

Review

Combined Effect of Ionizing Radiation and Alkylating Agents on Cancer Induction

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Human beings are exposed to numerous natural and man-made agents that are potentially carcinogenic. Therefore, cancer risk by ionizing radiation (IR) should be assessed as a result of combined exposures with other agents. These agents include genotoxic and non-genotoxic chemical carcinogens such as, tobacco, hormones, viruses, metals etc. Carcinogenesis is a multi-step process that accumulates several genetic and epigenetic changes of oncogenes and tumor suppressor genes. For agents having similar biological function and affecting the same step of carcinogenesis, additivity is generally expected, while for agents acting at different rate-limiting step, combined exposure is expected to be deviated from additivity. Conceptually, carcinogens are classified as initiator and promoter. IR could function at several steps as initiator, promoter or both. In order to predict the mode of combined action of IR with other agents, the sequence and time interval of the exposures, the dose, and the type of exposure (acute or chronic) are the critical factors. In this review, we focus on the combined effect of IR and alkylating agents. The data in the literatures and in our laboratory on mouse thymic lymphomas indicate that combined effect of these two genotoxic agents is synergistic, additive or antagonistic, depending on the dose and the sequence. Mechanistic approach determining frequency and spectrum of cancer-related genes and loss of heterozygosity (LOH) shows that role of IR differs in combined exposures depending on the dose. At low dose range, in general, the combined effect may not deviate from additivity. More information on the mode and the mechanism of low-level exposures, which occasionally encountered in environmental and occupational situation, are required for reaching a unifying concept.

Key words: combined effect, ionizing radiation, alkylating agent, carcinogenesis

Introduction

Human beings are exposed to numerous natural and man-made agents that have potent carcinogenic activity. The increase in number of these agents has given rise to growing concerns about the cumulative risks of mixed

exposures. Historically, national and international regulatory agencies have set standards for individual hazardous substances. Recognizing that this approach may not be appropriate, U.S. Environmental Protection Agency (EPA) published general guideline for the risk assessment of chemical mixtures (Guidelines for the health risk assessment of chemical mixtures, 51 Fed. Reg. 34014–34025, 1986). Currently, EPA uses dose-additive and response-additive model in chemical mixture risk assessment. Ionizing radiation (IR) is now of great concern because of an increased prevalence of medical and industrial use. Since the number of man-made agents is rapidly increasing, the effect of IR should be assessed as a result of combined exposures with these agents. The combined effect may be greater or smaller than the sum of the effect of single exposure. This review attempts to summarize the combined effect of IR with genotoxic chemicals, especially alkylating agents, as an example of evaluation for the cancer risk of IR in the environment with numerous chemicals mixtures.

The Mode of Combined Effect of Carcinogens

One of the basic concerns for the combined effect in our life surrounded by numerous carcinogens is whether the effect of combined exposure is simple sum of the effect of each carcinogen. In case of combined exposure of two agents whose dose response curves are linear, the mode of combined effect could be classified into additive, synergistic (or supra-additive) and antagonistic (or sub-additive) effects. These modes reflect a combined effect equal to, greater and smaller than the sum, respectively. For the agents with non-linear dose response, the identification of interaction is more complicated. For an upward bending dose response or dose response with a

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threshold, additional increment per the second dose of the same agent would be larger than that of the first dose. Thus, the term of “envelope of additivity”, which covers the range of additivity, is defined. The greater effect of “envelope of additivity” could be considered as synergism and the smaller effect of envelope could be considered as antagonism (Fig. 1). Therefore, the dose effect relationship is critical to judge the existence of interactions. Epidemiological study on A-bomb survivors, the dose response for solid cancers after exposure to radiation is linear (L) and that for leukemia is linear quadratic (LQ) (Fig. 2) (1). Dose response relationship shows threshold exceptionally for skin cancer at around 1 Gy (2). Several tumor models in animals, such as mouse skin tumors, bone tumors, ovarian tumors and thymic lymphoma, and rat kidney tumors also give threshold (3). Thus, the mode of com-

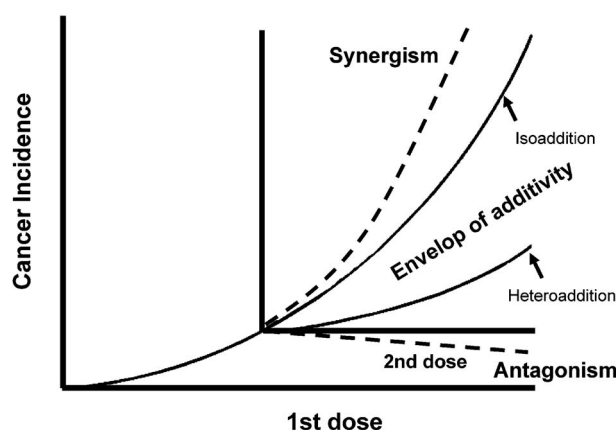


Fig. 1. Combined effect of two agents having non-linear dose-response relationship. Isoaddition is given when two agents have function, and heteroaddition is given when these act independently.

bined effect may be tissue dependent.

It is generally accepted that carcinogenesis is a multi-step process. It consists of initiation, which is defined as genomic alteration of oncogenes and tumor suppressor genes, promotion with clonal expansion of initiated cells, which leads to further accumulation of mutations, and progression, which is characterized by the acquisition of malignancy. Multi-stage cancer model was proposed by Armitage and Doll, which was the first attempt to develop a biological model of carcinogenesis (4). Then, Knudson, Moolgavkar and Venzon proposed a two stage stochastic model, considering clonal expansion of initiated cells, cell death and differentiation (5,6). We have recently applied two-stage model for the combined exposures of two agents, which are presumed to act at both stages (7). On a mechanistic level, synergism can be seen when each agent acts at different rate-limiting step of multi-step process or at different molecular target corresponding to rate-limiting step (8). When both agents affect the same step, combined effect is expected to be additive. Antagonistic effect could be observed when the agent could enhance the capacity for DNA repair or biological defense system against oxidative stress or induce apoptosis of initiated cells.

The Risk Factors that Interact with Radiation in Cancer Induction

Human epidemiological data have demonstrated several examples of combined effect of radiation and other physical, chemical and biological factors such as smoking, diet, ultraviolet (UV) radiation, virus and exogenous hormones (Fig. 3) (9).

A large body of information on uranium miners has provided an estimation of lung cancer risk in combined exposure to radon and smoking (10). It should be of

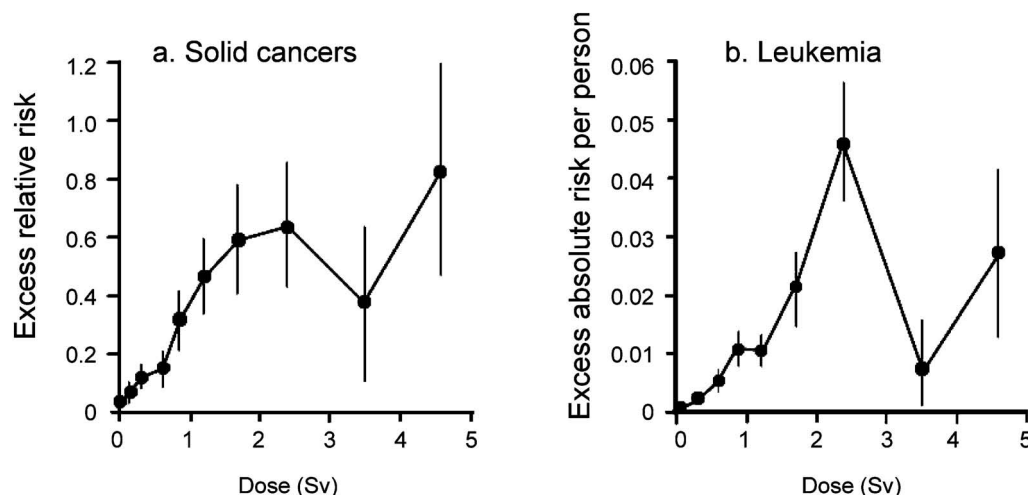


Fig. 2. Dose response for mortality of solid tumors and leukemia in A-bomb survivors. A linear relationship with no threshold is fitted for solid tumors (a), while a linear-quadratic model with upward curvature is best described for leukemia (b). Exceptionally, dose response of non-melanoma skin cancer shows curvilinearity with a possible threshold of 1Sv. Redrawn from the data in reference (1).

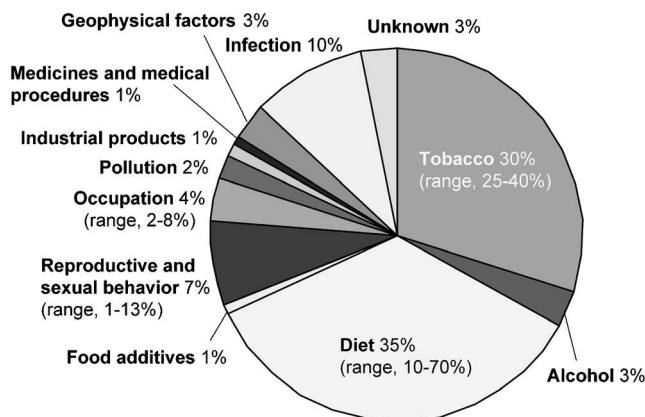


Fig. 3. Proportion of cancer deaths attributable to various environmental factors. Redrawn from the data in the reference (9).

note that tobacco smoke itself is a mixture of more than 4,000 genotoxic and non-genotoxic substances and some natural radionuclides such as ^{210}Po and ^{210}Pb . Importantly, nitrosamine and polycyclic aromatic hydrocarbons may function as initiators and catechols, phenols and terpenes may do as promoting agents. Reduction of risk of cancer development with increasing time after quitting smoking is understood because of the reduction of promotion (11). Lubin *et al.* (12) reviewed 11 studies of underground miners and concluded that combined effect of radon and smoke on lung cancers was more than additive but less than multiplicative. The absolute risk of lung cancer in smokers was about three times higher than that in non-smokers.

UV radiation is recognized as an important initiator of human skin cancer. Skin color and age at exposure are the critical determinants for this cancer. The skin cancer risk of IR was particularly pronounced on the face, where there would be more UV exposure. These findings suggest that UV exposure levels or sensitivity to such exposure interact with ionizing radiation (13). Not only exogenous but also endogenous hormones are potent growth stimulators and play a role as promoters in breast carcinogenesis. These include estradiol-17 beta (E2), prolactin, diethylstilbestrol (DES) and androgens. Virus may also have a role in the development of human cancers. Statistically significant interaction between A bomb radiation and hepatitis C virus infection is demonstrated in the etiology of hepatocellular carcinoma (14).

Although epidemiological data are important to identify the combined effects, they have limited potential to dissect the interactions completely because human being are exposed numerous known and unknown mixture of agents. Therefore, knowledge on combined effect has been confirmed and provided by animal experiments. Although animal experiments have

disadvantage to extrapolate the data to humans, they have advantage over epidemiological studies in that they retain control of the dose and population (age, gender, genetic background and so on). So far, numerous chemical agents have been examined using mice and rats if they interact with radiation to induce cancers. Chemical carcinogens may be classified into either genotoxicants or non-genotoxicants. Genotoxicants directly act on DNA molecules, thereby forming small or bulky adducts, strand breaks, and DNA-protein cross-links. Non-genotoxic chemicals may affect cell proliferation, differentiation and senescence. The experimental data on combined exposures are mostly accumulated for alkylating agents such as *N*-methyl-*N*-nitrosourea (MNU), *N*-ethyl-*N*-nitrosourea (ENU), 1,2-dimethylhydrazine (DMH), diethylnitrosamine (DEN) etc (15-22).

Combined Effect of IR and Alkylating Agents

Murine T-lymphomagenesis is one of the most extensively studied model for research of combined effect of IR and alkylating agents. Weekly lower doses (12×0.25 Gy; 4 Gy in total) concurrently combined with butyl-nitrosourea (BNU) enhanced lymphoma development, while intermediate doses (12×0.5 Gy) had no effect and high doses (12×0.75 Gy) delayed it (16). There appeared an inverse relationship between lymphomagenesis and dose, which might be ascribed to cell killing. Another study revealed that the incidence of lymphoma increased to 92% after ENU was preceded by 4 Gy from whole body irradiation, whereas single treatment with ENU induced lymphomas in 20% of mice and 4 Gy irradiation alone had little effect (18). Cell kinetics analysis indicated that 4 Gy irradiation was followed by regeneration of cells within a few days and maximum induction of lymphomas was given at the peak of DNA synthesis. This suggested that the role of IR was to provide susceptible subpopulation for the subsequent ENU treatment. Urethane has been also used for the combined treatments. X-rays (11×0.4 or 0.8 Gy; 4.4 or 8.8 Gy in total) every 4 days induced lymphomagenesis, which was enhanced by simultaneous treatment with urethane even at non-effective doses by itself in C57BL mice. Urethane also augmented the induction of lymphoma by X-rays in BALB-c mice (19). We recently examined T-lymphomagenesis of mice after weekly exposure of IR at doses of 0.2-1.0 Gy for 4 times followed by ENU in drinking water for 4 weeks (Kakinuma unpublished data). Combined exposure to ENU and high doses (4×1.0 Gy; 4 Gy in total) enhanced and accelerated T-lymphoma development compared to ENU alone. Surprisingly, low doses (4×0.2 Gy) reduced and delayed it, suggesting a protective role of low-dose IR for ENU-induced lymphomagenesis. Similarly, incidence of brain tumors, which were

induced by ENU treated *in utero*, decreased after combined treatment with pre-exposed X-rays (1 and 2 Gy), showing antagonistic effect. The reduction of tumor incidence corresponded with the inductive effect of X-irradiation on *O*⁶-alkylguanine-DNA alkyltransferase (ATase), suggesting a protective role of IR by inducing ATase for subsequent ENU treatment (20). Induction of ATase by IR has been frequently observed in several tissues *in vivo*. Interestingly, small but significantly higher increase in ATase activity was achieved when mice were exposed at a low dose rate (0.015 Gy/min) compared to a high dose rate (0.5 Gy/min), suggesting a protective effect of chronic exposure for alkylating agents (21). Collectively, these studies point out that the dose and dose rate are critical determinants for the mode of combined effect.

The sequence of exposure of two agents is also important. C57BL/6 mice were received X-irradiation (5 × 0.9 Gy with every 5 day) followed by urethane (5 × 20 mg, intraperitoneally injection) or urethane followed by radiation (22). Augmentation of lymphomagenesis by urethane was only obtained when urethane followed X-irradiation, but not when the sequence was reversed. This indicated that IR acted as initiator and urethane as promoter in lymphomagenesis. This is in good contrast to that urethane acts as an initiator in skin carcinogenesis, while for the lungs it is a complete carcinogen (23,24). The same agent plays different roles in carcinogenesis depending on the tissues.

DNA Damage and Molecular Signature Induced by IR and Alkylating Agents

DNA is a principal target of IR. IR induces several types of DNA damages including single- and double-strand breaks (dsb), base damage, and cross-links with protein. IR is considered to initiate carcinogenesis through generating DNA deletion and/or rearrangement caused by DNA dsb. On the other hand, molecular studies of induced somatic mutation show that majority of alkylating agents act through inducing point mutations. It is therefore expected that distribution of molecular changes in DNA differs between tumors induced by IR and those by alkylating agents.

Inactivation of TP53 is strongly suspected to contribute to the early development of human cancers. There are evidences that exogenous genotoxic agents are associated with the specific mutation spectrum of the *TP53* (*p53*) gene in human cancers. For instance, aflatoxin B1 (AFB1), a fungal derived contaminant of grain and peanuts, induces human hepatocellular carcinomas, acting with HBV (25). A specific *TP53* mutation is reported in hepatocellular carcinoma from hepatitis B virus positive patients having AFB1 contaminated food in certain areas of China (26). This is the AGG to AGT transversion at codon 249. The evidence for an in-

creased amount of AFB1-N7-guanine adduct in urine support the targeting the last nucleotide of codon 249 by AFB1 (27). Another example of *TP53* fingerprint is CC to TT double mutation in UV-induced skin cancer. C to T transition is also associated with UV irradiation (28). These mutations correspond to the two major types of DNA damages induced by UV radiation; cyclobutane pyrimidine dimers and (6-4) photoproducts.

Radon is a carcinogen of lung cancer. Lung squamous cell carcinomas in uranium miners showed a *TP53* hotspot mutation in codon 249; 16 out of 52 tumors harbored AGG to ATG mutation, suggesting radon-associated mutation (29). However, the following study could not confirm such specific mutation (30). This discrepancy is possibly ascribed to the difference in cancer histology and the exposure to mycotoxins in the former study. On current knowledge, it is considered unlikely that unique *TP53* mutation exists in radon-induced lung cancer.

Animal experiments have an advantage for the finding distinct molecular signature associated with the cause of cancer because of the ability to exclude the exposure to other carcinogens. UV-induced skin tumors have been confirmed to characteristic to harbor C to T and CC to TT mutation in the *p53* gene in hairless mice (31). Treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) induces a signature mutation of G deletion from GGGA sequence in rat colon tumors (32). Nitroso-compounds such as MNU or ENU are good inducers of point mutations. MNU efficiently induces rat mammary tumors and mouse thymic lymphomas. All mammary tumors, which were induced by MNU, contained *H-ras* mutation at codon 12 GGA to GAA (17). Eighty percent of mice developed lymphomas after MNU treatment, and they harbored *K-ras* gene mutation in codon 12 GGT>GAT (33). Likewise, *K-ras* point mutation was found in a half of ENU-induced lymphomas in B6 mice, most of which were GGT to GAT at codon 12 (34). In contrast, only 13% of X-ray-induced lymphomas contained *K-ras* mutation. The G to A transition mutation may result from the formation of *O*⁶-methyl- or *O*⁶-ethyl-guanine, which are extremely mutagenic (35). Mutation spectrum of ENU-induced tumors, however, differed among the genes examined. Although G to A transition was main spectrum of mutation in *K-ras* gene, T to A was dominant for *p53* and T to C for *Ikaros* (Table 1). The cells with these type of mutations in these genes may be selected because of their advantage for survival and growth (36). It is to be mentioned that mutation spectrum is also highly dependent upon the balance of repair capacity. The mutation of *Ikaros* in radiation-induced lymphomas induced in *Mlh1* deficient mice was completely different from that in wild-type mice. Almost all mutations in *Mlh1* deficient lymphomas were frameshift

Table 1. Mutations of the *Ikaros*, *p53* and *K-ras* genes in X-ray- and ENU-induced thymic lymphomas in B6C3F1 mice

	<i>Ikaros</i>		<i>p53</i>	<i>K-ras</i>
	X-rays	ENU	ENU	ENU
Null mutation	5			
Alternative splicing	4	1*		
Insertion	1			
Point mutation				
G > T				4
G > A	2		2	4
T > C	5	4		
T > A			4	
others	1	1	2	1
Number of lymphomas examined	37	27	27	27

*: The point mutation at splice-donor site within intron 4.

Thymic lymphomas were induced by repeated exposure of X-rays (4×1.6 Gy) or by ENU (200 ppm in drinking water).

The data were taken from the references (34,36).

mutation at the mononucleotide repeat, which was rarely observed in radiation-induced lymphomas in wild-type mice (37). Therefore, one-to-one correspondence between chemical exposure and mutation spectrum is not always observable.

Loss of heterozygosity (LOH) has been examined in many cancers as a possible localization of tumor suppressor genes. Since LOH could be generated by deletions and recombination, radiation-induced tumors were considered to harbor frequent LOH. Others and we have extensively studied the distribution of LOH in radiation-induced lymphomas (38,39). We found a significant increase in the frequency of LOH in the centromeric region of chromosome 11 in radiation-induced lymphomas, compared to spontaneous or ENU-induced lymphomas in B6C3F1 mice (Fig. 4). We mapped the *Ikaros* gene in this region and found numerous aberrations of *Ikaros* sequence and expression (39,40). Mice with heterozygous *Ikaros* point mutation or dominant negative isoform are reported prone to the development of IR-induced thymic lymphoma (Table 1) (41,42). Thus, *Ikaros* is a critical tumor suppressor gene for the genesis of thymic lymphomas. Interestingly, ENU-induced lymphoma also harbors *Ikaros* point mutation (T to C transition), but it did not accompany the loss of wild-type allele (36). Further accumulation of data on LOH in radiation-induced tumors is required to conclude that existence of radiation-induced molecular signature.

Mechanism of Combined Effect of IR and Alkylating Agents

The interaction of combined exposure takes place at molecular, cellular and tissue levels. At low dose ranges, damages induced by IR may accumulate independently

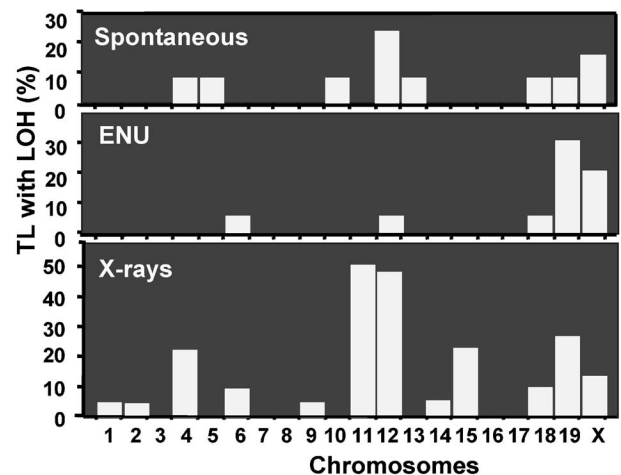


Fig. 4. Distribution of LOH in spontaneously developed, ENU-induced and X-ray-induced thymic lymphoma in B6C3F1 mice. The data are derived from the reference (39).

of those by alkylating agents. Therefore, the biological effect of combined exposure to IR and other agents is generally expected to be additive. In some cases as described above, IR induces several repair enzymes, some of which have protective activities for chemically-induced damages (21). High dose IR can kill the target cells or provide the environment to expand the preneoplastic subpopulation. Several growth factors or growth promoting cytokines are induced by high dose radiation. High dose X-rays enhance the expression of IL-1 beta and IL-7 in normal spleen cells (43) and fetal thymus (44), respectively. It is known that thymic lymphoma is developed in the unirradiated thymus transplanted into thymoectomized, irradiated mouse (45,46). This evidence is interpreted as that IR provides the tumor-promoting microenvironment for pre-existing pre-lymphoma cells in unirradiated thymus (47). Transforming growth factor-beta (TGF-beta) is the most potent known inhibitor of the proliferation of normal epithelial cells, and TGF-beta can act as an anti-tumor promoter. Advanced breast cancer cells, contrarily, are mostly refractory to TGF-beta-mediated growth inhibition. Recent observations indicate that IR can cause stromal fibroblasts to activate TGF-beta, thereby providing growth advantage for malignant cells over normal cells (48).

The available data on the mutations in tumors developed after combined exposures to carcinogens are quite limited. Rat mammary tumors induced by MNU harbor *H-ras* codon 12 mutation, while DMBA-induced tumors show activation of *H-ras* codon 61. The mammary tumors induced by both MNU and DMBA show predominantly G to A mutation in *H-ras* codon 12 (49). When MNU is combined with IR, mammary tumors with *H-ras* mutation are more frequent and develop

Table 2. Effects of IR and/or MNU treatment on the development of mammary adenocarcinomas carrying *H-ras* mutation in rats

Group	Tumors examined	Adenocarcinoma with <i>H-ras1</i> mutation			Adenocarcinoma without <i>H-ras1</i> mutation		
		Number (%)	Number per rat	Frequency [$\times 10^{-3}$; per rat per week]	Number (%)	Multiplicity	Frequency [$\times 10^{-3}$; per rat per week]
Control	3	0 (0%)	0	0	3 (100%)	0.08 \pm 0.05	2 \pm 1
γ -Rays	23	0 (0%)	0	0	23 (100%)*	1.15 \pm 0.27*	31 \pm 7*
MNU	54	29 (54%)*,†	1.61 \pm 0.56*,†	42 \pm 14*,†	25 (46%)*	1.39 \pm 0.37*	37 \pm 12*
Combined	76	47 (62%)*,†	2.24 \pm 0.74*,†	82 \pm 33*,†	29 (38%)*	1.38 \pm 0.36*	45 \pm 13*

*†p < 0.001 vs. control and γ -rays, respectively.

The data were taken from the reference (50).

significantly earlier than those when MNU is administered alone (Table 2) (50). *H-ras* mutation is not seen in IR-induced tumors. We have now analyzing the LOH and mutation of *ras* or *Ikaros* in the mouse thymic lymphomas and rat mammary tumors induced by IR and alkylating agents. The result will add further information on the role of these two agents in carcinogenesis.

Recent mouse models for detection of mutation *in vivo* give important results for consideration of the mode and the mechanism of combined exposures. Combined action of B[a]P and amosite (asbestos) caused a synergistic increase in mutation rate in the lung of *lambda-lacI* transgenic rats (51). In combination of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), chronic IR did not result in any obvious combined effect in the *gpt* selection, while the combined exposure suppressed large deletions in *Spi*-selection (52). We have recently found that combined exposures of ENU with high dose exposures increased mutation rate in a synergistic manner, while low dose IR decreased mutation rate (Yamauchi *et al.*, unpublished). Time course dependent changes in the mutant rate and its spectrum in the tissues and tumors induced by combined exposures with those by single exposure will shed light on the mechanism of combined effect. The descriptive approaches must be supplemented by the use of mechanism based cancer model (8).

Extrapolation to Low Dose

For risk assessment for human health, combined effect of numerous carcinogens at low doses is particularly relevant. However, many experiments have used acute, high doses of IR and other agents. It is not available how these data could be extrapolated to low and chronic exposure conditions. It is occasionally observed that many genotoxic agents have non-linear dose response. For low dose and dose rate, the linear term of dose response tends to remain. Under these conditions, the interaction of two agents decreases, and additivity results. At low doses, the interaction associated with compensatory cell proliferation, which usually

occurs after high dose exposure, is unlikely to take place. Non-genotoxic substances, which act as tumor promoter, have threshold dose and the effect at less than threshold dose will not be manifested. Low dose IR might have capability to induce repair system for other carcinogens, resulting in antagonistic response. Recently, it is reported that irradiation of non-transformed cells with low doses lead to stimulation of intercellular induction of apoptosis of neighboring transformed cells *via* reactive oxygen species, which was induced by TGF-beta (53). These results suggested that low dose radiation had potential on anticancer defense mechanism. Taken together, although synergistic effect of combined exposure might be common at high dose and dose rate, large deviation from additivity cannot be expected at low dose and dose rate relevant in occupational and environmental condition.

Combined Effect on Fetal and Infant Animals

For several decades, evidences have been accumulated that young children are more susceptible to cancer-causing agents than adults. The risk from childhood exposures to environmental chemicals and IR is thought to be heightened for the following two reasons. First, children's rapidly growing organs are vulnerable to carcinogen-induced changes. Secondly, children's behavior makes them prone to high exposures; they crawl on the ground, and they inhale more air per unit body than adults. Therefore, EPA has recently assumed that children under age 2 are 10 times more susceptible to carcinogens, and children aged 2–15 are 3 times more vulnerable than adults (Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens. EPA/630/R-03/003F, 2005).

In the viewpoint of combined effect, it is a concern whether or not the early-life exposure to one carcinogen may have impact on the subsequent exposure to another carcinogen. Hoyes-KP *et al.* examined the effect of early-life exposure to IR on the development of adulthood cancer (54). They showed that exposure to IR at the fetal, neonatal and juvenile stages of development

induced residual haematopoietic damage and increased oncogenic susceptibility to adulthood exposure to MNU. It was of interest that the neonatal stage was the most sensitive for induction of lymphoid malignancy while fetal stage was the most sensitive for the induction of myeloid leukemia. Mice irradiated on day 15 of gestation with 0.2 or 0.4 Gy X-rays in combination with postnatal exposure to ENU showed the greater-than-additive effect (55). This suggests that low-level of prenatal and perinatal X-irradiation leads to a lasting sensitivity towards a subsequent carcinogenic stimulus.

Recent growing use of interventional and fluoroscopic imaging in children represents a great benefit for diagnosis and treatment of benign conditions. Along with an increase in medical use for children, however, comes concern about the late effect of IR, especially cancers development. Therefore, the information on the effect of childhood IR exposure combined with later exposure to other carcinogens such as tobacco smoke will be necessary for the risk assessment for children.

Conclusion

Combined exposures are characteristic of life. Synergistic combined effects are common at high dose exposure, but the deviation from additivity is not expected at low dose exposure of genotoxic and non-genotoxic agents. The idea supports for the current approach of the risk assessment for mixture of carcinogens, which is based on the linear dose response and additive model. However, the agents that are exposed at high dose and function at different carcinogenic step may show synergy when combined with IR. These include tobacco smoke and, possibly, daily diet. Systematic quantitative assessment and mechanistic understanding of combined exposures is needed for reliable risk estimation.

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