the satisfactory propagation of the angiogenic signal (Roy et al., 2008); in fact, NADPH oxidase has been proposed as a target for anticancer therapy (Ushio-Fukai & Nakamura, 2008).

Similarly, it has been demonstrated that EGF stimulates ROS production in cells and that inhibition of this production leads to a weakening of the signaling system of the corresponding factor (Mills et al., 1998). A ROS mediated signaling cascade has also been reported to be activated following stimulation of the c-Met/HGF system; this cascade has been found to be associated with the crucial role of the receptor in the development of metastasis (Ferraro et al., 2006).

All of these biological effects occur at low to moderate concentrations of ROS. For this reason, redox regulation is essential for the maintenance of an optimal level of oxidation which permits precise signal transduction and the appropriate cellular response.

4. Glutathione metabolism in colorectal cancer

Cells are exposed to oxidative stress which is generated by normal metabolism and also by exogenous factors, such as ionizing radiation, some chemotherapeutic drugs and xenobiotics. The oxidative modification of cell components via ROS is one of the most potentially damaging processes for normal cellular activity (Halliwell, 1991). However, ROS
are well recognized for playing a dual role. Thus, a number of studies have provided convincing evidence that, depending on the level of oxidative stress, ROS can function as pro-life signals in certain contexts (as mentioned above, low or mild increases in ROS play a pivotal role in many physiological reactions, such as the regulation of transcription factors and cellular signaling pathways) (Maellaro et al., 2000) and pro-death signals in others (high concentrations of ROS can induce apoptosis) (Le Bras et al., 2005). Consequently, the maintenance of the redox status is a key factor for cell survival, in the case of both normal and cancer cells.

In order to maintain redox balance and also to protect themselves from oxidative stress, cells possess powerful redox regulation systems, known as the “redox buffer”, including GSH and thioredoxin (TRX), as well as antioxidant enzymes, such as superoxide dismutase (SOD), catalase, GSH peroxidase (GPx) and thioredoxin reductase (TrxR). In addition, cells also have available other non-enzymatic antioxidants which are obtained via the diet, among which are ascorbic acid (vitamin C), α-tocopherol (vitamin E), flavonoids, carotenoids and selenium.

Intracellular redox homeostasis is sustained primarily by GSH, the most prevalent intracellular non-protein thiol. In fact, the ratio between its reduced and oxidized states (GSH/GSSG) is considered to be an indicator of the redox status of the cell. GSH is intracellularly synthesized from the three amino acids glutamic acid, cysteine and glycine; it possesses an unusual γ peptide bond between glutamic acid and cysteine, and has a thiol group on the latter aminoacid. The biosynthesis and degradation of GSH occurs within the γ-glutamyl cycle, in which GSH is transported to the extracellular space and γ-glutamyl-aminoacids are transported to the intracellular space. GSH is synthesized from glutamate by two consecutive reactions which are catalyzed by the γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase enzymes. GSH can be exported outside the cell, although its constituent aminoacids can be reincorporated into the cell, thanks to a transpeptidation reaction catalyzed by the γ-glutamyl transpeptidase (γ-GT) enzyme, which is a glycoprotein localized on the outer surface of the plasma membrane. Transpeptidation occurs in the presence of aminoacids, giving rise to γ-glutamyl-aminoacids and cysteinylglycine (Cys-Gly). The γ-glutamyl-aminoacids are transported into the cell, whereas in the case of cysteinylglycine, bond breakage by means of a dipeptidase is first required. This dipeptidase is present on the outer surface of the plasma membrane, thereby allowing the incorporation of the peptides into the cell. The γ-glutamyl-aminoacids are the substrate of the γ-glutamyl cyclotransferase enzyme, which transforms the glutamyl residue into 5-oxoproline, liberating the remaining aminoacids. Next, by means of the 5-oxo-L-prolinase (5-OPase) enzyme, 5-oxoproline is transformed into glutamate and this reaction involves the consumption of ATP. The cycle is completed with the action again of γ-GCS and GSH synthetase (Fig. 1) (Meister & Anderson, 1983).

Due to its structural characteristics, GSH participates in numerous processes which are essential for cell physiology. GSH and its related enzymes are involved in cell proliferation and participate in the cell cycle, in the synthesis of proteins and in DNA synthesis and repair (Higuchi, 2004). In addition, its capacity as a reducing and antioxidant agent renders GSH an essential component for the maintenance of the integrity of the protein and lipid components of the cell, as well as a substrate for antioxidant GSH peroxidase enzymes, a selenium-dependent system. As indicated previously, another of its important functions consists in the protection of the cell from free radicals, endogenous and exogenous toxic substances, and carcinogens. GSH also defends the cell against the effects produced by
radiation and some chemotherapeutic drugs, such as alkylating agents. The formation of GSH S-conjugated products generated during intracellular detoxification may occur due to the non-enzymatic reaction of exogenous electrophilic compounds or to the action of GSH S-transferase (GST) enzymes. GST conjugates can then be eliminated via an ATP-dependent GS-X pump.

In addition to its essential role in normal growth, GSH is also involved in cell differentiation. Thus, it has been reported that as the cell progresses from proliferation to differentiation, cellular GSH content decreases. For example, it has been observed that butyrate-induced differentiation of the HT-29 human colon cell line is associated with reduced levels of cellular GSH (Bernard & Balasubramanian, 1997). These findings led to the notion that thiol status may be dependent on cellular energy metabolism. In this regard, the tumor cells have a very high cellular metabolism and, consequently, they generate high levels of ROS. Here, we should underline the importance of regulation of redox balance for the survival of malignant cells; the activation of redox regulatory systems, in which GSH plays an important role, could be considered to be the first line of adaptation of cancerous cells to oxidative stress. In fact it has been reported that non-differentiated and highly metastatic melanoma cells have a significantly higher GSH content than non-tumorigenic melanocytes (Thrall et al., 1991). Moreover, it has been demonstrated that whereas elevation of intracellular GSH is associated with mitogenic stimulation (Palomares et al., 1997), GSH depletion decreases the rate of cell proliferation and inhibits cancer growth (Del Olmo et al., 2000).

Increased levels of ROS in cancerous cells may have profound consequences, including enhanced cell proliferation, increased incidence of mutations and genetic instability, and reduced sensitivity of cells to anticancer agents, leading to resistance. In the case of CRC, intense oxidative stress and significant oxidative DNA adducts have been found during all stages of colorectal carcinogenesis (Schmid, et al., 2000). In fact, these DNA adducts, as well
as GST polymorphisms, have been suggested as molecular biomarkers for the detection of early CRC and the prediction of the clinical effectiveness of chemopreventive drugs (Garcea et al., 2003). Elevated GST expression (Naidu et al., 2003) and a significant increase in GSH levels (Balendirian et al., 2004) have been found in CRC; these are often associated with an increased resistance to cancer chemotherapy drugs via GSH conjugation. Elevated GSH levels may also be related to γ-GCS, another GSH-related enzyme whose levels have also been found to be elevated in CRC (Tatebe et al., 2002). Finally, it should be remembered that GSH and its related enzymes is only one of the redox regulation systems which are implicated in CRC, since an increased expression of TRX-1 in human CRC has been found to be associated with reduced survival times of patients (Raffel et al., 2003).

In summary, the GSH system involves complex and dynamic processes in which several related enzymes participate. Although it may be difficult to know a priori what type of GSH metabolism a given CRC may have, the fact that some CRC cells contain high levels of GSH has led to the suggestion that it may be an important factor in limiting the therapeutic efficiency of conventional cancer treatment.

5. The influence of glutathione metabolism in the response to chemotherapy

A common cause of treatment failure in CRC is chemoresistance. This resistance to current cytotoxic therapies limits their success in the majority of advanced cancer patients. This is particularly true in the case of liver metastases. GSH is able to modulate cell susceptibility to chemotherapy. In particular, GSH plays an important role in the protection against cell injury caused by various anticancer agents (see Balendirian et al., 2004 for review), and elevated GSH levels render tumor cells resistant to chemotherapeutic drugs. In the particular case of CRC, there is also evidence that the GSH status of colon cancer cells is a critical determinant of cell damage by various agents. Indeed, it has been proposed that elevated intracellular GSH levels may be a cause of acquired resistance to 5-FU, platinum agents and camptothecins. In this regard, it has been suggested that the increased levels of antioxidant enzymes in response to the generation of ROS by 5-FU, a standard drug for the treatment of this disease, may underlie the acquired resistance to this anti-tumor agent (Hwang et al., 2007). In in vitro studies, we have shown that treatment with 5-FU produced the greatest antiproliferative effect after 24 hours of incubation and that later, once drug treatment had been stopped, the growth of tumor cells rebounded (Palomares et al., 2009). This finding may be due to the recovery of GSH levels after the initial 5-FU-induced reduction, which has also been suggested by other authors (Chen et al., 1995).

It has also been reported that GSH may modulate the cytotoxicity of platinum agents (Sadowitz et al., 2002). However, intracellular GSH levels do not appear to influence the cell growth-inhibiting activity of these compounds in cells not previously exposed to platinum complexes (Boubakari et al., 2004), suggesting that GSH may be more relevant in acquired resistance. Furthermore, several authors have reported the influence of GSH on sensitivity to camptothecins (Yoshida et al., 2006). In order to decrease the resistance of tumor cells to chemotherapeutic drugs, many GSH-based therapeutic strategies have focused on lowering GSH levels, principally via the use of agents which reduce this tripeptide or inhibit its synthesis. The agent which is most frequently used to reduce the levels of this thiol, not only in basic research but also in clinical assays, is L-buthionine-[S,R]-sulfoximine (BSO) (Fig. 2). We found that this potent
inhibitor of γ-GCS enhances the sensitivity of tumor cells to treatment with ionizing radiation and cytostatic drugs, such as alkylating agents (Palomares et al., 1999). We have also found that the reduction in GSH content (around 52%) produced by BSO significantly inhibits the proliferation of colon cancer WiDr cells (Palomares et al., 2009).

Fig. 2. Inhibition of γ-glutamylcysteine synthetase by L-buthionine-[S,R]-sulfoximine (BSO).

However, the anti-tumor efficacy of BSO is accompanied by increased toxicity, due to the fact that BSO exerts its effects in a non-selective manner. In fact, it reduces GSH levels in both tumor and normal cells and thus sensitizes both cell populations to the toxic effect of anticancer agents. This finding has been reported in clinical studies, such as that of Bailey et al. (1998), who upon combining BSO and melphalan, found an important increase in medullar toxicity with respect to that produced by the administration of the alkylating agent alone. Thus, BSO treatment produces toxicity at the level of the immune, gastrointestinal, urinary and central nervous systems. This toxicity limits, de facto, the therapeutic potential of BSO and of other non-selective GSH reducing agents.

One of the principal reasons for the limited effects of chemotherapy is the insufficient therapeutic index of available drugs. This index could be increased by regimes which protect healthy tissues against toxicity and at the same time enhance the sensitivity of tumor tissue to anticancer agents. Since GSH is highly relevant in protecting both normal and tumor cells, one way of achieving this objective would be to selectively modulate GSH levels. An increase in GSH levels or in the capacity of normal cells to synthesize GSH, would enhance their resistance, leading to a protector effect. In contrast, a reduction in GSH content or in the capacity of tumor cells to synthesize this tripeptide would enhance sensitivity to the effects of anti-tumor agents. In this regard, it was suggested many years ago that agents which induce a selective modulation in GSH levels could be beneficially added to conventional treatments in order to enhance the anti-tumor efficacy of radiotherapy and/or chemotherapy (Russo et al., 1986).

Selective modulation of GSH as a therapeutic strategy requires an in-depth knowledge of the physiological differences in GSH synthesis and metabolism between healthy and tumor cells, as well as of the level of expression of GSH-related enzymes. In this regard, lower expression of the 5-OPase enzyme has been found in some tumor cell lines in comparison to healthy cells, leading to the suggestion that this enzyme may be a key player in obtaining the required selective modulation of GSH (Chen & Batist, 1998).
Within the γ-glutamyl cycle, 5-OPase catalyses the hydrolysis of 5-oxo-L-proline to L-glutamate, one of the three aminoacids which participate in GSH synthesis, joining in this way the reactions of GSH synthesis and metabolism in this cycle (see Fig. 1). It has also been observed that L-2-oxothiazolidine-4-carboxylate (OTZ) – an analog of 5-oxo-proline – also acts as a substrate of 5-OPase, thereby converting this cysteine prodrug into S-carboxycysteine, hydrolyzing it subsequently to cysteine and CO2 (Fig. 3).

Some studies have found that in contrast to BSO, OTZ treatment is selective, increasing GSH levels in healthy tissue and reducing it paradoxically in tumor tissue (Chen & Batist, 1998). These authors have suggested that OTZ, by competing with 5-oxo-L-proline for 5-OPase, could exert two different effects on GSH levels, depending on the level of expression of the 5-OPase enzyme and on the quantity of aminoacids necessary for the synthesis of the said tripeptide. In this way, OTZ would increase intracellular levels of GSH in healthy cells by means of increasing the contribution of cysteine – in normal conditions, the limiting aminoacid in GSH synthesis in these cells - (Meister A, 1983), but would reduce GSH content in tumor cells by means of the inhibition of glutamate synthesis from 5-oxo-L-proline. In fact, it has been observed that in tumor cells and in other cells under conditions of oxidative stress, glutamate is the limiting factor in GSH synthesis (Kang, 1993).

Fig. 3. Metabolism of L-2-oxothiazolidine-4-carboxylate (OTZ) and of 5-oxo-L-proline by means of the 5-OPase enzyme.

In in vitro studies, OTZ has been found to be useful as a protector in human lymphocytes against toxicity due to nitrogenated mustard, or in cultures of human fibroblasts against radio-induced toxicity. In in vivo studies using mouse models, OTZ has demonstrated its efficacy in protecting against liver damage produced by alcohol, by reducing the degree of cystitis induced by cyclophosphamide (CY) or of hepatotoxicity produced by acetaminophen, among others. OTZ has been used in diverse clinical assays for the treatment of a variety of pathologies associated with ROS generation and with reduced GSH levels. Diseases in which OTZ treatment has been successfully employed include acute respiratory distress syndrome (Morris et al., 2008), amyotrophic lateral sclerosis (Cudkowicz et al., 1999) and atherosclerosis (Vita et al., 1998). Patients subjected to peritoneal dialysis (Moberly et al., 1998) and patients infected with the AIDS virus (Barditch-Crovo et al., 1998) have also benefited from this treatment.
Paradoxically, in contrast to the effect produced in healthy tissue, OTZ reduces GSH levels in some human tumor cell lines including breast adenocarcinoma and ovary adenocarcinoma (Chen & Batist, 1998). In the same way, we have also demonstrated the selective character of GSH modulation by OTZ, *in vitro* as well as *in vivo*. Thus, OTZ was found to reduce the intracellular content of GSH in melanoma cells, producing reduced proliferation and increased chemosensitivity, whereas it increased GSH levels in peripheral blood mononuclear cells, exhibiting a corresponding cytoprotector effect (Del Olmo et al., 2000, 2006; Bilbao et al., 2002).

Several authors have pointed to the usefulness of GSH modulating agents as an adjuvant in chemotherapy treatments for CRC. Regarding 5-FU, a number of studies support the therapeutic use of antioxidant compounds in combination with this drug. Thus, therapy with high doses of antioxidants such as pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC) seem to enhance the therapeutic efficacy of 5-FU (Bach et al., 2001).

NAC is a prodrug of cysteine, which is an essential element for GSH synthesis. It was developed to avoid the important toxicity produced as a consequence of the direct administration of this aminoacid. The mechanisms of protection of this thiol against mutagenesis and carcinogenesis are related to a large number of biological effects, including antioxidant activity, involvement in DNA repair mechanisms, modulation of gene expression and of signal transduction, immunological activity, regulation of cell survival and of apoptosis, inhibition of cell transformation, of invasion and metastasis and of angiogenic activity, among others (Morini et al., 1999). However, some authors have reported contradictory effects of NAC in its anticancer action. Moreover, it has been demonstrated that antioxidant protection therapy in cancer patients should be used with caution, since it can give rise to counterproductive effects (Brizel & Overgaard, 2003). The reduction in the concentration of free radicals due to the excessive administration of antioxidants can stimulate the survival of damaged cells, enhancing the neoplastic stage, thereby promoting carcinogenesis more than inhibiting it. Furthermore, we and others have demonstrated that the increase in GSH levels induced by NAC is not specific to normal cells; rather, this can also occur in tumor cells, such as melanoma, increasing its proliferative capacity and protecting it against the cytotoxic effects of acrolein, one of the active metabolites of CY (Del Olmo et al., 2000).

It has also been found that the reduced levels of GSH induced by BSO and OTZ, lead to an increased cytotoxic effect of 5-FU in different human CRC cells (Meurette et al., 2005; Palomares et al., 2009). It has also been observed that BSO enhances the activity of SN-38 (an active metabolite of the anticancer drug irinotecan) in WiDr colon cancer cells (Caramés et al., 2010), and in cell lines of ovary cancer resistant to cisplatin, as well as of breast cancer. BSO is also capable of reverting resistance to SN-38 in leukemia cells with increased GSH levels (Yoshida et al., 2006). This increased anti-tumor effect of SN-38 may be related to the reduced activity of the transcription factor NF-κB, which is dependent on the intracellular redox status and thus sensitive to a reduced content of intracellular GSH. In fact, SN-38 is known to activate NF-κB and so the pharmacological inhibition of this NF-κB signaling pathway can enhance the anti-tumor activity of SN-38 in colon cancer cells *in vitro* and of irinotecan *in vivo* (Lagadec et al., 2008).

Regarding platinum compounds, various authors have demonstrated that GSH participates in the detoxification of these agents and that reduced GSH levels sensitize cancer cells to the cytotoxic effects of these anti-tumor agents (Jansen et al., 2002). We have found that both BSO and OTZ increase the efficacy of oxaliplatin in the WiDr human colon cancer cell line.
Thus, reduced GSH levels mediated by BSO or OTZ lead to an increase in cytotoxicity induced by drugs which are more frequently used nowadays for CRC therapy, with an additive effect being observed in the antiproliferative effects of these combinations.

6. The influence of growth factors in the sensitivity of tumor cells to chemotherapy

Research on the phenomenon of chemotherapy resistance has traditionally focused on the tumor cells themselves. However, it has become apparent that the tumor microenvironment may also influence chemoresistance in an important way. In this regard, it is necessary to underline the fundamental role of GFs in cancer biology and in the formation of metastasis, since they control critical functions in cancer cells, such as proliferation, angiogenesis and the inhibition of apoptosis. Thus, GFs, due to their capacity to modulate the sensitivity of tumor cells to cytotoxic drugs, have become important targets for the development of new anti-cancer therapies, either as individual agents or in combination with conventional chemotherapy, with the aim of enhancing the efficacy of anti-cancer drugs.

It has been demonstrated that the presence of GFs significantly reduces the cytotoxic activity of a number of commonly used drugs. In this regard, some authors have pointed out that HGF protects tumor cells against the cytotoxicity and apoptosis induced by DNA-damaging agents, such as ionizing radiation or adriamycin (Shen et al., 2007), and that it may contribute to the resistance of RMS cells to conventional treatment (Jankowski et al., 2003). Similarly, it has been suggested that this factor could induce resistance to cisplatin in lung cancer cells (Chen et al., 2008). Nevertheless, in contrast to expectations, it has also been observed that HGF sensitizes ovary cancer cells to the drugs paclitaxel and cisplatin (Bardella et al., 2007). These findings indicate that HGF effects depend on the targeted tumor type. Indeed, various other studies have reported the effect of VEGF in reducing the efficacy of endocrine therapy in breast cancer (Qu et al., 2008). It has also been found that VEGF diminishes the response to drugs in myeloid leukemia (De Jonge et al., 2008) and that doxorubicin exerts a milder inhibitory effect in the presence of VEGF overexpression in soft-tissue sarcoma (Zhang et al., 2006). Regarding EGF, it has been widely demonstrated that this GF reduces the response of tumors, such as human breast carcinoma, to cytotoxic compounds and to radiotherapy (Schmidt & Lichtner, 2002).

In the case of CRC, we (Palomares et al., 2009) and others (Sun & Tang, 2003; Allendorf et al., 2004) have demonstrated that HGF, EGF and VEGF significantly reduce the efficacy of drugs currently used in CRC. In particular, the increased expression of HGF and VEGF results in fluoropyrimidine-based adjuvant chemotherapy being less effective, increasing the risk of recurrence. In relation to EGF, it has been shown that its receptor, EGFR, increases resistance to 5-FU. Moreover, 5-FU itself induces the activation of EGFR, which protect colon cancer cells against chemotherapy (Hiro et al., 2008). Moreover, it has been reported that SN-38, through a mechanism involving ROS, induces the activation of EGFR and EGFR, and this could contribute to resistance to irinotecan (Kishida et al., 2005). These data suggest that inhibition of the EGFR signaling pathway could revert resistance to treatment with the fluoropyrimidines and irinotecan. On the basis of this hypothesis, some authors have carried out assays using tyrosine kinase inhibitors of EGFR, such as gefitinib (Stebbing et al., 2008), as well as inhibitors of the Src tyrosine kinase (Ischenko et al., 2008).
The molecular mechanisms underlying GF-mediated resistance continue to be largely unknown. On the one hand, GFs induce cell proliferation and the activation of anti-apoptotic signaling pathways, via proteins such as Bcl-XL, thereby contributing to the resistance to apoptosis in CRC cells following treatment with 5-FU, oxaliplatin and irinotecan (Schulze-Bergkamen et al., 2008). In addition, it has also been suggested that GFs may also induce an increase in the repair of damaged DNA (Hiro et al., 2008). On the other hand, it has been observed that the EGFR-Src-STAT3 oncogenic signaling pathway plays an important role in CRC, contributing to proliferation, cell survival and treatment resistance (Hbibi et al., 2008). In fact, it has been demonstrated that this pathway is activated in response to treatment with topoisomerase I inhibitors, such as camptothecins, reducing DNA damage and enhancing cell survival (Vigneron et al, 2008).

Moreover, as we have recently shown, GFs give rise to an increase in GSH levels which, as mentioned earlier, is an important mechanism of cell defense against oxidative stress and against the effects produced by radiation and by some chemotherapeutic agents; this increase in GSH levels has been correlated with diminished 5-FU anti-tumor activity in colon cancer cells (Palomares et al., 2009). In this regard, it has been reported that the combination of an EGFR inhibitor with doxorubicin leads to enhanced cytotoxic effects via the generation of oxidative stress, due to ROS induction and reduced GSH content in rat hepatoma cells (Ortiz et al., 2008).

Additionally, it has been suggested that GF-induced increases in intracellular GSH levels and the activation of the redox-sensitive transcription factor NF-κB could play a major role in inducible chemoresistance. This cell survival transcription factor, which is subject to regulation by GSH (Lou & Kaplowitz, 2007), has been shown to be constitutively activated in many colon cancer cells. NF-κB has been shown to be associated with the proliferation of tumor cells, with invasion, angiogenesis and the production of metastasis (Bours et al., 1994). It has been demonstrated that HGF, via the PI3K/Akt signaling pathway, leads to the activation of NF-κB, by means of which cells are protected against Adriamycin and irinotecan (Fan et al., 2005). In the same way, the transmission of the proliferative signal induced by EGF is also mediated by the activation of NF-κB (Sethi et al., 2007), which plays an important role in the regulation of EGFR ligands via a ROS-mediated mechanism (Murillo et al., 2007). Moreover, NF-κB activation in response to exposure to anti-cancer drugs has been shown to be one of the mechanisms of tumor resistance to chemotherapy, as has been reported in the cases of 5-FU and irinotecan (Ahn et al., 2008). In contrast, inhibition of NF-κB has been shown to enhance the sensitivity of colon cancer tumor cells to HT-29 and 5-FU (Voboril et al., 2004).

Overall, these data indicate that GFs play a critical role in the resistance of colon cancer cells to chemotherapeutic agents. In consequence, these factors are potential therapeutic targets for increasing the anti-tumor activity of cytotoxic drugs.

**7. New therapeutic strategies to enhance the response of CRC to chemotherapy by reversion of the growth factor pro-tumour effects**

Based on the aforementioned data, GFs have been identified as important targets to be considered in the development of new anticancer drugs and, consequently, many experimental studies have been carried out to evaluate the effects of blocking GF effects on tumor cells. These attempts could be classified into three categories, according to the mechanism chosen to avoid the GF stimulation of these cells. The first approach was to
administer monoclonal antibodies (MoAb) against one or several GFs, and the results have been quite exciting. Another idea was to produce MoAb against the membrane receptors for different GFs, and again the results have been very promising. In fact, several of these MoAb have already entered the armamentarium for cancer therapy and others are currently at different stages of clinical trials. The third exciting arm of these GF-based therapies consists of the so-called “small molecules” which block the activation of the intracellular part of GF receptors.

The combination of conventional cytotoxic drugs with new agents that specifically interfere with GF signaling pathways presents the advantage of avoiding crossed resistance, since these approaches are directed against different cell targets and have different underlying mechanisms of action. In this regard, many studies have indicated that inhibitors of GFs or of their receptors enhance the efficacy of conventional cytotoxic agents (Wanebo & Berz, 2010). The GF inhibitors bevacizumab and cetuximab are particularly noteworthy. Currently, bevacizumab is used in combination with regimes which contain 5-FU (FOLFOX or FOLFIRI) as a first line therapy in advanced or metastatic CRC (Giantonio et al., 2007; Tol & Punt, 2010). On the other hand, combined therapy consisting of irinotecan and cetuximab is indicated after progression in patients who have previously received 5-FU based therapy (Cunningham et al., 2004).

Other agents, such as gefitinib, have been found in preclinical studies to exhibit synergistic inhibitory effects when administered in combination with different cytotoxic drugs. For example, some authors have observed that gefitinib and irinotecan act synergistically in WiDr cells, as a result of the inhibition of the survival signal induced by irinotecan via the phosphorylation of EGFR (Koizumi et al., 2004). Similarly, in vitro studies have shown that the combination of gefitinib and oxaliplatin has a synergistic effect in colon cancer cells due, at least in part, to the fact that the EGFR inhibitor reduces the activity of \gamma\-GT. This enzyme, which participates in the \gamma\-glutamyl cycle, helps to salvage extracellular GSH and contributes to redox control by providing a substrate for GSH synthesis during oxidative stress, thereby preventing apoptosis, as we have showed previously (Castro et al., 2002). Reduced \gamma\-GT activity thereby leads to increased cellular oxaliplatin accumulation and platinum-DNA adducts (Xu et al., 2003).

However, these anti-tumor agents also have their inconveniences. They induce diverse side effects which complicates their clinical use (Mulder et al., 2011). Also, as happens with other chemotherapeutic agents, the development of resistance to these GF-based agents has already been reported (Giaccone & Wang, 2011). For these reasons, it is important to continue the search for new therapeutic strategies which could be used in combination in order to enhance the efficacy of GF-related targeted agents. In this sense, the therapeutic biomodulation of GSH metabolism may hold promise for the improvement of the efficacy of anticancer treatments. Many lines of evidence indicate that this may be an effective approach to treating cancer: i) the fact that tumor cells are under high levels of oxidative stress may represent a great opportunity given that it means they are particularly vulnerable to further increases in ROS levels; ii) colon cancer cells contain particularly high levels of GSH; iii) GF-induced signal transduction pathways are redox sensitive, and accordingly, alterations in cellular GSH content may affect the growth of GF-sensitive cells; and iv) the fact that NF-κB is involved in GF-dependent proliferation and that the activity of this transcription factor might also be subject to regulation by GSH suggests that depletion of cellular GSH could interrupt NF-κB activity and consequently lead to growth inhibition.
In this regard, we have recently demonstrated that GSH-induced depletion by BSO or OTZ abrogated the growth-promoting effects of GFs in WiDr colon cancer cells (Palomares et al., 2009). Similarly, other authors have demonstrated that BSO inhibits GSH upregulation induced by HGF, thereby blocking its mitogenic effect (Yang et al., 2008) and the protection against apoptosis afforded by this factor. It has likewise been reported that BSO interferes with EGF-induced proliferation and that extended exposure (for 48 h or more) of cells to BSO induces cell death, probably via a necrotic mechanism (Carmona-Cuenca et al., 2006). Regarding VEGF, it has also been reported that BSO treatment reverses increased GSH activity induced by this factor and, in this way, its vasculoprotective function (Kuzuya et al., 2001). In contrast, decreased GSH levels produced by BSO have been shown to promote the autocrine secretion of VEGF (Sreekumar et al., 2006).

Thus, the two effects derived from the biomodulation of GSH intracellular content with BSO or OTZ, i.e. i) the reversion of the pro-tumor effect of GFs and ii) the enhanced efficacy of chemotherapy, may contribute to enhancing the therapeutic benefit of chemotherapy treatment. In fact, we have shown that, in the presence of GFs, the combination of either of the GSH modulators with chemotherapeutic drugs produced greater anti-tumor activity than the cytotoxic drugs alone. Thus, we found that both BSO and OTZ completely reverted the resistance (due to the presence of GFs) of WiDr colon cancer cells to 5-FU, a finding which holds promise for more successful anticancer treatment, particularly after surgical resection of hepatic metastases (Palomares et al., 2009). Indeed, 5-FU activity was enhanced by 40% following the addition of GSH modulators. The activity of oxaliplatin was also found to be significantly enhanced (by nearly 25%). Moreover, combined therapy with SN-38 was found to produce the optimal chemotherapeutic combination; thus, OTZ pretreatment combined with SN-38 resulted in an increase of almost 70% in the cytotoxic activity of SN-38 (Caramés et al., 2010). To this benefit, we must also add the advantage of OTZ with respect to BSO, i.e. the selective reduction of GSH levels in tumor cells, protecting healthy cells, as mentioned above.

Other interesting approaches to the GF problem in cancer therapy have been developed. Thus, as cell proliferation and differentiation are deregulated in tumor cells, the induction of cell differentiation with retinoids could help to neutralize the pro-tumor effect of GFs. The mechanisms of action which underlie the effects of retinoids include the activation of nuclear retinoic acid receptors (RAR), but also, curiously, the induction of enhanced ROS levels (Palomares et al., 2006) and a direct interaction of retinoids with the GSH-dependent protein kinase C, a key regulatory enzyme in signal transduction (Radominska-Pandya et al., 2000). In this sense, we have analyzed the effect of all-trans-retinoic acid (ATRA), a well known pro-differentiating agent, on the growth-promoting effect of GFs in two tumor models. This drug was found to reduce the proliferative rate of RMS (García-Alonso et al., 2005) and CRC cells (Martínez-Astorquiza et al., 2008), and hindered or completely abolished the stimulus produced by serum obtained from hepatectomized rats, and by a wide variety of GFs (HGF, VEGF, PDGF, EGF, bFGF). Furthermore, we also found that cells cultured in medium containing ATRA do not develop resistance to the drug, and these ATRA-preexposed cells responded to subsequent ATRA treatments in the same manner as non-treated cells. However, we observed that the antiproliferative effect of ATRA in vitro is not permanent: forty eight hours after removing the drug from the culture medium the cells recovered their normal proliferative rate (Díaz et al., 2009). Nevertheless, in in vitro studies, we found that ATRA did not interfere with the antiproliferative effect of chemotherapeutics...
drugs, such as 5-FU; moreover, when both drugs were administered together, an additive effect was observed (García-Alonso et al., 2010).

In order to corroborate these findings *in vivo*, we designed an experimental model in which daily intraperitoneal doses of ATRA were administered for two weeks, starting three days before a partial hepatectomy was performed in animals bearing liver metastases. These *in vivo* experiments confirmed the efficacy of ATRA in reducing the proliferative rate of tumor cells. In rats bearing RMS S4MH liver metastases, the mean number of liver metastases, as well as their mean size, were significantly reduced and significantly longer survival was achieved. Using this tumor model, we also analyzed the synergistic effect of ATRA with commonly used chemotherapeutic agents such as CY. Once again, animals treated with ATRA+CY presented a significant reduction in the mean number of liver metastases and also an increase in survival compared to animals treated with CY alone. Similar experiments were carried out with the murine CC-531 colon cancer cell line, and similar, albeit not so dramatic, results were found (unpublished data). Thus, the mean number of liver metastases was unmodified by ATRA, but the mean size of the liver foci was significantly reduced, suggesting that tumor progression had been retarded. However, survival remained unaltered. Regarding drug tolerance, ATRA was well tolerated by the animals, with no repercussion on hematological cell counts, serum enzymes or weight gain. These findings point to the need to enhance ATRA effects via other mechanisms. In this regard, it has recently been shown that the selective COX-2 inhibitor celecoxib, increased the expression of RARbeta in human colon cancer cells, as well as sensitivity to ATRA through COX-2-independent mechanisms (Liu et al., 2010).

Novel synthetic derivatives of ATRA have been developed recently and examined in clinical trials (Sogno et al, 2010). However, these trials involve administration of the drug as a conventional chemotherapeutic agent (Kummar et al, 2011). In the light of the above, it is apparent that retinoids (or pro-differentiating agents in general) should be tested as a complementary treatment, and administered as part of a combined therapy during the early postoperative period, when their action would be most effective. Otherwise, it is unlikely that significant improvements will be found in patients treated with these agents in monotherapy.

Overall, and in the light of the important role of GFs in tumor recurrence following surgical resection of hepatic metastases, the use of GSH modulators and pro-differentiating agents seems to hold promise as a novel therapeutic strategy for metastatic CRC, by reversing GF pro-tumor effects and improving the efficacy of chemotherapy.

8. Conclusion

Growth factors play a pivotal role in the regulation of CRC progression and metastasis. They are involved not only in promoting tumor growth, but also in reducing the responsiveness of tumor cells to cytotoxic compounds. The mechanisms of action underlying GF effects include the redox state of the tumor, in particular GSH metabolism, and the level of expression of related enzymes. The biomodulation of GSH metabolism via agents such as BSO or OTZ, could reverse the growth-promoting effects of GFs and enhance the therapeutic benefit of chemotherapeutic drugs. The use of pro-differentiating agents may also represent a promising anti-tumor strategy to block the pro-tumor effects of GFs. The development of more effective retinoids, used either alone or preferably in combination with other drugs, may also provide more effective anti-tumor benefits. These new types of
strategies to neutralize the pro-tumor effects of GFs may well be crucial in the treatment of metastatic disease and the prevention of the recurrence of liver metastases arising from CRC.

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10. References


Growth Factors and the Redox State as New Therapeutic Targets for Colorectal Cancer


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Colorectal cancer is a common disease, affecting millions worldwide and represents a global health problem. Effective therapeutic solutions and control measures for the disease will come from the collective research efforts of clinicians and scientists worldwide. This book presents the current status of the strides being made to understand the fundamental scientific basis of colorectal cancer. It provides contributions from scientists, clinicians and investigators from 20 different countries. The four sections of this volume examine the evidence and data in relation to genes and various polymorphisms, tumor microenvironment and infections associated with colorectal cancer. An increasingly better appreciation of the complex inter-connected basic biology of colorectal cancer will translate into effective measures for management and treatment of the disease. Research scientists and investigators as well as clinicians searching for a good understanding of the disease will find this book useful.

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