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DNA Mismatch Repair and p53 Play Independent Roles in DNA Damage-Induced Apoptosis

By Ming Zeng, MD, PhD, Latha Narayanan, MS, and Peter M. Glazer, MD, PhD

THE DNA MISMATCH REPAIR (MMR) SYSTEM HAS BEEN THOUGHT TO PLAY A KEY ROLE IN MAINtaining genome integrity, primarily by correcting base pair mismatches and other DNA polymerase errors. However, when bacteria were used to study alkylation damage, it was found that MMR factors were implicated in the cellular response to DNA damage.[1] A similar role for MMR-mediated cellular response to DNA damage also was found in mammalian cells. Cells deficient in MMR were found to have resistance to alkylating agents.[2,3] This effect was subsequently observed to apply to a variety of DNA-damaging agents, including several used in cancer therapy, such as cis-platinum and temozolomide.[4,5] On the basis of available data, we proposed a mechanism of cellular responses to alkylating agents via MMR-mediated cytotoxicity.

MMR-Mediated Cytotoxicity

To test whether other DNA-damaging agents, such as ionizing radiation (IR), also require MMR in cellular response, Fritzell and colleagues used different MMR-deficient cells to study the cellular response to high-dose rate IR.[6] The clonogenic survival data suggest a role for the MMR factors MSH2, MLH1, and PMS2 in the cytotoxicity of IR;[6] results showed a small but statistically significant increase in clonogenic survival after IR of MMR mutant cells compared to wild type. These studies were carried out in immortalized cell lines established from transgenic mice in which the MSH2, MLH1, and PMS2 genes were mutated by targeted disruption in mouse embryonic stem cells.[7]

Recently, DeWeese and associates, focusing on cells from MSH2 knockout mice, reproduced and extended the observations to show that at low-dose rates, the survival differences

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between wild-type and MSH2-deficient cells were even larger than the differences seen at high-dose rates.[8] In addition, Zhang and colleagues found that IR-induced apoptosis was reduced in MSH2-nullizygous mouse embryo fibroblasts compared to wild type.[9]

MMR-Associated Apoptosis

On the basis of these results, a model was proposed that applies radiation-induced oxidized bases to the same MMR-mediated pathway of cytotoxicity as the alkylated bases. However, several other studies using MMR-deficient, human cancer-derived cell lines or immortalized MSH2-deficient mouse lines failed to find substantial or consistent differences in radiation response.[7,10] These conflicting reports complicate the understanding of the mechanism of MMR-mediated cellular response to ionizing radiation.

The mechanism by which the MMR complex may influence damage response is not yet fully understood, and different hypotheses have been developed to interpret the available data. Two hypotheses focus on how the signal is initiated and mediated downstream, eventually resulting in cell death or successful repair of the lethal/sub-lethal damage. One proposes that the MMR complex recognizes base damage and then initiates a cycle of futile repair,[11] leading to gaps and breaks that ultimately may signal apoptosis. The other hypothesis proposes that the recognition of damage by the MMR complex directly initiates a signal transduction pathway, thus triggering apoptotic pathways. Both hypotheses support the central role for MMR in cellular response to certain types of DNA damage, but debate how the initial signal starts.

The evidence supporting a central role for signal transduction in the MMR-mediated damage response includes the finding that MSH2 function is required in the DNA damage response for both oxidative agents and IR. The role of the MMR complex in the recognition and processing of oxidatively damaged bases has been suggested by the results of several studies. For example, Ni and coworkers observed binding of MSH2/MSH6 complexes to DNA containing 8-oxo-guanine,[12] and DeWeese et al reported increased accumulation of 8-oxo-guanine in MSH2-deficient mouse cells.[8] Two studies of mutagenesis in yeast also indicated a role for MMR in mutagenesis related to oxidative base damage.[12,13] Previous studies of MMR-associated apoptosis have identified MSH2 and MLH1 as key mediators of the process. For example, MSH2-deficient cells exhibited reduced apoptosis after IR in two studies.[10,11]

In one study, simple overexpression of MSH2 or MLH1, but not PMS2, MSH3, or MSH6, induced apoptosis in human cells.[11] These latter observations suggested a special role for MSH2 and MLH1 factors in the apoptotic response, raising questions as to the particular role of PMS2 and other factors in induced apoptosis. In addition, work by DeWeese et al implicated MSH2 in an exaggerated response to IR when delivered at low-dose rates.[8] Zeng and associates showed that PMS2 also plays a role in the different effects of low-dose rate IR.[14] These latter observations suggest, as above, that the effect of the low-dose rate IR is mediated via recognition and processing by the MutSa (MSH2/MSH6) and MutLa (MLH1/PMS) complexes, not simply by MSH2 or MLH1 alone. These results also directly demonstrate that PMS2 plays a role in damage-induced apoptosis, suggesting that formation and normal functioning of the MutSa and MutLa complexes are required for MMR-dependent, IR-induced apoptosis.

MMR-Mediated Cellular Response Pathway

To further define the MMR-mediated cellular response pathway, it was shown that the MSH2/MSH6 and the MLH1/PMS2 complexes are required for the phosphorylation of p53 at serines 15 and 392, following treatment of cells with alkylating agents.[15] Consistent

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with this observation, an MLH1-dependent induction of p53 following IR was observed in human colon cancer cell lines,[10] and a MMR-deficient lymphoblastoid cell line showed reduced accumulation of p53 following temozolomide exposure.[16] MMR-dependent induction of p53 also was seen in response to a variety of carcinogens.[17] These observations raise the important question of the proposed role of p53 in the MMR-mediated damage response pathway for a variety of reasons: p53 is mutated in a large number of human cancers and is involved in cell cycle regulation, transcription, and apoptosis.[18,19] Evidence also implicates p53 as a central factor in the cellular response to IR, leading to cell cycle checkpoint activation and apoptosis.[20] However, recent work using a series of Chinese hamster fibroblast and human lymphoblastoid cell lines suggests that the MMR-mediated apoptotic response to the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine may be independent of p53,[21] which raises questions regarding the functional importance of MMR-dependent signaling of alkylation damage through p53 phosphorylation.

Irradiation-Induced Apoptosis Assays

To further examine the potential relationship between MMR and p53 and their putative interdependent role in the cellular response to IR, Zeng and colleagues used genetically well-defined cells to study the question. They interbred mice carrying targeted disruptions at the PMS2 and p53 loci to produce primary embryo fibroblasts with defined genotypes at these loci (including wild type, p53 null, PMS2 null, and double null).[14,22-24] First, the role of p53 in the MMR-mediated response of cells to IR was investigated using an apoptotic assay as an end point to study cellular response. Primary cells carrying targeted disruptions of p53 and/or the MutL homologue MMR gene PMS2 were used to perform IR-induced apoptosis assays. The results showed that deficiencies in either p53 or PMS2 genes were associated with reduced levels of IR-induced apoptosis compared to the wild type, and were consistent with roles for both factors in the cellular response to IR. In cells deficient in both p53 and PMS2, even lower levels of apoptosis were observed, indicating that MMR and p53 mediate IR-induced apoptosis via separate and apparently additive pathways.

Secondly, by extending these results to an examination of clonogenic survival, it was found that lack of PMS2 rendered cells more resistant to IR regardless of p53 status. These results indicate that the MMR-mediated apoptotic and cytotoxic response to IR does not depend on p53, and were consistent with a recent report that the MMR-mediated apoptotic response to N'-methy1-N'nitro-N-nitrosoguanidine is not dependent on p53.[21] Moreover, a recent study in MSH2- and p53-deficient mice and mouse cells suggested that temozolomide-induced apoptosis mediated by MSH2 may proceed via both p53-dependent and p53-independent pathways.[25] In addition, Wu and associates reported that the induction of p53 in response to selected carcinogens was dependent on functional MMR and that the MMR-dependent apoptotic response to chemicals was mediated through both p53-dependent and p53-independent pathways.[17] Finally, a report from Hickman and Samson further supports the observation of p53-independent MMR-mediated pathways.[21] Whether these results reflect inherent differences between cellular responses to IR vs. various chemicals or to differences in the sensitivities of the assays remains to be determined. Whether cell cycle checkpoint regulation is associated with MMR-mediated signaling through p53 also remains to be established.

Nevertheless, it has been shown that p53 phosphorylation is affected by certain MMR factors,[15] and there does seem to be some cross-talk between the MMR and the p53 pathways. Although it is possible that some small degree of IR-induced apoptosis depends on this cross-talk, Zeng's report suggests that the majority of the detectable IR-induced, MMR-dependent apoptosis and cytotoxicity is independent of it. IR generates a large number of lesions in DNA, including double-strand breaks, single-strand breaks, and a wide variety of base and sugar damage. It is likely that cell death from strand breaks is independent of MMR. However, IR-induced base damage, or at least some subset of it, may be subject to MMR recognition.[6] On the basis of the emerging model for the alkylation damage-response pathway,[26] it is further proposed that this recognition initiates a signal transduction pathway that leads to apoptosis. More and more data suggest that this pathway does not require p53, although MMR recognition of base damage may signal p53 for other purposes. Aside from p53, recent work suggests that MMR-associated signaling involves a number of other factors, including c-abl and p73.[26] p73 is a homologue of p53, and one possibility is that MMR signals apoptosis following IR via a p73-dependent pathway. Such a role for p73 in the case of cis-platinum exposure was proposed.[26]

Conclusion

Aside from the well-established concept that p53 can mediate apoptosis following IR, emerging data also support the concept that MMR can trigger apoptosis in a p53-independent pathway. Additional research to more fully understand the MMR-mediated apoptotic signaling

is very important, not only to enhance molecular understanding of DNA repair and apoptosis, but also for the future development of better and more effective cancer treatments. *(Dr. Zeng is a Resident and Ms. Narayanan is a Research Associate in Therapeutic Radiology, and Dr. Glazer is Professor of Therapeutic Radiology and Genetics, Yale School of Medicine, New Haven, CT.)* ❖

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Polyunsaturated Fatty Acids and Skin Cancer: Part II

By Anthony P. Albino, PhD and Leonard A. Cohen, PhD

TO DATE, THREE FUNDAMENTAL OBSERVATIONS HAVE emerged that clearly support the thesis that n-3 PUFAs have clinical potential as an adjuvant cancer therapeutic modality: 1) n-3 PUFAs can be directly cytotoxic to tumor cells independent of other agents;[1,2] 2) n-3 PUFAs can have synergistic or additive antitumor effects when used in combination with conventional chemotherapeutic agents;[2-4] and 3) n-3 PUFAs can reverse intrinsic tumor cell drug resistance to several chemotherapeutic drugs (e.g., vincristine, doxorubicin, and cis-platinum).[2,5]

Mechanisms of Action

Developing a mechanistic basis for these multifactorial antitumor roles has been the focus of a large body of research.[2,6] These studies have shown that n-3 PUFAs can exert their effects by a number of basic mechanisms. They can directly alter the composition and, thus, the basic structure of the cell's bilipid plasma membrane, allowing facilitated entry of chemotherapeutic drugs (e.g., adriamycin, doxorubicin, and vincristine).[5-7] n-3 PUFAs can increase the generation of damaging reactive oxygen species (ROS) via membrane lipid peroxidation by drugs like doxorubicin. They may alter gene expression or change the composition and bioactivity of downstream metabolites of PUFAs (i.e., the eicosanoids). These types of alterations can, in turn, induce tumor cell cytostasis or cytotoxicity by excessive DNA damage, induction of apoptosis, suppression of tumor cell-stromal matrix association, inhibition of tumor-driven angiogenesis, and alterations in key cell cycle regulatory proteins.

Antitumor Potential of PUFAs

Cell cycle deregulation, in particular, is a hallmark of transformed cells. How PUFAs affect the cell cycle is obscure. In melanoma, as in other cell types, normal transition from one phase of the cell cycle to the next is regulated at checkpoints, which are, in part, governed by cyclin-dependent kinases (CDKs) that are assembled with partner cyclins.[8] Active CDK-cyclin complexes are further regulated by phosphorylation-dephosphorylation events that are mediated through CDK-activating kinases and phosphatases. CDKs also can be inactivated through physical binding with CDK inhibitory proteins (CKIs).[8] D- and E-type cyclins are important in cell cycle transition from G1 to S.[9] The cyclin D-CDK complex is believed to function by specific phosphorylation of the RB product.[9] Hypophosphorylated pRB binds members of the E2F transcription factor family, leading to inactivation of E2F function and reduced expression of genes critical for S-phase events (e.g., dihydrofolate reductase, DNA polymerase α, and cyclins).[10] Hyperphosphorylation of pRB disables its E2F-binding, thereby influencing the passage of cells from G1 into S and cellular proliferation.

Thus, pRB is the master switch regulating cell cycle progression and its continuing phosphorylation parallels cell transit through G1 and S.[10] The initial phosphorylation carried out by the Cdk4(6)/cyclin D complex is required for cell passage through the G1 restriction point.[10] Although many tumor types have disruptions in the pRB pathway, virtually 100% of invasive and metastatic melanomas have detectable defects in one or more of the important regulators of the pRB regulatory circuit (i.e., the cyclin-dependent kinase inhibitor 2A [CDKN2A] or p16INK4a gene; the CDKN2B or p15INK4b gene; and the D-type cyclins and their functional partners Cdk4 and Cdk6).[11,12] The most common defect in melanoma cells is the loss or mutation of the p16/CDKN2A gene; although there is only minimal dysfunction of pRB itself, with most melanoma specimens and established cell lines expressing normal RB protein.[12,13] Defects in the pRB circuit appear to play a major contributory role in inducing uncoordinated tumor cell proliferation, a fundamental biological trait that differentiates the melanoma cell from the usually non-proliferative benign melanocyte.

Presently, little is known about the effects of fatty acids on cell cycle control, and we only recently have begun to elucidate the mechanism by which docosahexaenoic acid ([DHA] found in high levels in cold water fish such as mackerel, menhaden, salmon, and tuna) inhibits the growth of human melanoma cells.[14] The molecular events by which DHA suppresses the growth of melanoma cells was examined in detail in two melanoma cell lines: one refractory (SK-Mel-29) and the other sensitive (SK- Mel-110) to the inhibitory effects of DHA. Exponentially growing melanoma cell lines were exposed in vitro to DHA. Then the cell lines were assessed for inhibition of cell growth; expression of cyclins and cyclin-dependent kinase inhibitors in individual cells by flow cytometry and immunocytochemistry, using specific monoclonal antibodies to cyclin D1, cyclin E, $p21^{WAF1/CIP1}$, or $p27^{KIP1}$; and expression of total pRB^{T} independent of phosphorylation state and hypophosphorylated pRB^{P-} in fixed cells by flow cytometry

and immunocytochemistry, using specific monoclonal antibodies to pRB^{T} or pRB^{P-}, respectively.

Upon treatment with increasing concentrations of DHA, cell growth in seven of 12 melanoma cell lines was inhibited, whereas cell growth was minimally affected in the other five cell lines. Two melanoma cell lines were examined in detail, one that was resistant (SK-Mel-29) and one that was sensitive (SK-Mel-110) to the inhibitory activity of DHA. SK-Mel-29 cells were unaffected by treatment with up to 2 mcg/mL DHA. No appreciable change was observed in cell growth, cell cycle distribution, the status of pRB phosphorylation, cyclin D1 expression, or the levels of the CKIs, p21 and p27.

In contrast, SK-Mel-110 cell growth was inhibited by DHA with the cells accumulating either in G1 or S phase (0% in SK-Mel-29 vs 41.2% in SK-Mel-110). Moreover, considerable death occurred by apoptosis. In addition, DHA treatment resulted in increasing numbers of SK-Mel-110 cells expressing hypophosphorylated pRB (from 12% to > 40%), whereas the levels of cyclin D1 and p21 changed little.

Potential Clinical Applications

This study showing that DHA inhibits the growth of cultured metastatic melanoma cells provides information as to potential mechanistic interactions of fatty acids with specific components of the cell cycle machinery. The finding that DHA-induced growth inhibition correlates with a quantitative increase in hypophosphorylated pRB suggests a cross-talk mechanism between fatty acid metabolism and the pRB pathway. More importantly, these data suggest that reactivation of pRb by n-3 PUFAs may prevent the abnormal proliferation of melanoma cells. Determining the mechanism by which n-3 PUFAs can inhibit melanoma growth will be an important first step in the rational use of these PUFAs as antitumor agents in combination with conventional chemotherapeutic drugs.

For example, one of the most potent single-agent drugs for the treatment of melanoma is interferon-α (IFN-α). The interferons are a family of naturally occurring, small proteins with molecular weights of approximately l5,000-21,000 Da. They are produced and secreted by virtually all eukaryotic cells in response to viral infections or to various biologic and synthetic inducers.[15] Three major classes of interferons have been identified: alpha, beta, and gamma.

Interferons induce their cellular activities by binding to specific membrane receptors on cell surfaces. IFN-α-2b, like the naturally occurring alpha or leukocyte interferons, demonstrates potent antiproliferative and antiviral properties. IFN-α has shown both immunomodulatory and antiproliferative effects in metastatic melanoma. IFN-α-2b recently has been approved by the FDA as the first effective adjuvant therapy for the treatment of the "high risk for recurrence" melanoma patient.[16] The antitumor activity of IFN-α can be affected by direct and indirect mechanisms. For example, IFN can induce cytostatic or cytotoxic effects by increasing the length of the cell cycle, by affecting the expression levels of various critical oncogenes (i.e., c-myc, c-fos, or c-H-ras genes), by inhibiting the induction of enzymes critical for cell survival, or by impacting the antitumor actions of host cytotoxic T-cells.[15,17,18] Each of these mechanisms would be capable of augmenting or complementing the several types of antitumor actions of n-3 PUFAs discussed above.

Thus, in theory, a combination therapy using both IFN-α and n-3 PUFA supplementation should provide a more potent antitumor effect than either agent alone. We have preliminary in vitro data showing that treatment of melanoma cells with DHA and IFN-α has synergistic inhibitory effects on their growth and motility.

Ratio of n-3 to n-6 PUFA

An important aspect of any dietary trial using PUFAs is to control the n-3:n-6 ratio. The clinical objective of n-3 PUFA supplementation is to increase the overall n-3:n-6 PUFA ratio and to decrease the production of arachidonic acid and the subsequent formation of specific eicosanoid metabolites (which include the prostaglandins, leukotrienes, and thromboxanes).[19] PUFAs are converted to metabolites via a series of enzymatic reactions that add more double bonds to the molecules (mediated by desaturase enzymes) and elongations that extend the length of the carbon chain (mediated by elongase enzymes).[20] (*See Figure.*) These reactions ultimately convert dietary n-6 linoleic acid to arachidonic acid (20:4n-6) and n-3 alpha linolenic acid to eicosapentaenoic acid (EPA) (20:5n-3). The subsequent metabolism of these PUFAs produces a different spectrum of eicosanoids because of the competition that exists between the n-3 and n-6 PUFAs for the Δ^4 and Δ^6 desaturases (n-3 PUFA having greater affinities for the enzyme active sites). Thus, increasing the dietary intake of n-3 PUFAs (e.g., alpha linolenic acid, EPA, or DHA), while simultaneously increasing the n-3:n-6 ratio, reduces the desaturation of linoleic acid and, therefore, the production of arachidonic acid.[21] Simply increasing n-3 levels or decreasing n-6 levels without changing the overall n-3:n-6 ratio has been shown not to reduce the levels of arachidonic acid or its metabolites in human tissues.[22]

Altering the spectrum of eicosanoids is important because of their differing physiologic effects on tumor cells.[23] In a study designed to examine the effect of PUFA on susceptibility to lung metastases in mice, Abbott and associates used experimental diets in which the predominant lipids were either n-3 PUFAs or n-6 PUFAs.[24] The n-3 (fish oil) diet was found to be protective, whereas the n-6 (corn oil) diet was not. The superiority of the fish oil diet was attributed to its higher n3:n-6 ratio and a concomitant decrease in arachidonic acid formation. Other studies also stress the importance of overall n-3:n-6 ratio in the diet rather than the absolute amount of n-3 PUFAs as a primary determinant of eicosanoid biosynthesis.[25] In rats, it was found that the suppression of eicosanoid biosynthesis from arachidonic acid by n-3 PUFAs showed no dose response if the n-3:n-6 ratio was left unchanged.[25] However, a shift in the ratio from 0.3 to 0.6 was the determining factor in inhibiting the production of eicosanoids. A study designed to examine the interactions between fish and vegetable oils in affecting rat leukocyte phospholipid PUFAs and leukotriene production showed that EPA-rich dietary fish oil supplements achieved a greater reduction in leukotriene B-4 synthesis when the linoleic acid content of the total fat intake approached that of olive oil rather than safflower or corn oil.[26]

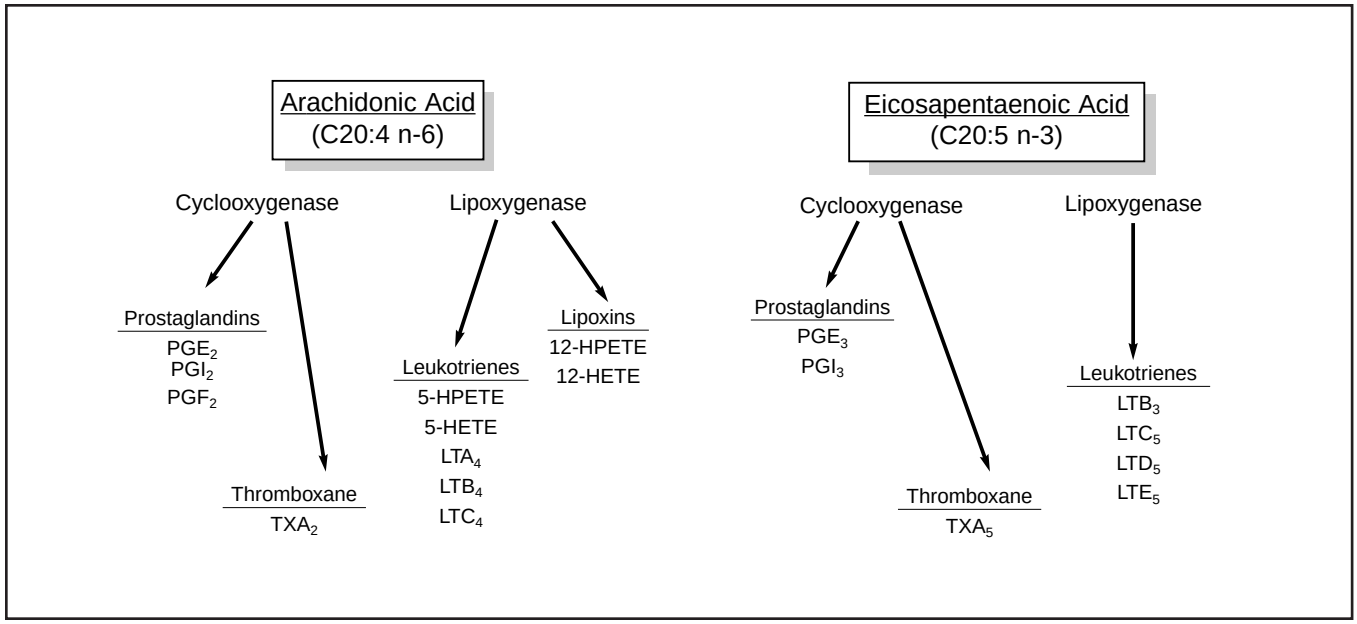
While there are few published data concerning the impact on oncogenesis of altering the n-3:n-6 ratio in humans, epidemiological observations support the supposition that a significant reduction in breast cancer risk occurs when the n-3:n-6 ratio is raised.[27] Lands and colleagues have observed an overall inverse relationship between cancer and dietary n-3:n-6 ratio.[28] In Japan, a downward shift in the estimated dietary n-3:n-6 ratio from a mean value of 0.34 in 1960 to 0.26 in 1985 was observed, with the concomitant increase in risk of breast and colon cancers. In 1985, the corresponding value for the United States was only 0.12. Rose has recently reviewed a large number of studies that used animal models to investigate the relationship of PUFAs and mammary and prostate cancers.[29] He concluded that n-6 PUFAs stimulate mammary carcinogenesis and tumor growth and metastasis, whereas n-3 PUFAs exhibit inhibitory effects. Studies of prostate cancers are less advanced, but the available data suggest a similar stimulatory role for n-6 PUFAs and an inhibitory role for n-3 PUFAs. Thus, based on accumulated data, Rose makes a cogent argument for clinical intervention trials designed to reduce total fat intake and increase the n-3:n-6 PUFA ratio in groups at high risk of developing cancer, and in cancer patients in remission, with the objective of preventing disease recurrence.

Conclusion

The observation that the capacity of PUFAs to promote or suppress tumor development and progression depends more on the n-6:n-3 ratio than on the total amounts of these PUFAs in the diet indicates that n-6 and n-3 PUFAs have different, but overlapping, physiologic functions. Altering the balance of these fatty acids can affect mechanisms that control critical biological characteristics of tumor progression, such as tumor cell

Figure

Eicosanoid Pathways



proliferation and metastatic potential. Manipulating the quantity and composition of dietary fatty acids may prevent and/or retard both localized growth and the development of distant metastases of a wide range of common cancers, including non-melanoma and melanoma skin cancers.

We further hypothesize that the addition of specific n-3 PUFAs to the diets of patients with advanced cancers, in conjunction with conventional therapeutic modalities, can provide a more efficacious therapeutic treatment than standard therapy alone. Controlling dietary n-3 PUFAs in patients at high risk for tumor recurrence and metastasis represents a conceptually new approach to improve clinical outcomes in patients with advanced disease. In addition, it affords patients a potential psychological edge in being able to control and participate in their own treatment using familiar substances that appear naturally in the food supply. *(Dr. Albino is the Director of Research and Dr. Cohen is the Section Head, Nutrition and Endocrinology, The American Health Foundation, Valhalla, NY.)* ❖

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Apo2L/TRAIL Induction by Ionizing Radiation Can Be an Important Contributor to Apoptosis

By Alex Almasan, PhD

THE SUCCESS OR FAILURE OF CLINICAL CANCER TREATments depends in great part on their ability to induce programmed cell death (apoptosis). Because apoptosis is deregulated in most cancers, apoptotic-modulating therapies offer an attractive opportunity for many tumors. For most cancers, ionizing radiation is a major modality of treatment. The radiation response of mammalian cells includes apoptosis, a process which requires activation of multiple genes; however, their role in radiation-induced apoptosis is poorly understood. One important pathway to apoptosis is mediated by surface-receptor signaling, which depends on ligand-receptor interactions for cell death effector activity. These cell surface receptors are part of the tumor necrosis factor (TNF)-receptor family and include Fas (also known as CD95) and death receptor (DR) 4 and DR5 molecules.[1] Engagement of these receptors by Fas ligand (FasL), or the apoptosis-inducing ligand Apo2L (also known as TRAIL) can lead to cell death.[2,3]

Apo2L as an Antitumor Agent

Apo2L has emerged as a powerful antitumor agent, as evidenced by increasing interest in its potential use in cancer therapy. The DR4 and DR5 receptors for Apo2L contain a cytoplasmic "death domain" capable of engaging the cell suicide apparatus through the adaptor molecule, Fas-associated death domain protein (FADD).[4] Most importantly, it was reported that while many human tumor cell lines are sensitive to cell-surface or soluble Apo2L, the majority of normal cells are not. This apparent protection of normal cells from the cytotoxic effect of Apo2L is believed to be based on a unique set of decoy receptors (DcR); these cells either lack the DcR1 or have a truncated DcR2 and are unable to signal, but compete instead for receptor binding to Apo2L. An alternative view is that levels of an intracellular inhibitor, FLICE-inhibitory protein (FLIP), may provide resistance in normal cells.[5] The mechanism of activation of this receptor-mediated cell death pathway during cancer therapy is still not well understood.

A recent study has shown Apo2L mRNA induction following γ-irradiation of a variety of human T lineage-derived normal and tumor cells.[6] Increased Apo2L protein levels also were found in these cells. Radiation also activated the Apo2L death receptor DR5, but only in those cells which harbored a wild-type p53. Similar to radiation treatment, exogenously added Apo2L induced the typical cellular and molecular events known to be associated with apoptosis, such as phosphatidylserine exposure on cell membranes and activation of caspases.

These events constitute a signaling cascade consisting of regulator caspases, such as caspase 8 and 9, and effector caspases, such as caspase 3, 7, and 6.[7] It has been clearly demonstrated using caspase cleavage site substrates and inhibitors, and by monitoring the cleavage of caspase cellular substrates, such as poly(ADP-ribose)polymerase (PARP) protein in vivo, that at least four known caspases are activated following exposure to radiation.[8] Caspase 8 is an apical caspase known to be the first caspase activated in receptor-mediated apoptosis. This recent study showed a substantial decrease in the levels of pro-caspase 8 protein, as well as an increase in caspase 8 enzymatic cleavage activity, indicating that the caspase cascade is activated by Apo2L through caspase 8.[6]

Proteolytic activation following cleavage of Bid, a Bcl-2 family pro-apoptotic protein also known to contribute to receptor-mediated apoptosis by inserting into

mitochondrial membranes, indicates the requirement for mitochondria in this process. Mitochondria serve as stores for apoptogenic molecules, such as cytochrome c, which once released into the cytosol further contribute to caspase activation and apoptosis.[9] Bid levels were significantly reduced upon Apo2L treatment, indicating processing of the full length Bid to its active proteolytic fragment. Activation of caspase 8 and caspase 3 indicate a caspase-dependent apoptosis, with Bid most likely contributing to the amplification of the caspase cascade. The functional significance of Apo2L for radiation-induced apoptosis was demonstrated by a significantly higher cell survival of those cells expressing the dominant negative Apo2L receptor DR5, which blocks Apo2L signaling.[8] This finding is consistent with a recent suggestion that radiation kills lymphocytes by a Fas-independent mechanism.[10] Apo2L induction in T cells could be most important because radiation could selectively kill tumor cells, which express only the DR5 and/or DR4 receptors, but not normal cells, which also express decoy receptors or FLIP.

Apo2L as an Adjuvant Therapy

While tumor-specific, Apo2L kills only about 60% of tumors. For the remaining tumors, a second therapeutic agent could be used in combination with Apo2L for an effective response. A recent study used clonogenic assays to show that low levels of purified, recombinant soluble Apo2L enhanced the lethality of therapeutic doses (1-2 Gy) of γ-irradiation.[6] This indicates that production of Apo2L may cooperate synergistically with the cytotoxic effect of radiation, and that combinations of Apo2L and radiation may become a powerful tool in clinical therapy. The observation that radiation can induce Apo2L, and that low-dose radiation can cooperate synergistically with Apo2L in enhancing cell death, may have implications for clinical therapy.

These findings are reminiscent of the previously reported synergistic or additive cell killing between TNF and radiation.[11] Gene therapy approaches have been proposed based on radiation-responsive promoters driving TNF expression,[12] and TNF expression has been shown to sensitize certain radiation-resistant tumors. Indeed, some tumor cells also may be sensitive to the death effects of Fas or TNF (applied alone or in combination with radiation). However, the therapeutic use of TNF or FasL has been hampered by severe side effects, since systemic administration of TNF causes a septic shock-like response, likely mediated by the inflammatory response triggered by activated nuclear factor-κB (NFκB), and Fas causes hepatotoxicity. Compared to TNFα and Fas, Apo2L is promising to be a safer agent, because most normal cells seem to be resistant to it and it activates NFκB only weakly, if at all.

Moreover, unlike TNFα and Fas, systemic administration of Apo2L has no toxicity in mouse and non-human primates.[13,14] In contrast to TNF and FasL, Apo2L is present in most human tissues, indicating that it is not cytotoxic in these tissues. However, it is not expressed in brain and liver, which may be one reason why some sources of Apo2L can induce apoptosis in primary hepatocytes.[15] It is important to note that other sources of Apo2L, which in fact are the ones soon to be tested in humans, are not cytotoxic to these cells, indicating that cells may respond differently to different sources of Apo2L. This has become a very controversial issue that may delay the use of Apo2L for clinical therapy until it is resolved satisfactorily. However, if this issue is addressed, understanding the regulation of Apo2L gene expression in brain, liver, and cancer cells may provide the basis for clinical application of Apo2L as an antitumor agent.

In addition to radiation, which induces Apo2L mRNA and protein in lymphoma and leukemia, interferons (IFN) also induce Apo2L in multiple myeloma cells (unpublished results). IFNs are a family of pleiotropic cytokines that play an essential role in the antiviral and antitumor host defense and have been widely used clinically to treat certain types of cancers, including myeloma. Cell death induced by radiation, IFNs, and Apo2L can be prevented by blocking the Apo2L receptor DR5, demonstrating the functional significance of Apo2L in mediating the cytotoxic effects of these therapeutic agents. This Apo2L induction is transcriptional and is mediated through 5' flanking DNA sequences likely to be responsible for radiation or induction of Apo2L by IFN.[16]

Conclusion

The observed synergy between Apo2L and therapeutic doses of radiation can form a basis for developing strategies for pharmacological intervention, with a potential for clinical application. In particular, the specificity of Apo2L cytotoxicity for tumor cells and its systemic distribution reaching metastases,[17] and the surgical precision with which IR now can be delivered could constitute a very attractive combination for enhancing the clinical response of tumors resistant to radiation therapy. Importantly, radiation therapy usually requires the function of the p53 tumor-suppressor gene for antitumor activity; however, more than one-half of human tumors acquire inactivating p53 mutations, thereby becoming resistant to therapy. Apo2L induces apoptosis independently of p53, and thus, may offer a complementary

approach to conventional cancer therapy. Further studies are needed to elucidate the mechanism by which Apo2L can induce apoptosis in combination with radiation or chemotherapy. *(Dr. Almasan is Assistant Staff, Department of Cancer Biology, Lerner Research Institute, and Department of Radiation Oncology, The Cleveland Clinic Foundation, Cleveland, OH.)* ❖

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Funding News

The National Science Foundation
Major Research Instrumentation Program

The National Science Foundation (NSF) Office of Integrative Activities is continuing the Major Research Instrumentation (MRI) program in 2001. The program is designed to increase access to scientific and engineering equipment for research and training, which generally is too costly for support through other NSF programs. The program is intended to support the acquisition through purchase, upgrade, or development of major state of the art instrumentation for research at U.S. institutions. Maintenance and technical support associated with these instruments also is supported. Proposals may be made either for single instruments or for large systems of multiple instruments. This proposal is not intended for general-purpose equipment such as commonly available computer networks. In addition to supporting the purchase of existing sophisticated equipment, this grant also may be used to support the development of new instruments or software simulations with enhanced performance (i.e., increased accuracy, reliability, resolving power, throughput speed, sample capacity, flexibility, breadth of application, user friendliness, and cost).

Researchers at U.S. institutions of higher education and non-profit institutions are eligible. An institution may submit up to three proposals, at least one of which must be for instrument development. Funding is available in the range of $100,000 to $2,000,000. The deadline for application is Feb. 7, 2001. Further information is available at http://www.nsf.gov\pubs\2001\nsf017\nsf017.htm#section1.

National Cancer Institute:
Development of High-Yield Technologies
for Isolating Exfoliated Cells in Body Fluids

The National Cancer Institute has announced a program to develop novel technologies for capturing, enriching, and preserving exfoliated abnormal cells in body fluids or effusions for biomarker studies. Because current methods of cytopathologic analysis are limited by the high ratio of normal exfoliated cells to cancer cells, there is a very real need to enhance analysis sensitivity. The development of enrichment methods for the routine detection of small numbers of exfoliated cells or subcellular material in biologic fluids would represent an important advancement in early detection. Such detection methods could be very useful for screening of colon, lung, prostate, oral cavity, esophageal, stomach, cervical, or bladder cancers. Molecular and genetic abnormalities within these exfoliated cells, such as microsatellite instability, could be used to detect and identify precancerous lesions or very early stage cancer if highly sensitive technologies are made available.

Applicants are encouraged to address the technology of enriching and isolating exfoliated cells as well as their viability and usefulness for cytologic and molecular studies. This program announcement will utilize the R21 NIH grant mechanism. The total project period should not exceed two years. The size of the award is variable, although direct costs are limited to $100,000 per year or $125,000 per year for consortiums. Applications are open to domestic and foreign for-profit and not-for-profit organizations. The grant applications cycles twice annually, with application receipt deadlines of April 10 and November 15. Letters of intent are due on March 6 and Oct. 11, 2001. Further information is available at http://grants.nih.gov\grants\guide\pa-files\PAR-01-019.html. ❖

CME Questions

6. The DNA mismatch repair (MMR) system is responsible for:
- a. correcting base pair mismatches.
- b. correcting chromosomal translocations.
- c. correcting chromosomal deletions.
- d. maintaining nuclear matrix protein integrity.

7. Evidence that n-3 PUFAs have potential as adjuvant therapeutic agents include which of the following findings?
- a. n-3 PUFAs can be directly cytotoxic to tumor cells.
- b. n-3 PUFAs can have synergistic or additive antitumor effects when used in combination with conventional chemotherapeutic agents.
- c. n-3 PUFAs can reverse intrinsic tumor cell drug resistance to several chemotherapeutic agents.
- d. All of the above

8. Cell surface receptors that are part of the tumor necrosis factor (TNF)-receptor family include:
- a. CD95.
- b. DR4.
- c. DR5.
- d. All of the above

9. Cell surface or soluble Apo2L:
- a. is cytotoxic to the majority of normal cells.
- b. is cytotoxic to many cancer cells.
- c. is bound by decoy receptors DcR8 and DcR9.
- d. is antagonistic to the effects of radiation therapy.

In Future Issues: Alcohol Consumption and Breast Cancer