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FREE-RADICAL PROCESSES IN MULTISTAGE CARCINOGENESIS

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Rodent and human cells in culture, transformed *in vitro* by radiation or chemicals into malignant cells, afford us the opportunity to probe into early and late events in the neoplastic process at a cellular and molecular level. Transformation can be regarded as an abnormal expression of cellular genes. The initiating agents disrupt the integrity of the genetic apparatus altering DNA in ways that result in the activation of cellular transforming genes (oncogenes) during some stage of the neoplastic process. Events associated with initiation and promotion may overlap to some degree, but in order for them to occur, cellular permissive conditions prevail. Permissive and potentiating factors include free radicals, and thyroid hormone, and inadequate antioxidants. Protective factors which suppress the carcinogenic process include enzymatic and dietary antioxidants. These are constituitive under normal circumstances and can be induced under conditions of oxidative stress produced by a wide range of carcinogens.

KEY WORDS: Dietary factors, chemoprevention, antioxidants.

Epidemiologic studies and data from experiments *in vivo* and *in vitro* support the notion that cancer is a multi-step process, in that a series of specific events is required to transform a normal cell into a malignant one.[1]

The multi-step model of carcinogenesis involves initiation, in which irreversible genetic alterations take place, and promotion, in which the clonal population of initiated cells is expanded and ultimately progresses to a malignancy.

Within the past decade we have witnessed changes in our understanding of the molecular origins of cancer. Much of this progress stems from the discovery of specific genes, the oncogenes, which are present in the genomes of a variety of tumor cells and are responsible for specifying many of the malignant traits of the cells.[1] A number of oncogenes found in tumor cells or in cells transformed *in vivo* or *in vitro* play a central role in carcinogenesis. This has been underscored by the ability of these genes to confer a malignant state on normal cells when introduced into the normal cells by means of transfection.[2,3]

While DNA is the target in carcinogenesis, the ultimate course and frequency of the neoplastic processes are determined by an interplay of endogenous and exogeneous factors. These include permissive factors such as hormones which may act as co-transforming agents and potentiate carcinogenesis.[1] The permissive factors are balanced, under normal conditions, by cellular protective factors which suppress the carcinogenic process at its various stages and antagonize the action of the permissive factors.

ANTIOXIDANTS AS PROTECTIVE FACTORS IN TRANSFORMATION

The interaction of cells with radiation, both X-ray and ultraviolet (UV) light, as well as with a variety of chemicals, results in an enhanced generation of free-oxygen species and free-radical intermediates.[1] The result is a loss in the optimal cellular balance between the oxidative challenge, a source of DNA damage, and the inherent mechanisms that protect the cell from excess oxidative stress. These protectors include enzymes (SOD, catalase, peroxidases, transferases) and thiols. Also included are a variety of nutrients that directly or indirectly prevent peroxidation and autoxidation of proteins and lipids in cell membranes as well as in the nucleus. These include vitamin A, β-carotene, vitamin C, selenium, and vitamin E.[3,4]

In recent years, increasing evidence has implicated free-radical mechanisms in the initiation and promotion of malignant transformation *in vivo* and *in vitro*. Much of the evidence has come from the fact that the agents that scavenge free radicals directly or that interfere with the generation of free-radical-mediated events inhibit the neoplastic process. We have shown in hamster embryo cells that SOD inhibits transformation by radiation and bleomycin and suppresses the promoting action of TPA.[5] Catalase had no effect as an inhibitory agent in this cell system, perhaps because of the inherent high level of the enzyme in the hamster cells.[5] SOD had a more dramatic inhibitory effect when maintained on the cells throughout the experiment, suggesting that later stages in the transformation process are influenced by free radicals.

SELENIUM AND VITAMIN E

Other agents that qualify as important antioxidants are various examples of nutrients which are critical in controlling free-radical damage, namely, selenium, a component of glutathione peroxidase, and vitamin E, a powerful antioxidant and a component of the cell membrane. We examined the single and combined effects of selenium and vitamin E on cell transformation induced C3H/10T-1/2 cells by X-rays, benzo[a]pyrene, or tryptophan pyrolysate and on the levels of cellular scavenging systems and peroxide destruction. Incubation of C3H/10T-1/2 cells with 2.5 μM Na_2SeO_3 (selenium) or with 7 μM α-tocopheral succinate (vitamin E) 24 hours prior to exposure to X-rays or the chemical carcinogens resulted in an inhibition of transformation by each of the antioxidants with an additive-inhibitor action when the two nutrients were combined.[1,8] Cellular pretreatment with selenium resulted in increased levels of cellular glutathione peroxidase, catalase, and nonprotein thiols (glutathione) and in an enhanced destruction of peroxides. Cells pretreated with vitamin E did not show these biochemical effects, and the combined pretreatment with vitamin E and selenium did not augment the effect of selenium on these parameters. The results support our earlier studies showing that free-radical-mediated events play a role in radiation and chemically induced transformation. They indicate that selenium and vitamin E act alone and in additive fashion as radio-protecting and chemopreventing agents. Selenium confers protection in part by inducing or activating cellular free-radical scavening systems and by doubling peroxide breakdown, thus enhancing the capacity of the cell to cope with oxidant stress. Vitamin E appears to confer its protection by an alternate complementary mechanism. Vitamin E acts as a chain-breaking antioxi-

dant inhibiting the lipid peroxidation and the formation of malonaldehyde, a compound with oncogenic potential.[6]

Selenium acts as a true protector. Time-course experiments indicate that the addition of selenium at various exposure to X-rays results in a suppressive action which diminishes with time.

An important determinant in the efficiency of cellular protection by inherent antioxidants lies in the interaction among various factors. The metabolic functions of vitamin E and selenium are interrelated, and selenium plays a role in the storage of vitamin E. Vitamin E action is also closely related to that of vitamin C, which appears to increase its antioxidant effect.

INTERACTION OF VITAMIN E AND VITAMIN C

The synergistic interaction between vitamins E and C as antioxidants are known.[7] Vitamin C spares vitamin E by reducing the vitamin E radical to regenerate vitamin E. Vitamin E scavenges lipid radicals to interfere with chain propagation. The resulting vitamin E radical is reduced by ascorbate to regenerate vitamin E.

We conducted studies to test if vitamin E and vitamin C were synergistic in their capacity to prevent transformation. We pretreated cells with vitamin E, 7 μM, and vitamin C (0.1 mg/ml), alone or in combination. Our work indicates that vitamin E and vitamin C act in concert to inhibit transformation in a manner that appears to be synergistic in nature.[8]

The role of vitamin E in protecting the organism from oxidative stress is a critical one. As observed from experiments *in vitro* and from human studies, vitamin E in its capacity as an antioxidant can protect against neoplastic development. Because vitamin E is consumed in linear fashion during oxidative processes, and exposure to a variety of environmental carcinogens (radiation, chemicals, ozone) increases oxidant stress, it is critical for us to maintain a high level of vitamin E in our tissues. This would be of great value for enhancing our defense against the malignant process induced by the plethora of cancer-causing oxidants around us.

Ozone as a Carcinogen and Co-carcinogen

The identification of environmental pollutants with carcinogenic potential is of obvious relevance to human health. Ozone, a reactive species of oxygen, constitutes the largest atmospheric pollutant in industrial areas and creates a potential health hazard to man.[9] It is a key oxidant in photochemical smog and is formed by the action of sunlight on nitrogen oxides and hydrocarbons emitted from automobiles. Furthermore, O_3 is used as a disinfectant and in cosmetology, and it is produced in u.v. lamps and is present in planes flying above 30,000 feet.[10] In urban areas where smog prevails (e.g. Southern California) peak levels of O_3 may exceed 0.5 p.p.m. (reviewed in [10]).

Though not a free radical in itself, O_3 interacts with a wide variety of organic molecules to produce toxic free radical intermediates, some possessing carcinogenic potential.[10]

The toxicity of ozone depends upon its ozonation and oxidative properties. While the precise mechanisms of ozone damage are unclear, a variety of mechanisms have been proposed. Of these, the most compelling are the following, which implicate cell

membranes as primary sites of ozone toxicity and suggest that ozone damage is in part induced via free radical processes.

a) Ozone exerts its toxicity by oxidation of low molecular weight compounds containing thiol, amine, aldehyde and alcohol functional groups and by oxidation of proteins.

b) Ozone acts by initiating peroxidation of polyunsaturated fatty acids present mainly in the cell membrane. The peroxides and secondary reactive oxygen species which ensue produce their toxicity by damaging the integrity of the cell membrane and other molecules.

Both soluble peptides, such as glutathione, and proteins in lipid bi-layers provide potential targets for ozone action. Protein modification takes place via oxidation of amino acids chain.

One of the major actions of ozone resides in its ability to peroxidize polyunsaturated fatty acids and produce malondialdehyde, which reacts with thiols, cross-links DNA and histones and acts as an initiator in mouse skin carcinogenesis.[11]

Direct oxidation of amino acids and proteins by high ozone levels of oxidation by secondary reaction products of polyunsaturated fatty acids (PUFA) peroxidation can inhibit a variety of cellular protective systems. These include glutathione, a scavenging thiol, glutathione peroxidase, superoxide dismutase and catalase, which detoxify peroxides and enzymes which supply reducing co-factors such as glucose-6-phosphate dehydrogenase. Both thiols and enzymes may be restored metabolically to control levels or rebound to higher protective levels following intermittent or continuous ozone exposure.

Following exposure to high levels of ozone, the relative importance of PUFA peroxidation and the oxidation of proteins and small molecular weight compounds depends on many factors. These include membrane composition of PUFA and proteins, which determine ozone accessibility and degree of interaction and damage, enzymatic pathways to decompose peroxides, pathways to generate thiols, the presence of antioxidants to prevent peroxide formation and to partake in scavenging free radicals arising from secondary reactions.

We reasoned that O_3, in its ability to initiate cascades of free radical reactions which damage genetic integrity may act as an environmental direct carcinogen or co-carcinogen. The ability of O_3 to induce neoplastic transformation *de novo* by direct cellular exposure to the agent, as well as its possible role as a direct co-carcinogen with other environmental pollutants, is currently unknown.

We used primary cultures of diploid hamster embryo cells and the mouse C3H/10T-1/2 cell line. The two cell systems have been used extensively in radiation and chemical carcinogenesis studies an the culture conditions and transformation assays are well established (for review see refs. [5,15]).

We found that treatment of both cell types with 5 p.p.m. O_3 for 5 minutes resulted in enhancement of cell transformation compared to control untreated cells, and in activation of cellular oncogenes.[11,12]

We also found that malonaldehyde and malonaldehyde-like products were formed at higher levels in O_3, exposed cells as compared to controls.[11]

The finding that lipid peroxidation products are elevated in response to O_3 suggests a partial role for free radical-mediated reactions in O_3-induced neoplastic transformation. Further support comes from our ongoing work indicating that the antioxidant

Vitamin E (alpha tocopherol) inhibits O_3-induced transformation in the hamster and C3H/10T-1/2 cells.

CONCLUSIONS

One of the basic conundrums in carcinogenesis evolves from our ability to unequivocally distinguish primary events associated with initiation of malignant transformation from those that function as secondary events. Thus the role of oncogenes, mutations, gene rearrangements, amplification and other DNA alterations in transformation is yet unclear. The changes that take place give rise to abnormal expression of cellular genes.

We must always be cognizant of the fact that a variety of factors may modify the neoplastic process at its various stages of development. These constitute physiological permissive or protective factors. When permissive fators prevail, such as genetic susceptibility, optimal stage in the cell cycle, optimal hormonal control, or a particular stage in differentiation, initiation of transformation will take place. By contrast, if these permissive factors do not prevail, protective factors such as free-radical scavengers will inhibit to varying degrees the onset and progression of the neoplastic process. These may be inherent cellular factors or those added externally by dietary means acting as anticarcinogens. Thus, the interplay between inherent genetic and physiological factors, and lifestyle influences which either enhance or inhibit the neoplastic process, are critical determinants in the process of multi-stage carcinogenesis and in establishing the incidence of cancer

Different organs have a different content of inherent oxidants and these may vary from one species to another as well as from one individual to another. Thus, tissues and cells will vary in their response to oxidant stress. Adding external antioxidants may be effective in helping some cells mount a protective response while being ineffective in others.

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