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ACTION OF POLYPEPTIDE GROWTH FACTORS IN COLON CANCER; DEVELOPMENT OF NEW THERAPEUTIC APPROACHES

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1. ABSTRACT

There has been no significant improvement in the treatment of metastatic colon cancer over the last four decades. A major reason for therapeutic failure is the nonselective nature of conventional chemotherapy. Therefore, new selective therapeutic approaches must be explored to improve survival. Recent advances in cell and molecular biology have opened up new avenues of developing selective molecular therapy for colon cancer. In view of the heterogeneity of cells in colon cancer, it is likely that the use of more than one cellular target will be required to achieve a significant antitumor response. Mechanistic studies of how proliferation, differentiation and malignant properties are controlled by negative growth factors, positive growth factors and adhesion molecules have allowed the identification of several molecular targets of attack in colon cancer. Disruption of one or more of these targets should have a highly antiproliferative and/or cytotoxic effect on colon cancer cells. Additionally, disrupting the expression of these targets may augment sensitivity to conventional chemotherapy. In this article, we will discuss how polypeptide growth factors act in colon cancer cells, identify several molecular targets of attack and discuss strategies for selective disruption of these targets in colon cancer. Where appropriate, the biologic similarities or differences by comparison with other types of tumor will also be discussed.

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2. INTRODUCTION

Metastatic human colon cancer is a highly refractory malignant disease which afflicts both men women in equal proportion. The antimetabolite, 5-fluorouracil (5-FU), used to treat colon cancer over 4 decades ago, is still the drug of choice today for the treatment of metastatic disease. Despite some improvements in adjuvant therapy using 5-FU in combination with leucovorin or levamisole, almost half of all colorectal cancer patients will die of metastatic disease (1). Therefore, new therapeutic approaches must be explored to improve survival. An understanding of the biology behind malignant transformation and the mechanisms sustaining the transformed phenotype may provide avenues of therapeutic intervention.

Control of cellular proliferation, differentiation and adhesion are complex biological processess in which different biological systems interact to achieve control. Cell-matrix and cell-cell adhesion are important cellular processess in regulating cellular proliferation and differentiation (2-5). Extracellular matrix (ECM) adhesion molecules modulate gene expression in epithelial cells in a tissue specific manner (5-7). Disruption of these processes is a hallmark of malignant transformation and plays a critical role in tumor progression and the behavior of malignant cells (8-10).

Polypeptide growth factors constitute a potent class of extracellular and/or intracellular signal molecules in regulating cellular proliferation and differentiation (11-13). Aberrant expression of growth factors and/or aberrant responses to growth factors may circumvent the normal pathway of differentiation, leading to cellular transformation, tumor progression and maintenance of the transformed phenotype (11, 14). Transforming growth factor (TGF) beta constitutes a class of multi-functional

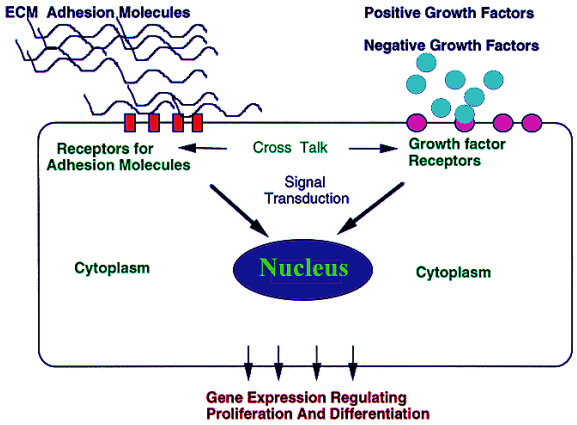

Figure 1. Interaction of ECM adhesion molecules with growth factors

polypeptide growth factors that regulate proliferation and differentiation in many cell types (15-17) and suppress malignancy in TGF-beta-responsive epithelial cancer cells (18-20). Loss of responsiveness to TGF-beta is thought to be a mechanism of escape from normal growth control in malignant cells (21-22). The ability of TGF-beta to induce a more benign and differentiated phenotype in malignant cells is partly attributable to its ability to upregulate the synthesis of ECM adhesion molecules (23-28). Thus, TGF-beta may be viewed as a class of negative growth factors.

The epidermal growth factor (EGF) family of polypeptide growth factors such as EGF and TGF-alpha, on the other hand, are potent stimulators of cellular proliferation and stimulate the malignant behavior of many epithelial-derived cancer cells (29-35). Thus, the EGF family of growth factors may be viewed as a class of positive growth factors. How ECM adhesion molecules and growth factors interact in controlling proliferation, differentiation and adhesion is depicted in Figure 1.

In this review, we will discuss how negative and positive growth factors act in colon cancer and review the rationale and strategy behind the development of new therapeutic approaches. Where appropriate, the biologic similarities or differences by comparison with other tumor types or other agents involved in regulating differentiation will also be discussed.

3. DISCUSSION

3.1. Adhesion molecules and TGF-beta

Fibronectin (FN) is a transformation-sensitive ECM adhesion molecule. The expression of FN in many transformed cells is lost (36-40). FN interacts with specific cell-surface FN receptor to initiate intracellular signal tranduction leading to the regulation of gene expression (41-45). A major species of FN receptor is the integrin alpha5beta1 which has been shown to act as a tumor suppressor (46-47). Interestingly, when transformed cells are treated with differentiation-inducing agents, these agents induce a higher level of FN and FN receptor expression in conjunction with the restoration of a more normal or benign phenotype to the transformed cells (36-38). In normal and malignant cell hybrids, the malignant phenotype is suppressed and the hybrids express a high level of FN by comparison with the malignant cells (48). However, blockade of FN synthesis in the hybrids abrogates the suppression of the malignant phenotype (48). The expression of FN and FN receptor is tightly regulated in the process of transformation and the induction of differentiation of the transformed cells. When mouse embryonic fibroblasts are transformed with a chemical carcinogen, the expression of FN and FN receptor is significantly reduced (36-38). Induction of differentiation of the transformed cells by treatment with differentiation-inducing agents restores normal growth control and cellular morphology to the transformed cells with a concurrent restoration of the expression of FN and FN receptor (36-38). Blockade of FN synthesis in the transformed cells downregulates the ability of differentiation-inducing agents to induce FN and FN receptor expression and circumvents the induction of normal cellular morphology (49). Thus, both FN and FN receptor play a functional role in chemical transformation and the induction of differentiation in transformed cells.

In this context, human colon cancer cells produce very little FN (50). The production of FN, however, is upregulated when colon cancer cells are induced to diffferentiate towards a more normal or benign phenotype by treatment with TGF-beta or other differentiation-inducing chemicals (23-28). Patients with FN-positive invasive breast carcinomas fare significantly better than patients with FN-negative tumors in terms of relapse-free survival. In addition, the FN staining pattern is independently correlated with relapse-free survival and nodal status (51).

Interestingly, contrary to the colon and breast tumor types, both androgen-responsive and non-responsive prostate cancer cells produce abundant amounts of FN (52-53). The biological role of these molecules in the malignant phenotype of prostate cancer cells is not known. However, it is now known that the ECM and stroma play a critical role in malignant transformation and progression of the prostatic epithelium (54). It is of interest to note that (contrary to colon and breast cancer) a high level of FN expression has been reported in prostate cancer tissue by comparison with normal prostate and hyperplastic tissues (55-56).

Three isoforms of TGF-beta (TGF-beta1, TGF-beta2 and TGF-beta3) are expressed in mammals and overall, the biological activities of these isoforms are very similar *in vitro* (57). Therefore, for the purpose of discussion, the generic term TGF-beta will be used in this article. TGF-beta interacts with a family of high-affinity cell-surface receptors through which it mediates its biological action. Most cell types, including human colon cancer cells possess such receptors for TGF-beta (57-60). The family of TGF-beta receptors interact to mediate the action of TGF-beta (61-65). The post-receptor or intracellular mechanisms through which

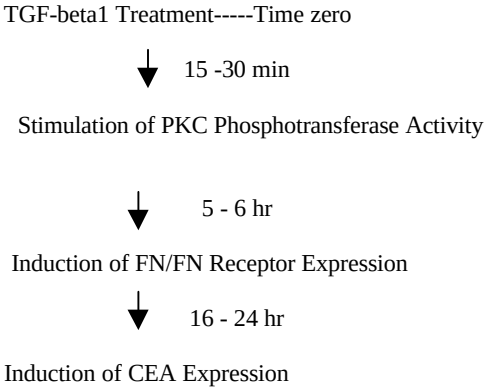

Figure 2. Intracellular events following TGF-beta binding to human colon cancer cells

TGF-beta acts, however, are poorly understood. Intricate and complex mechanisms appear to be involved in the action of TGF-beta. It has been proposed that multiple TGF-beta signal-transducing pathways exist (66). Both differences and similarities are likely to exist when the biological action of TGF-beta and its mechanisms of action are compared in different cell types (15, 66-72).

TGF-beta elicits diverse responses in TGF-beta-responsive human colon cancer cells. These responses include inhibition of proliferation, invasive capability, growth in soft agarose and tumor formation in athymic mice (18-20, 25, 73). At the molecular level, TGF-beta upregulates the expression of the adhesion molecules, FN and laminin (LM), the cell-surface receptors for these molecules, and the intercellular adhesion molecule, carcinoembryonic antigen (CEA) (23-28). In addition, TGF-beta upregulates the expression of epithelial-associated cytokeratins, reorganizes the cytoskeleton and induces increased adhesion and spread on the ECM (23-28). The net effect of TGF-beta action is the induction of a more differentiated or benign phenotype in colon cancer cells. Thus, how TGF-beta controls these cellular responses is of great interest to cancer biologists and such knowledge may provide leads in the development of novel therapeutics against colon cancer. It is hypothesized that the development of resistance to TGF-beta is a mechanism of tumor progression and escape from normal growth and differentiation control. Indeed, mutated TGF-beta type II receptor has been found to be associated with highly malignant TGF-beta-non-responsive human colon cancer cells (22) and suppression of TGF-beta autocrine activity promotes malignancy (18). The loss of responsiveness to TGF-beta may also be an important mechanism of escape from normal growth control in prostate and breast cancers (21, 74-75).

3.2. Mechanisms of action of TGF-beta in colon cancer, role of protein kinase C

Protein kinase C (PKC) is an ubiquitous calcium- and phospholipid-dependent protein kinase involved in transmembrane signal transduction (76). It may also play a role in growth control and carcinogenesis in the colon (77-79). At least 11 PKC isoforms have been identified and characterized (79-81). The functional significance of individual isoforms, however, is not well understood. TGF-beta stimulates a rapid rise in PKC phosphotransferase activity (69) and the kinetics of events that follow are depicted in Figure 2.

Earlier work has shown that chemical inhibitors of PKC were able to block the ability of TGF-beta to induce CEA expression, suggesting the functional role of this enzyme in the signal pathway of TGF-beta leading to the induction of CEA (69). Interestingly, blockade of FN induction (an earlier event) by expressing antisense FN RNA in the cells also blocked the induction of CEA, suggesting a functional role of FN in the induction of CEA (28). Exactly how FN interacts with the TGF-beta pathway leading to the induction of CEA is not known. FN can interact with other components of the ECM in regulating gene expression (82) or newly synthesized FN may act through a feed-back mechanism, interacting with the FN receptor and initiating signal-transduction cascades (41-45, 83) that are required for TGF-beta to mediate its action on the CEA gene.

Like the mouse embryonic fibroblasts described above, the expression of FN and FN receptor is tightly regulated in human colon cancer cells. Treatment of colon cancer cells with TGF-beta upmodulates the expression of both FN and FN receptor (27). Blockade of FN synthesis by the cellular expression of antisense FN RNA circumvented the ability of TGF-beta to upregulate the expression of both FN and its receptor (84). This makes sense from a cellular physiologic point of view. If the cells were to respond to the physiologic effect of an increased level of FN expression, the level of receptor expression needs to increase to accommodate the ligand. The mechanisms behind the coregulation of the FN ligand and its receptor by TGF-beta remain to be elucidated.

Recent work identifies PKCalpha isoform in controlling the adhesion response (induction of adhesion molecules and receptors for these molecules) to TGF-beta. Expression of antisense PKCalpha RNA (by transfection with an antisense PKCalpha expression vector) in human colon cancer cells downregulated PKCalpha protein expression and attenuated the ability of TGF-beta to upregulate the expression of FN, FN receptor and CEA (70). Interestingly, perturbation of PKCalpha did not interfere with the molecular pathway of TGF-beta leading to the inhibition of cellular proliferation (unpublished observation). These results suggest that PKCalpha is a focal point in controlling the adhesion signal pathway of TGF-beta but not its antiproliferative pathways. Further work is in progress to confirm the validity of this hypothesis.

We wish to point out that the induction of FN and CEA is not a unique property of TGF-beta. Many differentiation-inducing agents such as retinoic acid, difluoromethylornithine, dimethylformamide, sodium butyrate and sodium suramin will inhibit the malignant properties of human colon cancer cells with a concurrent

upregulation of FN and CEA synthesis (85). The mechanisms of action of these differentiation-inducing chemicals are probably quite different from that of TGF-beta, and the differentiation-inducing capability of these chemicals varies and is contingent upon the phenotype of the target cells (85). This is not surprising in view of the phenotypic heterogeneity of colon cancer cells.

3.3. PKC alpha - a potential therapeutic target

Alterations in PKC expression have been suggested to play a role in tumor progression and in the maintenance of the malignant phenotype (76-77, 86-89). In addition, PKC is implicated to play a functional role in multi-drug resistance (90-91). Specifically, PKCalpha activates the MDR-1 gene product, gp170, by phosphorylation and thus increases efflux of drugs from the cell (92). In the colonic epithelium, continuous activation of PKC by unsaturated diacylglycerols in the intestine may be responsible for intrinsic drug resistance (93). PKCalpha isoform is implicated to play a role in drug resistance in colon cancer cells (94). It has recently been shown that PKC alpha can be targeted to potentiate the responses of colon cancer cells to anticancer drugs. Expression of PKC alpha antisense RNA (but not beta or gamma isoforms) in human colon cancer cells resulted in down-modulation of PKC expression and increased the cytotoxic effect of 5-fluorouracil, mitomycin C and vincristine by several folds (95). Therefore, PKC alpha is a good target for the purpose of potentiating sensitivity to conventional anticancer drugs.

3.4. EGF family of growth factors and the potential of EGF receptor as a therapeutic target

Human colon cancer cells produce and respond to the EGF related family of polypeptide growth factors (32-33, 73, 96). The EGF family of growth factors consists of EGF, TGF-alpha, cripto and amphiregulin (29-31). These growth factors are potent stimulators of cellular proliferation, and overexpression of one or more of these growth factors or receptors for these factors have been implicated to play a causal role in the transformation and maintenance of the transformed phenotype (11-12, 14, 35, 97-102). EGF related growth factors mediate their action through a common receptor, the EGF receptor (EGFR) (29). Stimulation of the EGFR by growth factors induces proliferation and malignant cell behavior (e.g. propensity to grow in semi-solid medium and invasion of matrigel (32-34, 101)). In addition, highly metastatic human colon cancer cells express a relatively high level of EGFR (35).

Cells can respond to these growth factors via an autocrine mode (i.e. the cells secrete these factors and the factors then bind to the cell-surface EGFR thus, activating the EGFR); a paracrine mode (i.e. cells respond to factors produced by other cells via the cell-surface EGFR); or an intracrine mode (i.e. cells produce and respond to these factors inside the cells, bypassing the cell-surface EGFR) (12-13). Previous work shows that human colon cancer cell lines, including some highly aggressive lines, produce and secrete both EGF and TGF. Some cell lines utilize these growth factors via the cell-surface EGFR (i.e. susceptible to blockade by anti-EGFR antibodies), others do not (32) i.e. these cells may utilize growth factors via an intracrine mode. Subsequent work supported the hypothesis that these cells utilize growth factors in an intracrine manner because the intracellular expression of antisense EGFR RNA in these cells inhibited the proliferation and malignant behavior of these cells (33-34). Some human colon cancer cell lines are very sensitive to EGFR blockade by the intracellular expression of antisense EGFR RNA through transfection with an antisense EGFR expression vector (33-34), while others are relatively resistant. The expression of antisense EGFR RNA in the sensitive cells results in a significant downmodulation of EGFR protein and mRNA expression with a concurrent inhibition of cellular proliferation and malignant properties (33-34). Therefore, EGFR may be a good molecular target to be used against colon cancer.

The EGFR may also serve as a good target of attack for other epithelial cancer. Studies using human prostate carcinoma tissues and prostate carcinoma cell lines strongly implicate the participation of EGF-related molecules and EGF receptor in the pathogenesis of prostate cancer (103-104). In fact, the growth stimulatory effect of androgen in androgen-responsive prostate cancer cells is attributable to the EGF/TGF-alpha-EGF receptor loop (105-106) and EGF can activate the androgen-signaling chain in the absence of androgens. This loop also participates in the cellular proliferation of androgen-independent prostate cancer cells (107). In breast carcinoma, the expression of Her-2/neu, a member of the EGFR family of receptors, is significantly elevated and correlates with poor prognosis, tumor progression and drug resistance (108-109). EGF and TGF-alpha also act as positive regulators in breast cancer (110-111). Thus, Her-2/neu and EGFR are also good therapeutic targets for breast cancer.

3.5. Other potential therapeutic targets

Cyclin dependent kinase 2 (CDK2) is an intracellular signal-transduction molecule that functions in regulating cell cycle progression (112). It initiates cell cycle progression by phosphorylating specific cellular target molecules (112). Many growth factors mediate their effect through CDK2. Blockade of EGFR with anti-EGFR antibody blocks CDK2 activity and results in the induction of G1 arrest (113-114). Thus, disrupting CDK2 and EGFR may lead to a "double whammy" effect on the cancer cells: choking off the action of the EGF related family of growth factors and simultaneously blocking the action of CDK2, the activity of which is required for cell cycle progression. In addition, since CDK2 is a downstream target of the EGFR, cells that are resistant to EGFR blockade may have developed mechanisms of escape from CDK2 inactivation. Therefore, it is likely that cells that are resistant to EGFR blockade will be susceptible to the direct disruption of CDK2.

As discussed above, TGF-beta suppresses malignant tumor growth and induces differentiation in human colon cancer cells. Escape from TGF-beta control, through lack of TGF-beta receptor expression or expression of mutated receptor, is thought to play an important role in the tumor progression and malignant growth of colon

cancer (22). An important postreceptor target of TGF-beta is also CDK2. TGF-beta induces G1 cell cycle arrest by inhibiting the activity of CDK2 (115-116). Thus, disrupting CDK2 may mimic the effect of TGF-beta and furthermore it may also mimic TGF-beta effect in TGF-beta-resistant cells because of direct disruption of a downstream postreceptor target of TGF-beta. Thus, disruption of CDK2 and EGFR together may deliver a "triple whammy" effect to the cancer cells.

As discussed above, cell-matrix and cell-cell interactions play critical roles in controlling the normal growth and differentiation programs of epithelial cells (2-7) and that abnormal cell-matrix and/or cell-cell interactions is a hallmark of malignant transformation (8-11, 36-38). Interactions of extracellular matrix components with specific cell-surface receptors turn on intracellular signal transduction mechanisms leading to the control of gene expression in a tissue-specific manner (41-45). Focal adhesion kinase (FAK) is an intracellular signaling molecule (tyrosine protein kinase) associated with the intracellular tails of cell-surface integrin receptors that bind to extracellular matrix components such as fibronectin (117-119). When normal epithelial cells are detached from the matrix they undergo programed cell death (120). Activation of FAK allows epithelial cells to become immortalized and undergo malignant transformation (120). FAK has been shown to be overexpressed in colon cancer (121) and disruption of FAK expression by antisense FAK oligonucleotides is cytotoxic to highly malignant human rhabdomyosarcoma cells in culture (122). Therefore, we hypothesize that disrupting FAK expression will lead to growth inhibition and/or cell death in colon cancer.

3.6. Therapeutic Strategies

In view of the heterogeneity of colon cancer cells, and the many mechanisms that cancer cells use to circumvent normal cellular control, it is likely that a multi-targeting and multi-modal type of approach is required to achieve a significant antitumor response. We have discussed at least 4 molecular targets of attack in colon cancer. Some of these targets, (e.g. EGFR and FAK) though overexpressed in colon cancer, are also expressed in most normal tissues. Therefore, success is more likely, if a basis for selective disruption of these targets in colon cancer cells exists in the therapeutic design. In addition, disrupting the expression of these targets (a good example is PKC alpha) may augment sensitivity to conventional chemotherapy. Thus, the molecular approach has the potential to be used in a truly multi-targeting and multi-modal manner with conventional chemotherapy to induce a high level of antitumor response.

Over the last several years this laboratory has used antisense mammalian expression vectors, under the control of viral promoters, for the purpose of disrupting the expression and/or function of specific cellular molecules. To this end, we have been successful in disrupting the expression and function of adhesion molecules such as fibronectin (28, 84) and other important signal molecules such as protein kinase C isoform (70, 95) and EGFR (33-34). In each of these cases, we have demonstrated that following transfection with the antisense expression vectors, the cells expressed the antisense RNA species and that the effect of antisense RNA expression resulted in a downregulation of the corresponding mRNA and protein expression. Exactly how the antisense RNA work inside the cells in disrupting protein expression is not known. It is likely that the expression of the antisense strand RNA interferes with translation of the sense mRNA strand and/or the antisense strand binds to its complimentary sense strand leading to rapid degradation of the sense mRNA (123). Therefore, we propose to use this approach for the purpose of disrupting the expression of key molecular targets in colon cancer.

3.7. Basis of selectivity against colon cancer

CEA is preferentially expressed in colon cancer cells and other tumors of the gastrointestinal tract (124). CEA is also expressed, albeit at a low level, in the normal gastrointestinal tracts (125-126). CEA, however, is not expressed in the majority of normal tissues including the bone marrow, heart, kidney and liver (125-126) (the liver being the major site of metastasis for colon cancer). The CEA promoter has been shown recently to be capable of driving the expression of genes only in CEA-producing cancer cells (127-128); i.e. only cells expressing CEA have the capability, or possess the transcription initiation factors necessary for turning on the CEA promoter. Therefore, if the antisense expression vectors targeting EGFR, CDK2, FAK or PKC were constructed under the control of the CEA promoter, there is a good chance of achieving a good degree of selectivity against colon cancer cells. One may envision infusion of these "DNA drugs" which are selectively active in colon cancer even though they might be taken up by most normal tissues and may eventually be degraded.

3.8. Perspective

Recent advances in cell and molecular biology have opened up new avenues of therapeutic approaches in attacking colon cancer. Chances of success are much improved with a multi-modal approach. A multi-modal approach is particularly attractive if a basis of selectivity exists against colon cancer cells. In the context of the "antisense DNA drugs", this is only an embryonic beginning and much research needs to be performed to determine their efficacy and optimal administrative regimens. A key to success lies in the development of non-toxic drug delivery systems for systemic therapy or regional therapy of liver metastasis. The use of non-toxic cationic liposomes (129-131) for drug delivery is quite attractive and may allow frequent infusion of "DNA drugs" as needed to achieve a favorable antitumor response.

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