Diet, Gene Expression, and Apoptosis: Clues to Cancer Prevention?

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THE ROLE OF DIET IN THE INITIATION OF CANCER

The development of cancer results from the interplay between genetic factors and the environment, and dietary factors have been identified as modulators of almost every step in the process. The complexity of this interplay between genetic factors and the nutritional environment is summarized in Fig. 1. In its initial stage, the development of a tumor requires genetic damage, caused either by endogenous processes or by exogenous compounds that produce mutations or even gene deletions.

Free radicals are one of the endogenous mediators of oxidative DNA damage and mutations. They are produced by normal oxidative metabolism in cells, but their production is increased under pathologic conditions such as inflammation. The hydroxyl radical is considered to be the ultimate radical species responsible for DNA damage. There is evidence that the oxidative damage resulting, for example, in 8-oxoguanine and oxidized thymine bases, is involved in cancer induction and progression (1,2). Defense mechanisms protecting the DNA from the free radical attack include the antioxidant enzymes superoxide dismutase, catalase, and peroxidase, which require for their function trace elements such as selenium, zinc, and manganese. Other dietary constituents—for example, vitamin C and E, carotenoids, and flavonoids—also contribute to the antioxidative capacity of a cell or an organism.

In addition to those "classical" antioxidants, many other compounds, such as caffeine or diallyl polysulfides from aged garlic extracts (3,4), have been identified as antioxidants. However, under certain conditions and depending on a variety of factors, including the level of intake, antioxidants can also have pro-oxidant properties. In the presence of free transition metals such as iron or copper, oxidative DNA damage can be induced by, for example, ascorbic acid through acceleration of the Fenton reaction (5). The presence of metals has also been shown to accelerate DNA nicking by phenolic antioxidants (6).

Another important endogenous mechanism influencing the genetic events involved in tumor development is the biomethylation of DNA. About 3% of cytosine residues in mammalian DNA are methylated after replication, most probably to
FIG. 1. Processes that affect the alteration of DNA and hence promote the transformation of a normal cell into a transformed cell and a tumor. A large variety of endogenous as well as exogenous factors can interact with the DNA and cause alterations such as mutations or DNA adduct formation. Protective nutritional factors that modulate those pathways are, for example, antioxidants scavenging reactive oxygen species or compounds that promote elimination of toxicants by the "excretory metabolism." DNA caretaking systems provide efficient repair mechanisms for maintaining genome stability. If oncogenes are activated and/or tumor suppressor genes are inactivated permanently, however, a normal cell can transform finally into a metastatic cancer. Essentially every step in these processes of tumor promotion and progression can be influenced by dietary factors (see text).

compartmentalize the genome into active and inactive regions. Changes in the pattern and intensity of methylation are found as the earliest and most consistent molecular abnormalities in human cancers (7,8). Methylation of "CpG islands"—that is, regions rich in cytosine–guanine doublets—in promoter regions of genes is a common mechanism of epigenetic silencing in somatic cells. If this silencing process takes place in tumor suppressor genes, cancer initiation and promotion are increased (9). It is not yet understood whether certain promotors are predisposed to gene silencing by this process or whether it occurs by chance (9). However, the inheritance of DNA methylation patterns in humans has been described (10), and the extent to which genes are silenced by this epigenetic mechanism appears to vary with age (11). It is possible, therefore, that inherited variation in the genes that regulate epigenetic silencing, as well as alterations in the activities of the corresponding gene products with age, contribute to an individual's susceptibility to cancer.

Dietary factors that might interfere with the processes of DNA methylation are the so-called lipotropes, which encompass the methyl group donors methionine and choline, and the methyl group transfer factors folic acid and vitamin B₁₂. Diets low in lipotropes are often associated with a higher risk for cancer development and for the altered reactions that are involved in DNA stability and carcinogenesis (12). Apart from hypomethylation of DNA, the disturbance of the nucleotide pools that is
commonly associated with folate deficiency leads to uracil misincorporation into replicated DNA, and this might also contribute to mutagenesis (13,14). It must be stressed that antifolates used as chemotherapeutic agents act by generating a functional folate deficiency that is followed by enhanced mutagenesis, DNA damage, and apoptosis. An unfortunate consequence of this mode of action of antifolates and many other chemotherapeutic agents is that patients surviving this treatment are at increased risk of developing iatrogenic cancers because of enhanced rates of mutagenesis (15).

Exogenous genotoxic carcinogens encompass a wide range of chemically different compounds with the common property of forming chemical bonds with DNA, resulting in the generation of “DNA adducts” (16). Adduct formation can lead to base substitution, base deletions, or base additions and thus increased mutation frequencies. Mutations within a given gene often occur at “hot spots” determined by the sequence of DNA and by the chemical structure and reactivity of the adduct-forming agent (17). Although a single chemical will give rise to several different DNA adducts, it has to be assumed that a defined chemical has its individual “fingerprint” of DNA adducts generated. Molecular epidemiology tries to identify the mutational spectrum of certain cancers associated with an individual carcinogen to which subgroups of the population developing a particular kind of cancer have been exposed (18–20).

Most of the genotoxic carcinogens, such as polycyclic aromatic hydrocarbons, aromatic amines, and alkyl and aryl nitrosamines, are activated by enzymes that are normally involved in the metabolism, detoxification, and elimination of potentially toxic compounds (21). The procarcinogens are activated by phase I enzymes, most of which belong to the superfamily of cytochrome P450–dependent monooxygenases. The normal function of phase I enzymes is to insert an oxygen molecule into relatively inert and usually nonpolar substrates. These intermediates formed by oxidative metabolism are then conjugated by phase II enzymes with endogenous polar substrates—such as glucuronides, glutathione or sulfate—to allow the excretion of the now more polar products (22). Phase II enzymes can therefore reduce the cellular exposure to carcinogens, whereas phase I enzymes can increase it. There is some evidence that the genetic susceptibility to cancer may in part be determined by polymorphisms and altered functions of these enzymes. For example, it has been postulated that polymorphisms in the genes that code for N-acetyltransferases (NATs) confer a differential susceptibility to the effect of red meat consumption on the development of colorectal cancer (23). The strongest association between red meat intake and cancer risk was found for individuals with the “rapid acetylator” genotype, in whom activation of the heterocyclic amines formed during the cooking of meats and hydroxylated by phase I metabolism is more rapid. Other examples include the association between cancer risks and certain polymorphisms in xenobiotic metabolizing enzymes such as glutathione-S-transferase (24) or cytochrome P450 E2 (25).

While polymorphisms may determine how efficiently procarcinogens are toxified or detoxified, dietary constituents also alter the phase I or phase II metabolism of potentially hazardous compounds, by an acute effect on the enzymes or by actions at the
gene expression level. The isothiocyanate sulforaphane isolated from broccoli sprouts, for example, shows strong anticancer activity in a rat mammary tumor model by activating phase II enzymes (26), and isothiocyanates from cruciferous vegetables have been shown to increase the excretion of carcinogens in humans (27). Other isothiocyanates from Brassica vegetables or diallyl sulfone from garlic have been shown to inhibit phase I metabolism (28). Certain flavonoids identified in edible plants may serve as bifunctional inducers—that is, in addition to inducing phase II enzymes, they also can induce the procarcinogen activating phase I enzymes (29).

Based on their mechanism of action, compounds suppressing phase I enzymes or activating phase II enzymes can be regarded as “blocking agents”—that is, they are effective when given before or at the same time as the carcinogen. “Suppressing agents,” in contrast, are also effective when given after the administration of a carcinogen, because they may induce apoptosis to eliminate transformed cells or cause growth arrest and promotion of differentiation in initiated cells.

Although these mechanisms that determine the occurrence of mutations in the genome are crucial, DNA that has undergone mutational alterations is normally subjected to repair processes. Here, the tumor suppressor p53 plays a very important role. The p53 protein is part of the gene damage survey system, which is also linked to the control of the cell cycle. Arrest of an initiated cell by this control system allows DNA repair mechanisms to proceed, and if DNA damage has exceeded the capacity for adequate repair, apoptosis will be induced to eliminate cells that have accumulated too many mutations. Various gene products are involved in the repair process for eliminating errors introduced during replication. Beyond the DNA care-taking systems, base excision repair (acting on DNA damage as induced by x-rays, oxygen radicals, alkylating agents, and spontaneous reactions), nucleotide excision repair (acting on DNA damage as induced by ultraviolet light or polycyclic aromatic hydrocarbons), recombinational repair (acting on DNA damage as induced by x-rays or antitumor agents), and mismatch repair (acting on replication errors) take place (30). Inherited genetic defects in any of these mechanisms generally appear to predispose to cancer development by allowing the accumulation of mutations within a cell.

Similarly, as already mentioned, mutations in tumor suppressor genes or protooncogenes also predispose to tumor development, because tumor suppressors as well as the protooncogenes are involved in the pathways regulating cell proliferation (Fig. 2). Tumor suppressors normally inhibit these pathways by inducing cell cycle arrest or apoptosis, whereas the protooncogenes—especially when mutated—promote cell growth. Accelerated cell division rates caused by loss-of-function mutations in tumor suppressors or gain-of-function mutations in protooncogenes (converting them to oncogenes) predispose the cell to accumulation of mutations because the time for DNA repair is shortened.

Paradigms for tumor suppressors and (proto-) oncogenes are the p53 and ras genes, respectively. Both genes are found mutated in a large variety of human cancers with a high incidence. For example, mutations in the ras gene are found in 90% of pancreatic tumors and in 50% of colonic tumors. In lung tumors, as well as most other solid tumors, ras shows a mutation frequency of around 30% (31). The p53
FIG. 2. Accelerated cell growth by loss-of-function mutations in tumor suppressors and/or gain-of-function mutations in protooncogenes. Both the ras oncogene and the tumor suppressor p53 show a high incidence of mutations in a large variety of human cancers. Ras carrying a gain-of-function mutation is permanently activated by losing its ability to hydrolyze guanosine triphosphate (GTP). The p53 with a loss-of-function mutation loses its ability to induce the expression of p21, which promotes cell cycle arrest, or of Gadd45, which enables DNA repair. Also, mutated p53 is no longer capable of inducing apoptosis in cells with excessive DNA damage.
gene bears mutations in 70% of all tumors (32). Ras belongs to the family of small cellular guanosine triphosphate (GTP) binding proteins (G proteins) and serves a signal transduction molecule with multiple functions including the stimulation of cell proliferation. The nonmutated ras hydrolyzes GTP after activating downstream signaling molecules, and becomes inactivated upon GTP hydrolysis. Gain-of-function mutations as found in ras prevent the hydrolysis of GTP and consequently keep ras in an activated state (33). The tumor suppressor p53 monitors genomic integrity and therefore has been called the "guardian of the genome"; p53 itself is involved in proofreading (34) and when DNA damage has occurred, it transcriptionally activates genes such as p21, coding for a cyclin-dependent kinase inhibitor that contributes to cell cycle arrest, or Gadd45, coding for a protein that increases the accessibility of damaged DNA to proteins involved in repair processes (35). The mechanisms whereby p53 induce apoptosis are poorly understood. However, it has been shown that p53 localizes to mitochondria and is involved in the onset of the apoptotic process by causing changes in mitochondrial membrane potential, leading to the release of cytochrome c, an important activator of the apoptotic cascade (36).

ROLE OF DIETARY COMPONENTS IN THE PROMOTION AND PROGRESSION OF CANCER

One of the mechanisms whereby proteins involved in controlling cell growth can acquire increased activity is by a sporadic gain in function mutations as described above. Increased activity, however, may also result from overexpression due to amplification or translocation of the corresponding genes. Gene amplification refers to increased copy numbers, usually as a result of end-to-end replication of a gene at the same chromosomal location. Such an amplification is found in almost 30% of breast carcinomas for the erb-B protein, a plasma membrane receptor for heregulin, which is a growth factor structurally related to epidermal growth factor (Fig. 2) (37). Overexpression of erb-B is associated with clinical outcome in cancer mainly because of its mitogenic effects, but also through an erb-B–induced increase in the production and secretion of vascular endothelial growth factor, which stimulates the angiogenesis necessary for progressive tumor growth (38). A paradigm for translocations associated with cancer development is Burkitt lymphoma. Here, chromosomal translocations fuse the c-myc gene with genes coding for immunoglobulins (39); accordingly, c-myc is placed under the control of promotors normally activated only during infections. Indeed, there is a strong association between the occurrence of infections with Epstein–Barr virus and Burkitt lymphoma (39). The normal cellular function of the c-myc protein is to act as a transcription factor participating in many cellular functions, such as replication, growth, metabolism, and differentiation. The constitutive expression of myc family members caused by such translocations seems to be a key event in the genesis of many cancers (40).

It was believed for a long time that genomic alterations—including mutations, amplifications, and translocations—must occur in selected genes in the initiation phase to provide a growth advantage for cells carrying these alterations in the promotion
phase. In the worst case, this of course would allow the accumulation of further genomic alterations in the progression phase and hence promote cancer development. It becomes increasingly obvious, however, that many compounds are capable of inducing cancer—at least in animal models—without showing any genotoxicity. These compounds may act by stimulating proliferation, either by inducing regenerative proliferation because of their toxicity or by affecting proliferative pathways. These nongenotoxic substances, however, can enhance mutagenesis indirectly by reducing the time available for the cell to repair the replicated DNA. Consequently, cellular hyperproliferation is crucial in cancer development, especially in cells that already have suffered DNA damage (41,42).

A huge variety of food ingredients has been shown to affect cell proliferation rates in either direction. They therefore contribute in the promotion phase to cell transformation and may either reduce or increase the risk of cancer development and progression. Various studies—mainly in animal models—have shown that a high intake of dietary fat accelerates the development of breast cancer. In particular, the content of n-6 fatty acids such as linoleic acid is associated with increased rates of tumor formation, whereas the n-3 fatty acids such as eicosapentaenoic acid and docosahexaenoic acid do not affect tumor development but can inhibit the promoting effects of n-6 fatty acids (43). Another potent suppressor of mammary tumors in animal studies is conjugated linoleic acid, a good source of which is milk fat (44). In humans, however, there is evidence that increased body weight is a more critical variable for cancer development in the mammary gland than fat intake per se or the fatty acid pattern (45). Overnutrition in early life causes rapid growth, which results in early menarche, and consequently, the mammary tissue is confronted with high levels of growth-stimulating estrogens for a longer time. Overnutrition and a high fat consumption in later life results in a breast cancer promoting hormonal imbalance by increasing the levels of free steroid hormones associated with visceral obesity (46).

While certain dietary factors promote the proliferation of cells in mammary tissue by enhancing the circulating estrogen levels, there are also dietary ingredients that can suppress these effects. Phytoestrogens, found in vegetables such as soya, are weak estrogens but in competition with more potent endogenous estrogens show a net antiestrogenic activity. They also act as aromatase inhibitors, thus reducing the synthesis of estrogens (47). These actions of the phytoestrogens have been related to a reduced incidence of breast cancer associated with a diet rich in soya products (47).

It has been suggested that certain diets also act as tumor promoters in other types of cancer such as colon cancer, where high intakes of fat and phosphate have been linked to colonic hyperproliferation (48) and colon cancer development (49). Because there are dietary factors that increase the proliferation rate of colonic cells, there are also compounds that block uncontrolled tissue growth. Butyrate, produced by the gut microflora from fermentable dietary fiber, is one of the key factors shown to suppress growth of cancer cells by increasing the expression of proteins that permit cell cycle arrest. The induction of the cyclin-dependent kinase inhibitor p21, in particular, seems crucial for the growth-inhibiting effects of butyrate (50).
Selected flavonoids found in a large variety of vegetables and fruits can also cause cell cycle arrest. Their actions are linked to the increased expression of cell cycle regulators, such as p21, or the reduced expression of different cyclins or proteins affecting proliferation, such as cyclo-oxygenase-2 (COX-2) (51,52). The effects of these plant components on COX-2 expression could provide an important chemopreventive mechanism in colon cancer development because COX-2 is overexpressed in about 90% of all human colorectal cancers; moreover COX-2 inhibition by long-term treatment with non-steroidal anti-inflammatory drugs is considered to be a chemopreventive strategy (53,54). The mechanism whereby enhanced COX-2 expression promotes cancer development is probably through an increase in cell proliferation reflecting increased production of growth-promoting prostaglandins (55).

**APOPTOSIS: THE CLUE TO CANCER PREVENTION?**

Apoptosis is the process of programmed cell death that serves as a mechanism for regulating cell numbers during development, as well as in the adult organism, and for eradicating cells that have sustained damage to their DNA (56). Approximately 50 to 70 billion cells perish every day in an adult because of apoptosis. Cell death in self-renewing tissues, such as the skin, gut, and bone marrow, is necessary to maintain tissue and organ structure and functionality. Unlike accidental cell death caused by infarction or trauma, apoptosis represents a physiologic process culminating in the fragmentation of cells that are cleared by phagocytosis in neighboring cells without causing inflammatory reactions or tissue scarring.

Apoptosis of a cell is triggered by external plasma membrane–associated receptors as well as by internal sensors (Fig. 3). The ability of tumor cells to resist apoptosis in response to the death signals has become a central theme in the understanding of how tumors develop (57). For both chemoprevention and cancer treatment, the induction of apoptosis in precancerous or cancerous cells may be more important than inhibiting proliferation or inducing differentiation in such cells. This is because under certain unfavorable conditions, cell cycle arrest can provide an escape mechanism for apoptosis, as is often found in cancer treatments with cytostatic drugs (58,59).

On a morphologic basis, apoptosis is characterized by membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation, DNA degradation, and the engulfment of the generated corpses by neighboring cells (60). Most of the morphologic changes are caused by a set of proteases that are specifically activated in apoptotic cells (61). As these death-inducing proteases possess a cysteine in their active site and cleave their substrates C-terminally at an aspartate residue, they are called *caspases* (cysteine proteases that cleave after aspartate). Caspases can autocatalytically cleave and activate themselves as well as other members of the caspase family. Once the first caspase in the apoptotic pathway has been activated, it processes downstream caspases and initiates a cascade of amplifying events that lead to the apoptotic death of a cell (62). The substrates of the downstream effector caspases are proteins that are either activated by the cleavage, such as the caspase-activated DNase, or inhibited, such as the inhibitor of caspase-activated DNase, the poly-ADP ribose polymerase or nuclear lamins.
Various caspase substrates have been identified that induce each of the classic features of apoptosis (63). Two major apoptosis pathways have been defined in mammalian cells, the Fas/TNF-R1 death receptor pathway and the mitochondrial pathway (Fig. 3) (61). The first mechanism is triggered by the Fas ligand or tumor necrosis factor α (TNF-α) binding to the extracellular domain of the death receptors. Binding induces receptor clustering and the formation of a death inducing signaling complex (DISC). Through the adaptor molecule FADD (Fas-associated death domain protein), DISC recruits multiple procaspase-8 molecules, resulting in the activation of this upstream caspase. The mitochondrial pathway is triggered by intrinsic proapoptotic stimuli such as DNA damage and by extracellular or environmental factors such as cellular stress. The different triggers that converge in the mitochondrial pathway
often act through the activation of proapoptotic members of the bcl-2 family, such as bax or bak, or through the inhibition of its antiapoptotic members, such as bcl-2 or bcl-XL (64). Whereas bcl-2 and bcl-XL remain constantly attached to the mitochondria, many proapoptotic members can shuttle between the cytosol and mitochondria (65). The cytosolic forms represent inactive proteins that are activated by proteolysis, dephosphorylation, and probably several other mechanisms, resulting in recruitment to the mitochondria in order to exert their proapoptotic effects.

The release of cytochrome c from the mitochondria is the critical step in the mitochondrial pathway, which is determined by the relation of the activities of pro- and antiapoptotic proteins at the mitochondrial surface. When released, cytochrome c associates with Apaf-1 (apoptotic protease activating factor-1) and then pro-caspase-9 is recruited and activated in the presence of adenosine triphosphate (ATP) or deoxyadenosine triphosphate (dATP). As a part of the so-called apoptosome, caspase-9 activates the effector-caspase-3, the point at which the mitochondrial pathway and the death receptor pathway converge (Fig. 3) (61). It has to be stressed that this model is an oversimplification of the apoptotic machinery—for example, caspase-3 activation can be antagonized by inhibitors of apoptosis (IAPs), which themselves are antagonized by Smac/DIABLO protein released from mitochondria. Moreover, downstream of caspase-3, the apoptotic program branches into a multitude of pathways. There is also a third apoptosis pathway that is initiated by stress in the endoplasmic reticulum, including disruption of calcium homeostasis. As a result caspase-12 is activated and most probably also converges on caspase-3 activation (66).

The complexity of apoptosis means that a multitude of proteins with impaired regulation during cancer development could serve as targets for treatment. Reduced sensitivity toward apoptotic stimuli, often observed in neoplasms, could result from loss-of-function mutations or reduced expression of proapoptotic proteins, or alternatively from gain-of-function mutations or enhanced expression rates of antiapoptotic proteins (67,68). Interestingly, tumor development is often associated with overexpression of the Fas ligand in combination with a loss-of-function mutation in the Fas receptor of the transformed cells. In this way, the cancer cell acquires two advantages: resistance to apoptosis and, through Fas ligand overexpression, the killing of bystander cells such as immune cells providing an immune escape mechanism (69).

Other proteins involved in the apoptotic machinery and often found to be overexpressed in various tumor types are the antiapoptotic proteins bcl-2 and bcl-XL, which exert their principal effects through stabilization of the mitochondrial membrane. Loss of Apaf-1 is also a relatively common event in malignant melanoma, and presumably also confers resistance to apoptosis (70). Alterations in the activities of proteins involved in the apoptotic machinery may not only enable precancerous cells to survive but may also play an important role in metastasis. Normally, detachment of cells from their neighbors or from the basal stroma is followed by spontaneous apoptotic suicide termed anoikis. The detachment deprives the cell of integrin- and cadherin-mediated survival signals, which, at least in part, account for the induction of apoptosis. Changes in integrin expression patterns and levels have been described during the progression of tumors from benign to malignant phenotypes and might contribute to the resistance of cells to anoikis (71).
MACRONUTRIENTS AND APOPTOSIS

It is becoming increasingly evident that diet has a major impact on cancer development, and that apoptosis—the dysregulation of which is crucial in allowing transformed cells to escape control—may directly or indirectly be affected by diet as well. One of the most striking findings is that apoptosis rates are increased by energy restriction (72). On a molecular basis, this increase in apoptosis is associated with an enhanced expression of p53 and a reduced expression of insulinlike growth factor 1 (IGF-1) (72,73). Besides the increased apoptosis, an increase in fidelity of DNA replication and enhanced DNA repair contribute to the maintenance of genomic integrity by low-energy diets. It is suggested therefore, that energy restriction retards tumor development as well as most physiologic indices of aging by maintaining the organism’s ability to deal with stress (72).

Effects of diet on apoptosis, however, cannot be predicted per se and strongly depend on the protein pattern expressed in the cell. This is convincingly demonstrated by overexpression of the antiapoptotic proteins bcl-X_L and Akt (74). Cells overexpressing Akt show an increased glucose transporter expression level as well as enhanced glycolytic activity associated with the requirement of high levels of extracellular nutrients to support survival of the cells. Bcl-X_L-overexpressing cells, in contrast, survive in a more vegetative state, with reduced glycolytic activity, and are consequently less dependent on extracellular nutrients (74).

In addition to the effects on apoptosis of the macronutrients (carbohydrates, fat, and proteins) as sources of cellular energy or delivery systems for structural components, dietary constituents might also affect apoptosis by other mechanisms. Prebiotic fructans, for example, have been shown to increase apoptosis in the rat colon, and this contributes to the growing evidence that fructans may have cancer-preventing properties (75). In this case, the production of butyrate from the soluble fiber seems to be crucial for the inhibition of early and late events in colon tumorigenesis by controlling the transcription, expression, and activity of the key proteins involved in the apoptotic cascade (76).

Dietary fat affects apoptosis not only because it is a high-energy substrate but also because of its fatty acid constituents. It has recently been shown that a high-fat diet (rich in saturated fatty acids) increases eicosanoid production by increased COX-2 expression and causes a reduction in apoptosis. These effects are associated with increased colon cancer development and are not observed with a diet rich in n-3 fatty acids or a low-fat, corn oil–based diet (77). Further studies showed that consumption of a fish oil–containing diet is associated with increased apoptotic cell death, and it was suggested that n-3 polyunsaturated fatty acids can protect against carcinogenesis (78).

Among the amino acids, glutamine appears to play an important role in apoptosis. It serves as the preferred energy substrate in intestinal epithelial cells, and glutamine deprivation in the gut induces apoptosis, an effect not observed with cysteine or methionine deprivation (79). The effects of glutamine deprivation do not appear to be caused by lack of energy, because glutamine deprivation in leukemia and lymphoma cells is associated with apoptosis independently of the energy status, and glucose deprivation induces necrosis, not apoptosis (80).
The importance of nutritional factors in controlling cell proliferation and apoptosis rates has recently been shown in an exciting study in *Drosophila*. Both germline and somatic stem cells in the fruit fly adjust their proliferation rates to nutrient supply (81). Depending on the protein content of the diet, egg production rates varied almost 60-fold, reflecting a four-fold difference in proliferation rates and a 15-fold difference in apoptotic cell death (81). Moreover, it was found that an intact insulin pathway was necessary to fully up-regulate cell proliferation (81).

The finding that somatic stem cells can be drastically influenced by nutritional status may be of special importance in relation to cancer development, because somatic stem cells are probably the progenitors of transformed cells. In the case of colonic tissue, this was suggested on the basis of studies showing that the conversion of all cells in a human crypt to a radiation-induced mutant phenotype takes approximately 1 year (82). This is the time period necessary for a single stem cell to accumulate enough mutations to become transformed. On the other hand, this period is much longer than the lifespan of 2 to 3 days in a differentiated normal enterocyte. Intestinal stem cells are therefore very important, and it is suggested that the Wnt (Wingless-type) pathway may play a major role in the maintenance of the stem cell compartment in the colon (83). Wnt signaling stabilizes β-catenin, and alterations in this pathway are often found in adenomatous polyposis coli owing to the loss of heterozygosity in the adenomatous polyposis coli (APC) gene (84). Besides the loss-of-function mutation in APC, a stabilizing mutation in β-catenin can also activate the Wnt signaling cascade (84). B-Catenin transmits the signals of the Wnt pathway to the nucleus and amplifies the transcription of oncogenes (85). The importance of the Wnt pathway in the development of sporadic colorectal carcinomas implies that the maintenance of the stem cell compartment is crucial for the prevention of cancer.

An increase in stem cell numbers in response to nutritional factors may lead to the disruption of the spatial organization and control of cell proliferation, and consequently, proliferating cells are found in regions where no cell divisions normally occur. Subsequent mutations and clonal selection lead to a further increase in proliferation rates and reduced states of cell differentiation, allowing the accumulation of mutations and finally enabling cells to invade the mesenchyme and cause metastasis (83).

**MICRONUTRIENTS AND APOPTOSIS**

Vitamins and a limited number of minerals are essential for human life. They are necessary to maintain fundamental functions of the body such as growth, metabolism, and cellular integrity. Apoptosis is also dependent on the supply of micronutrients. It has been shown that the beneficial effects associated with decreases in energy intake described above only occur in the presence of sufficient micronutrient density (86). In the absence of proper nutrition, the sensitivity to carcinogens appears to be enhanced.

With regard to the effects on apoptosis, the most extensively studied micronutrients are vitamin A metabolites and selenium. Recently, retinoids selective for the retinoic acid receptor-α have been shown to induce expression of the membrane-bound tumor selective death ligand, TRAIL (tumor necrosis factor related apoptosis
inducing ligand), in promyelocytic leukemia cells. This activates apoptosis by binding to the Fas/TNF-R1 death receptor (Fig. 3) (87). In other cancer cells, retinoic acid stimulates apoptosis by triggering the mitochondrial death pathway involving the activation of the proapoptotic bax and the release of cytochrome c, followed by caspase-9 activation (Fig. 3) (88).

Because selenium is the constituent metal in glutathione peroxidases, its availability contributes to the detoxification of reactive oxygen species. Thus selenium is a critical nutrient with regard to oxygen radical scavenging. However, it also plays an important role in the apoptotic process—its monomethylated forms in particular seem to promote its proapoptotic effects, and this can also be observed in cells with a p53-null phenotype (89). A novel chemopreventive mechanism is proposed involving the catalysis by selenium of the reversible cysteine/disulfide transformations that occur in a number of redox-regulated proteins, including transcription factors (89) that are also important in the apoptotic pathways.

Other micronutrients can affect apoptosis rates and could therefore interact in the process of carcinogenesis. For example, the calcium content of the diet has been shown to alter apoptosis rates, and a high calcium intake may reduce the formation of aberrant crypt foci in the colon (90). Antioxidant vitamins, such as ascorbic acid and tocopherol, have been shown to inhibit radiation-induced apoptosis (91), proving that apoptosis can be modulated by micronutrients in both directions. These antiapoptotic effects of antioxidants are important in anticancer treatment, where efficient apoptosis is crucial. Depletion of the nutritional antioxidants has been shown to be beneficial in a transgenic mouse brain tumor model by increasing the rate of apoptosis (92). However, not all antioxidants inhibit apoptosis per se, because vitamin E was shown to induce apoptosis in colon cancer cells by increasing the expression of p21 through a mechanism involving C/EBP-β (a member of the CCAAT/enhancer binding protein family of transcription factors), independent of p53 (93). It has to be assumed, therefore, that various different tissue-specific conditions determine the effect of antioxidant micronutrients on cell death and apoptosis.

**NONNUTRITIVE FOOD INGREDIENTS AND APOPTOSIS**

Epidemiologic studies consistently show that diets rich in vegetables—and to a lesser extent in fruits—are associated with a relative low cancer risk (94). However, trials to identify single compounds responsible for this effect have so far failed to reproduce the earlier findings (95). This is perhaps not surprising, given that vegetables and fruits contain a myriad of nonnutritive compounds—that is, substances without a direct nutritional value but with the ability to affect cancer development. One class of these dietary constituents is the flavonoids. These consist of an O-heterocyclic ring that is fused to an aromatic ring, and by a CMC bond to a second aromatic ring. The group of the flavonoids encompasses about 5,000 related chemical structures and very few of their biologic activities have been investigated (96).

Apart from the strong antioxidative properties of most but not all of the flavonoids investigated so far, they also possess a multitude of other biologic functions that
could be relevant in cancer prevention. In particular, their inhibitory potency at various stages of tumor development in animal studies has attracted much attention (97). On a molecular basis, it has been found that selected flavonoids show pronounced effects on gene expression. For instance, individual compounds from the different flavonoid subgroups—such as the catechin epigallocatechin-3-gallate (EGCG) (98), the isoflavone genistein (99), the flavonol quercetin (100), and flavone (101)—have been shown to induce gene expression of the CDK inhibitor p21 (Fig. 4).

As pointed out above, expression of p21 is usually associated with cell cycle arrest, which allows control and repair of replicated DNA. We have shown recently that genes involved in the control of the apoptotic process are affected by the flavonoid flavone in colon cancer cells (51). The messenger RNA (mRNA) levels of the anti-apoptotic factors bcl-X\textsubscript{L} and NF-κB and of COX-2 were shown to be potently down-regulated by exposure of cells to flavone (Fig. 4). The expression of the antiapoptotic factors NF-κB and bcl-X\textsubscript{L} is associated with colorectal tumorigenesis and disease progression (102,103). Thus, their down-regulation by flavone might prove to represent an effective form of chemoprevention by reducing the threshold for apoptosis. As already mentioned, overexpression of the COX-2 gene is consistently found during neoplastic development in a variety of tissues, and enhanced prostaglandin formation along the dysregulated COX pathways has been shown to mediate tumor promotion in animal models. Prostaglandins are considered to enhance tumor growth by promoting angiogenesis and metastasis and reducing immunosuppression (104). Consequently, COX-2 has become a key target of pharmacotherapy, especially in the prevention and treatment of colorectal cancers (105). Inhibition of COX-2 enzyme activity is achieved by nonsteroidal antiinflammatory drugs, including the newly developed COX-2 specific inhibitors, while the dietary flavonoid flavone prevents the gene expression of the enzyme.

The effects of flavone on gene expression in colonic tumor cells consistently lead to DNA fragmentation and chromatin condensation as important markers of the execution of the final stages of the apoptotic process (51). At flavone concentrations providing maximal effects, almost 80% of the colon cancer cells undergo apoptosis when exposed to flavone, whereas only 40% of cells are killed under the same conditions on exposure to camptothecin, a classical cytostatic drug (Figs. 5A and 5B; See Color Plate 4).

Flavone has been shown to induce apoptosis only in transformed colonocytes, and not in nontransformed primary murine colonocytes (Fig. 5C) (51). A similar selectivity with regard to apoptosis induction was also shown for the green tea polyphenol EGCG (106). The constitutive expression of NF-κB and the binding of NF-κB to DNA cis-regulatory elements was inhibited by EGCG at much lower concentrations in the cancer cells than in the non-transformed cells (107). Moreover, the activation of NF-κB by stress stimuli such as TNF-α or lipopolysaccharide was also inhibited by EGCG more prominently in cancer cells than in normal cells (107). The ability to trigger apoptosis in transformed cells but not in nontransformed cells is a prerequisite for effective chemoprevention, because enhanced apoptosis rates in normal cells also contribute to cancer development. This is shown by the increased cancer risks often found in patients undergoing chemotherapy. As induction of apoptosis by
FIG. 4. Selected molecular effects of the flavonoid flavone in human cancer cells. Flavone increases the transcription of the cyclin-dependent kinase (CDK) inhibitor p21. Increased p21 levels cause inhibition of the CDK2–cyclin E complex that is crucial for S-phase entry in the cell cycle. As a result of increased p21 expression, flavone leads to cell cycle arrest. Growth inhibition may also be exerted by flavone through diminished cyclo-oxygenase-2 (COX-2) expression. COX-2 is responsible for the synthesis of prostaglandins. These have been shown to be involved in stimulation of cell growth and inhibition of apoptosis but may also play a role in angiogenesis and metastasis. The reduced mRNA levels of NF-κB observed by flavone exposure may inhibit the transcription of survival genes and thereby allow apoptotic signals to prevail. Moreover, flavone reduces the transcript levels of bcl-XL, an effect that presumably also occurs in the apoptotic activities of flavone that are observed in cancer cells. +, stimulation; −, inhibition.
FIG. 5. (A,B) Apoptosis in human colon cancer cells as mediated by flavone or camptothecin. Cellular DNA was stained with Hoechst 33258 for the detection of apoptosis (arrows) after exposure of the cells to camptothecin or flavone. The fraction of apoptotic cells as a function of camptothecin or flavone concentrations, as measured after 24 hours of incubation, is given in (C). (D) Effects of flavone and camptothecin on apoptosis in primary murine colonocytes. Caspase-3 activity, as a marker of apoptosis, was determined after 24 hours of incubation.

cytotoxic anticancer agents is not selective enough and thereby also affects non-transformed normal cells, the regenerative hyperproliferation may prime the normal cells to accumulate mutations that increase the risk of tumor recurrence (15).

FUTURE PERSPECTIVES: SINGLE NUCLEOTIDE POLYMORPHISMS AND COMPLEMENTARY DNA ARRAYS FOR BETTER CHEMOPREVENTION AND TREATMENT

Based on emerging new technologies, such as complementary DNA (cDNA) expression arrays or high-throughput analysis of single nucleotide polymorphisms (SNPs), new insights into the mechanisms of cancer initiation and progression are possible. Differential gene expression patterns between normal and transformed cells, and differences in amino acid sequences of proteins that are important for cell growth and apoptosis, are determined on a large scale (108-110). Simultaneous inspection of thousands of genes shows that cancers from different individuals differ extensively in their expression patterns. This result is, of course, not surprising because people differ in their genetic background and individual cancers in the same tissue differ in phenotype. This can only be explained by different activities of the
proteins caused by their different expression levels or by mutations in the different genes. Nevertheless, gene expression patterns allow clustering to be detected, which indicates the existence of subgroups of genes that may be regulated simultaneously. By identification of these subgroup patterns it is possible, for example, to distinguish two forms of lymphoma that differ dramatically in their survival rates (109).

With chemoprevention as the ultimate goal, high-throughput screening arrays could also be helpful in providing clues to the mechanisms whereby dietary components alter gene expression and thereby affect cancer initiation and progression. In addition to investigating the gene expression patterns, it will be necessary to define important SNPs. As discussed above, SNPs occurring in the promoter can alter its expression level, and SNPs in the coding region of a gene affecting the catalytic or regulatory domains of the protein can critically alter its function within the orchestra of cellular proteins. This may be directly or indirectly associated with an individual’s susceptibility to cancer. We are convinced that this will be one of the most important, most exciting, and most rewarding areas of postgenomic research.

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**DISCUSSION**

**Dr. Bachmann:** You showed an increase in NF-κB activation. If you turn that around, statins are known to inhibit NF-κB activation. Do you think that an overlimitation of cholesterol synthesis or of the intermediates of the colon cholesterol synthesis pathway might explain claims that a limitation of cholesterol intake might lead to increased tumor formation?

**Dr. Daniel:** We have learned recently that the statins may be a two-edged sword, and it is too early to make any clear statement about their role. The idea that cholesterol biosynthesis, as well as elemental cholesterol, is involved in colon carcinogenesis was put forward a while ago, and there are good models to explain how that could happen, although again, we don’t have any final proof. This raises an important question about the dietary factors that are now being introduced to reduce cholesterol absorption in the gut. These could result in a very high intestinal load of cholesterol, and even of some cholesterol metabolites that can be produced by bacteria in the colon—much higher than would normally be the case. We have been wondering whether this might have implications for colon carcinogenesis, but so far, this hypothesis has not been confirmed by animal data.

**Dr. Micheli:** You spoke about the effect of energy restriction on protein metabolism. I was puzzled that you said that energy restriction would increase protein turnover. Several of us here have measured the impact of energy intake on protein turnover in preterm babies and exactly the opposite occurs!

**Dr. Daniel:** I am aware of this experimental evidence. However, quite a number of the 6,500 genes in mouse muscle responded to energy restriction, and the mRNA levels and the enzymes involved in protein turnover were increased. There are other examples in which observed effects at the mRNA level do not match physiology and change opposite to the prediction. We can only say at present that the mRNA levels change but we cannot link this to the physiologic effects. We need to look additionally at the protein levels and, more important, at
the metabolite levels. Consequently, the next step is metabolome analysis, and we now have the technology to undertake this on a high-throughput level. This will hopefully give us clearer answers. By this, we are generating enormous databases, and there is a need for people that can relate the data to the biology. These databases will grow even larger if we achieve high throughput metabolic profiling.

**Dr. Pencharz:** Glutamine seems to be a popular topic—we actually call it vitamin G! The trouble is that as a fuel for the enterocyte, it has to be converted to glutamate. I'm always concerned when people present data showing that glutamine does something, because if you're talking about it as a fuel for the enterocyte, it's actually glutamate. Reeds has shown that if you replace glutamine with glutamate, you see the same effects on growth in the small intestine. I'm not sure whether the caspase 2 and 3 data you showed refer to the glutamine molecule itself, or to the energetics, in which case it's glutamate. Also, did they do proper controls with glutamate versus glutamine?

**Dr. Daniel:** Clearly, the effect was much larger than could be accounted for just by glutamine, so glutamate was also involved. It all comes down to whether you believe the glutamine story or not. It is certainly an excellent energy fuel, and some of the findings with glutamine supplementation look convincing. However, I spent lots of money trying to find an approach for utilizing peptides rich in glutamine that could be provided from the luminal side in animal models of methotrexate treatment and so on, and it didn't work at all, so I'm a skeptic about all the glutamine effects. I agree that the effects shown in various studies could be mainly by glutamate.

**Dr. Pencharz:** You have to be careful about the orientation of cells in culture. Dr. Reeds has shown clearly that glutamine is taken up from the blood side and glutamate primarily from the luminal side, so unless you have the cells properly oriented, you cannot interpret the experiments.

**Dr. Daniel:** In the experiments I described, it was mainly the uptake from the basolateral side, as the cells do not differentiate so well. Clearly all the transporters for glutamine on the basolateral side were present, and the cells took up huge amounts of glutamine.

**Dr. Batshaw:** With the urea cycle disorders, even when affected children are stable, the glutamine levels are severely affected, and of course, there are also children with glutamate dehydrogenase deficiency who will have very low glutamine levels. There are also animal models with high glutamine levels. I wonder whether those various models could be helpful in answering some of the questions you posed.

**Dr. Daniel:** As I say, I'm a skeptic about the glutamine effects myself, but at least they look convincing at cell culture level, and I agree there are nice models around that could be explored.

**Dr. Batshaw:** I wonder if you or Dr. Pencharz would be able to predict the possible long-term effects of either very high or very low glutamine levels?

**Dr. Pencharz:** I cannot predict that at this moment. I think that the uptake of glutamine is primarily related to amino acid metabolism within the enterocyte, whereas glutamate is the fuel that is provided luminally. What happens physiologically, and how we interpret these in vitro studies with transformed cells, and how they relate to human and animal biologies is anyone's guess at the moment.

**Dr. Bachmann:** May I ask Dr. Pencharz, in addition, did you try your experiments with ornithine as a control, because when you start to look at glutamate you move into the proline and ornithine story as well? So is it glutamine, glutamate, ornithine, or proline? We have to look at these effects very critically.

**Dr. Pencharz:** You are absolutely right. We have not done that yet.

**Dr. Daniel:** Glutamine/glutamate deprivation has been looked at in comparison with other
DIET, GENE EXPRESSION, AND APOPTOSIS 261

amino acids, and clearly the effect was on the glutamine-glutamate side, but they didn't look at ornithine in that study.

Dr. Clark: I think we have a responsibility to not lose sight of important biological questions. The calorie deprivation study (1) involved very severe energy deprivation, and the gene changes were relatively small. We must bear in mind that we have to ask good biologic questions and not simply jump on the bandwagon. The reported changes for mRNA levels were relatively small.

Dr. Daniel: You may have misunderstood me. I'm not saying that expression profiling necessarily provides the answer, but, as in other areas, the question is whether we want to explore it and whether we need to do it. You could ask the same questions about pharmacogenomics or toxicogenomics; so why not nutrigenomics? What I'm saying is that we should utilize these techniques to give us a better basis for understanding the effects of nutrition. I agree that a two-fold change in mRNA may not be considered significant, but there were ten-fold or 15-fold changes involved in the flavone effects I showed you.

Dr. Clark: I think you have to be very careful about -fold changes, because even with a many-fold change the effect may still be very small. We must also be wary of the severity of any restriction or intervention that is imposed experimentally. Can this really be achieved biologically? However, I agree with you that the techniques are exciting.

Dr. Bier: To follow up on your comments and those of Dr. Micheli, having lived through the era when we measured all sorts of enzymes in a pathway to try to determine the movement of material, we're now in the era of using changes in genes to estimate enzyme activity. We learned 30 years ago that just measuring the enzyme activity doesn't tell us the movement of material through a pathway unless we actually measure it in some kinetic way. We are a long way from doing that. My other comment is that when expression profiling is used in transgenic plants, the number of genes that respond is actually remarkably small considering the change this process induces.

Dr. Daniel: I agree, but here in plant biotechnology all those profiling techniques are used. As you know, we have a "novel food regulation" in the European Union (EU) where every transgenic material that goes into the food chain is evaluated for safety. One of the problems of this is the key statement that says "a novel food is a novel food if it is substantially different from a conventional one." When that was put in, I suppose nobody realized how difficult it would be to show "substantial equivalence." So, when is a transgenic tomato different from a normal tomato? There are hundreds of tomato varieties that change their content of nutrients, toxic alkaloids, and so on depending on where they grow, how much sunlight they get, and many other factors. As part of the safety assessment process, it is important to show what a normal tomato or potato is, although everybody thinks they know what a normal one is. Now that we have these new technologies, scientists are using them for safety assessments. We now verify "normality" in a potato, say, on the basis of its genome, of its proteome, and on the metabolome. I'm not saying that the answer should be based solely on this. It is just one of the approaches. If the transgenic procedure leads to expression of new proteins unexpectedly, you might need to worry about new allergens, for example, and then proteomics is a useful tool in safety assessment.

Dr. Bier: I'm afraid that these new methods will give us huge amounts of data but far less information, and certainly less precision.

Dr. Borum: You have nicely reviewed for us the correlations found between apoptosis and carbohydrate and amino acid metabolism. I would like to hear your comments about correlations of the ratios of other dietary components—not just energy—for example fatty acids.

Dr. Daniel: I cannot give you a scientific answer but I can talk about my beliefs. I stated
initially that we still have the genome of a hunter and gatherer. The 8,000 years that have passed since the Neolithic revolution have not really made a huge impact on the genome, although we have some examples of recent genetic alteration, for example in salt tolerance acquired by mutations in the angiotensin-converting enzyme gene. However, if you analyze the diet of a hunter—gatherer you find a quite different ratio of n-6 and n-3 fatty acids compared to today’s diet, and of course a much higher ratio of polyunsaturated to saturated fatty acids. The Neolithic revolution brought large quantities of saturated fat into our diet. Important fat components of the hunter’s diet were found in wild game. If you analyze the meat of wild game, you find that the ratio of n-6 to n-3 almost matches present dietary recommendation. Thus, wild game has a quite different fatty acid pattern and ratio compared to domestic animals. There is also the matter of dietary fiber. In the United States and Europe we have been telling people they have to have 30 g of dietary fiber every day—it’s good for your cholesterol and for protecting your colon from carcinogenesis. Then the large epidemiologic studies came out and told us that if there is an effect, it is only a moderate one. If you go back to the ancient diets of hunter—gatherers, or for example the diet of Aboriginal peoples of Australia—you find huge amounts of fiber, but not the fiber we are familiar with. The fiber in their diet is soluble fiber, composed of inulin, galacto- and fructo-oligosaccharides, and so on, which can very easily be hydrolyzed in the large intestine. Aborigines may eat up to 100 g a day of soluble fiber, whereas with the Neolithic revolution we replaced a large fraction of the soluble fiber in our diet by insoluble fiber. So my proposal is that we should look carefully into a moderate return to this earlier type of diet, which was responsible for molding our genome over thousands of years. Clearly, nutrition has been a very important genetic selector, so looking into the ancient diet could provide valuable information to guide us to a healthy diet for us now, even though the world has changed a lot.

**Dr. Wanders:** It has been shown that reactive oxygen species are involved in malignant cell transformation, and one hypothesis is that flavonoids are so potent because of their antioxidant properties. However, you mentioned that you tested various flavonoids and found no apparent relation between their antioxidant power and their effects in your tumor cell system. Could you explain that?

**Dr. Daniel:** What I can say is that the particular compound that had these very interesting effects on apoptosis is not an antioxidant. We also tested those with antioxidant activity, but those were much less effective. However, flavonoids have all kind of different cellular targets, which may have nothing to do with antioxidant activity. Moreover, there appears to be accumulating evidence that too many antioxidants, which are provided in all kinds of supplements, may not be desirable. We are talking about a balance here. For example, in cancer therapy, there is evidence that if the patient has a high intake of antioxidant compounds, the outcome of the treatment may not be as good as expected (2). This has its possible biochemical basis downstream in the cascade of apoptosis, where there is a step in which cells generate oxygen radicals within the apoptotic death pathway, and this might be important for the final execution of apoptosis. If you quench this process, you may have cells surviving that should not survive.

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• no TNF stimulation
• + TNF
COLOR PLATE 3

COLOR PLATE 4