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EXTENDED ABSTRACTS

Radiation in Realistic Environments: Interactions Between Radiation and Other Factors

American Statistical Association Conference on Radiation and Health
Beaver Creek, Colorado. June 26–30, 2004

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The 16th ASA Conference on Radiation and Health, held June 27–30, 2004 in Beaver Creek, CO, offered a unique forum for discussing research related to the effects of radiation exposures on human health in a multidisciplinary setting. The Conference furnishes investigators in health related disciplines the opportunity to learn about new quantitative approaches to their problems and furnishes statisticians the opportunity to learn about new applications for their discipline. The Conference was attended by about 60 scientists including statisticians, epidemiologists, biologists and physicists interested in radiation research. For the first time, ten recipients of Young Investigator Awards participated in the conference.

The Conference began with a debate on the question: ''Do radiation doses below 1 cGy increase cancer risks?'' The keynote speaker was Dr. Martin Lavin, who gave a banquet presentation on the timely topic ''How important is ATM?'' The focus of the 2004 Conference on Radiation and Health was *Radiation in Realistic Environments: Interactions Between Radiation and Other Risk Modifiers.*

The sessions of the conference included:

1. Radiation, Smoking, and Lung Cancer
2. Interactions of Radiation with Genetic Factors: ATM
3. Radiation, Genetics, and Epigenetics
4. Radiotherapeutic Interactions

The Conference on Radiation and Health is held bi-annually, and participants are looking forward to the 17th conference to be held in 2006.

DEBATE AND FOLLOW-UP LECTURE

David Brenner, Organizer

Eric Hall, Chair

Do Radiation Doses Below 1 cGy Increase Cancer Risks?

D. J. Brenner[a] and K. L. Mossman[b]

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Radiation doses below 1 cGy are frequently encountered in medical and occupational settings. For example, screening mammography involves breast tissue doses of approximately 0.5 cGy.

Brenner Position: We know there are significantly increased cancer risks when the general population is exposed to doses in the 0.5- to 10-cGy dose range, and we also know there are increased cancer risks after in utero exposure to 0.6 cGy.

Cancer incidence data for A-bomb survivors indicate a significant increase in cancer risk in a general population exposed to doses from 0.5 to 10 cGy (*1*). The signal-to-noise ratio will probably prevent us from ever getting statistically significant data for the general population at lower doses, irrespective of whether or not there are actually increased risks, so we need a different strategy. A logical approach is to increase the signal (e.g., by looking at a sensitive subpopulation, such as young people) and to decrease the background noise (e.g., by looking only at childhood cancer, which is rare). Whatever result we get in this situation can then be scaled back to normal populations, if desired. An end point that meets these criteria is childhood cancer after *in utero* exposure; Mole (*2*) has convincingly identified a population who were irradiated *in utero* between 1958 to 1961 with a mean dose of 0.6 cGy, and who had a significantly increased childhood cancer rate (odds ratio 1.23, 95% CI: 1.04–1.28). Mole pointed out that ''*This seems to be the only value for risk of cancer mortality after irradiation in utero based on independent determinations of dose and risk in nationwide samples of the same population of subjects. It is not based on extrapolation or on an unreliable dose response.*''

While these considerations seem to answer the subject of the debate, it is also worthwhile pointing out that 0.6 cGy of 80 kVp diagnostic X rays, as typically used in the *in utero* examinations, corresponds to a mean number of electron tracks per cell nucleus of about 1. In such a situation, there are quite plausible arguments for a linear extrapolation of risk to still lower doses. For example, if the dose were ten times less (0.06 cGy), the damage to each hit cell would be exactly the same—single/isolated electron tracks—but there would only be one tenth of the number of cells with this damage, suggesting that the risk would also be one tenth. Of course damaged cells can communicate with each other (bystander effects, instability effects, etc.), but the evidence we have suggests that cell-to-cell communication increases, not decreases, the risk.

Mossman Position: If risks exist below 1 cGy, they are too small to measure reliably.

Direct measurement of risks at very small radiation doses is difficult because of limitations of epidemiological studies to detect risk. Accordingly, risks are estimated by extrapolating from direct observations made at high doses to the low-dose region using predictive theories such as the linear, no-threshold theory. However, estimates are highly uncertain because the required dose extrapolation is very large.

The nonspecificity of radiogenic cancer, long latency, and large background rate of nonradiogenic cancer in the population necessitate the use of large epidemiological studies to quantify the carcinogenic effects of radiation in humans. At doses under 10 cGy, it is very difficult to detect a statistically significant increase in radiogenic cancer. The size of the population needed to detect an excess risk is inversely related to the square of the average population dose. A population of about 1 billion persons would be needed to detect a risk at 0.1 cGy. At 1 cGy a population of about 10 million persons would be needed [assuming a lifetime cancer mortality rate from all causes of 20%, a lifetime radiogenic cancer mortality risk of 5% per sievert (equivalent to 1 Gy assuming whole-body exposure to X or γ radiation), and 95% confidence]. It is interesting to speculate that had the highest dose to the Japanese survivors of the atomic bombings not exceeded 200 mSv (equivalent to 20 cGy), a statistically significant radiogenic cancer risk would not have been detected even though more than 60,000 survivors were involved.

Statistically significant radiogenic cancer risks have been observed at doses about 100 times higher than doses typically encountered in environmental or occupational exposure settings. The lowest dose associated with radiogenic cancer is about 10 cGy for thyroid cancer in children based on a pooled analysis of seven studies (*3*). Typical environmental and occupational doses are in the range of 0.1–0.5 cGy.

However, some have argued that there is credible epidemiological evidence of radiogenic cancer below 10 cGy. The principal source of data is the Oxford Childhood Cancer Survey (OCCS), which has reported a 40% increased risk of childhood leukemia and other cancers after 1–2 cGy intrauterine exposure (*4*). Although a statistical association is recognized, the causal nature of an association between *in utero* radiation exposure and childhood cancer and the level of risk remain uncertain. For example, the OCCS findings have not been corroborated in cohort studies (most notably Japanese children exposed *in utero* to atomic bomb radiation), and relative risks appear to be similar for all pediatric cancer sites, in contrast to findings in other radioepidemiological studies that show clear differences in relative risk by site.

Estimating low-dose risks using very large dose extrapolations strains the credibility of risk assessment. Accordingly, numbers of cancer deaths due to low levels of radiation exposure must be considered speculative; risk estimates at low doses have great uncertainties because they are derived theoretically. The possibility that there may be no health risks from radiation doses comparable to natural background radiation levels cannot be ruled out; at low doses and dose rates, the lower limit of the range of statistical uncertainty includes zero (*5*).

Summary

The lowest radiation dose associated with statistically significant increased risk remains controversial. Epidemiological studies are not powerful enough to detect risks at doses approximating 1 cGy in the general population, because the necessary large populations are not available. The published data that have been used to estimate low-dose risks are often equivocal. In evaluating risks at small doses, all published studies need to be considered unless there are scientifically defensible reasons for exclusion. Although unequivocal evidence of risk is unavailable at very low doses, this does not mean that increased risks do or do not exist. However, if there is a risk below 1 cGy, it is very small for any given individual—the controversial issue being the risk to a large population potentially exposed to these small risks.

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Radiation and Screening

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Screening, in general, may be defined as the use of some procedure in a population to detect a phenomenon that otherwise would not manifest itself until some future time or indeed may never manifest itself. In the public health context, screening usually refers to applying some sort of diagnostic procedure to a general population to detect an unsuspected disease or forerunner of disease in the hope that earlier intervention in the disease process may lead to an improved prognosis.

It is essential that any screening program be subjected to a detailed cost–benefit analysis before decisions are made regarding the desirability of such a program.

A number of such screening programs are of direct interest to radiation scientists because exposure to ionizing radiation is involved in the screening procedure, and this factor has to be taken into account in as quantitative a fashion as possible in any cost–benefit analysis. Examples of screening that involve exposure to ionizing radiation are screening for breast cancer by use of mammography and screening for lung cancer by means of chest X-ray films.

In conducting cost–benefit analyses of screening, the primary benefit of such programs is, of course, improvement in prognosis as a consequence of early diagnosis. However, to establish this requires a very careful assessment of the available evidence.

The most useful approach to evaluating the benefits of screening is the use of randomized controlled trials. These overcome many of the limitations of observational studies and thus are the preferred method of assessing benefit.

A number of randomized control trials of screening for breast cancer using mammography have been conducted (*1*), and the overall results of these trials are summarized in the meta-analysis shown in Table 1. The evidence from Table 1 shows that breast cancer mortality is reduced in groups receiving regular mammography, and the results are generally consistent over the study. This also applies to women who start screening under the age of 50, which has been a matter of some controversy.

In contrast to the situation with breast cancer in women, a number of studies of screening for lung cancer using various modalities have failed to show any benefit in terms of reduction in lung cancer mortality. A recent meta-analysis comparing more frequent to less frequent radiographic screening (*2*) is summarized in Table 2. It is obvious that, overall, there is no measurable benefit from routine screening for lung cancer by this modality in terms of reduction in mortality. This probably reflects the fact that lung cancers are usually more rapidly growing than breast cancers and survival rates from lung cancer are much poorer than survival rates from breast cancer. Thus the cost–benefit analyses for lung cancer stop at this point since there is no demonstrated benefit. Nevertheless, the radiation risks arising from chest X-ray films will be considered subsequently for demonstration purposes.

When considering the potential costs of a screening program, a number of factors have to be considered. These may be illustrated by consideration of a typical mammography screening program in women.

Detrimental factors that could arise from a mammography program include:

1. The psychological effect of false-positives, i.e., women who are told that they have some abnormality in their breasts and are in fear of having breast cancer until a definitive diagnosis (needle biopsy) is carried out. Since approximately four out of five tumors of the breast are benign, this represents a substantial burden of screenings.
2. The physical impact of false-positives: The procedure of a needle biopsy is low-risk, but any surgical intervention always carries some measure of risk. Of concern in this context, too, is the possibility that a diagnosis of malignancy in a tumor can be made, surgery be subsequently carried out, yet it is possible that some malignant tumors may never proceed to be life-threatening. The latter possibility is very controversial but may well apply to some diseases other than breast cancer.
3. Risk from exposure to ionizing radiation: This aspect will be examined in more detail, but obviously the concern is over the induction of breast cancer by ionizing radiation particularly when screening starts at an early age and particularly when frequency of mammography increases.
4. The impact of a screening program in terms of resource and financial costs upon the health-care system: Given the finite resources of any health-care system, it is necessary to consider the amount of benefit that will arise from any program in comparison to this cost to the health-care system to determine the most efficient allocation of resources within that system.

In summary, screening programs are of interest to radiation scientists in terms of assessing the risks when ionizing radiation is involved in the screening procedure. A careful understanding of the principles of cost–benefit analyses of screening programs is essential for evaluating the potential risks from radiation exposure during screening.

TABLE 1
Randomized Controlled Trials of Screening for Breast Cancer by Mammography in Women: Most Recent Results for All Ages Combined

Trial (dates)	Age at entry (years)	Follow-up (years)	Relative risk[a] (95% CI)
HIP (1963–1969)	40–64	18	0.77 (0.61–0.97)
Malmo (1976–1986)	45–69	12	0.81 (0.62–1.07)
Two-Country: Kopparberg, Ostergotland (1979–1988)	40–74	20	0.68 (0.59–0.80)
Edinburgh (1979–1988)	45–64	14	0.71 (0.53–0.95)
NBSS-2 (1980–1987)	50–59	13	1.02 (0.78–1.33)
Stockholm (1981–1985)	40–64	8	0.80 (0.53–1.22)
Gothenberg (1982–1988)	40–59	7	0.86 (0.54–1.37)
Combined			0.81 (0.70–0.94)
Combined − women screened <50 years			0.82 (0.71–0.95)

[a] For breast cancer mortality in groups receiving regular mammography screenings compared to groups without screenings.

TABLE 2
Relative Risk of Death from Lung Cancer Comparing Frequent Chest Radiographic Screening with Less Frequent Screening

	No. randomized		No. of lung cancer deaths		
Study	Radiographic screening	Control	Radiographic screening	Control	RR (95% CI)
North London	29723	25311	82	68	1.03 (0.74–1.42)
Czech study	3171	3174	247	216	1.14 (0.96–1.36)
Mayo Lung Project	4618	4593	337	303	1.11 (0.95–1.28)
Kaiser Permanente	5156	5557	44	42	1.13 (0.74–1.72)
Total	42668	38635	710	629	1.11 (1.00–1.23)

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RADIATION, SMOKING, AND LUNG CANCER

Ethel Gilbert, Organizer

Dan Stram, Chair

Joint Effects of Radiation and Smoking on Lung Cancer Risk among Atomic Bomb Survivors

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Results are presented on the joint effect of radiation exposure and cigarette smoking on lung cancer risks among A-bomb survivors, based on about 600 cases through 1994. Smoking information on about 45,000 persons derives from mail survey and clinical interviews in the Radiation Effects Research Foundation (RERF) cohort. Radiation and smoking effects on lung cancer are found to be significantly sub-multiplicative and quite consistent with additivity. More specifically, although for light smokers the apparent effects do not differ from multiplicative, for heavy smokers there is virtually no apparent radiation risk. The smoking relative risk, previously very low in studies of this cohort, is now similar to that found in Western populations. This increase is likely related to the scarcity of cigarettes during and soon after the war. The smoking relative risk depends little on sex. After adjusting for smoking, the radiation-related risks relative to background rates for nonsmokers are similar to that for other solid cancers: a sex-averaged excess relative risk (ERR) per sievert of about 0.9 with a female:male sex ratio of about 1.6. Adjusting for smoking removes a spuriously large female:male ratio in radiation relative risk due to the large interaction between sex and smoking level. The adjustment also removes an artifactual exposure-age effect in the radiation relative risk, opposite in direction to other cancers, which is apparently due to birth cohort variation in lung cancer rates.

The above results have been presented in detail in ref. (*1*). For the remainder of this abstract we consider more general statistical issues as we see them after this endeavor.

Analysis of radiation-related lung cancer risk without allowing for smoking is misleading, even though smoking level is not related to radiation dose. This is because interactions of dose with other factors are confounded with interactions of smoking and such factors. The phenomenon is most evident in regard to the sex ratio and exposure-age variations of excess lung cancer risk, and it is impossible to describe the risk levels without incorporating its dependence on these factors. It is sometimes too easy to forget that the excess risk per unit dose is not simply a number, but rather is a pattern depending on sex, exposure age and attained age. This makes analysis of joint effects of smoking and radiation effect challenging, and much of the effort in our project was in devising an approach to the problem, that is, suitable forms of descriptive modeling incorporating the major dependences on the factors other than radiation and smoking level.

It is interesting that allowing for smoking removes a radiation exposure-age effect in the relative risk (RR) for lung cancer that is opposite in direction to that seen for most other cancers. It can be seen mathematically that if the lung cancer birth-cohort trends for our cohort were mainly due to increased smoking, and if smoking and radiation act additively, this would explain the peculiar exposure-age effect in the radiation relative risk. Generally, as noted in ref. (*2*), apparent exposure-age effects seen in the RERF cohort are in a complicated sense confounded with birth-cohort trends in baseline cancer rates. Since these birth-cohort trends differ by culture, and by time even for Japanese, it is difficult to ascertain what are generalizable exposure-age effects. The nature of the confounding depends on whether factors responsible for the birth-cohort trends act additively or multiplicatively with radiation, and correspondingly whether one is considering exposure-age effects in the relative or absolute radiation risk. Although complicated, this is very important in generalizations regarding exposure-age radiation effects.

Smoking information was used in terms of a few levels of cigarettes per day, along with allowing roughly for attained-age and apparent birth-cohort variations in the smoking relative risk. The latter are probably not actual birth-cohort effects but are due to interactions between birth cohort and early-age smoking levels. More detailed information was available from the smoking surveys, including age of starting and stopping, and for many people repeated assessments of smoking levels. It is tempting to think that this more detailed information should be used. Of course it is fragmentary and of limited accuracy, but there are reasons to believe that aside from this, such detailed information is of limited value. Suppose one had complete and perfect information on each person's entire smoking history. Using this would necessarily entail modeling effects of all aspects of the smoking history, which would be difficult or impossible. Many may think that the primary value of detailed smoking history would be to compute pack-years of smoking, but we have reasons to suspect that this summary variable may not be so useful. A main issue is that it appears from various studies that the smoking RR may be fairly constant in age when the smoking covariable is represented as smoking level. If that were true, then using pack-years would introduce spurious age variation in the RR. Aside from this issue, it would certainly be difficult to model things such as the effect of cessation of smoking.

Achieving statistical discrimination between multiplicative and addi-

tive effects was barely possible, even though the heavier smokers had virtually no apparent radiation risk. Previous workers at RERF had been unable to make the discrimination, largely because apparent smoking RRs were of the order of 3 or so, rather than the values of 10–20 seen now and generally observed in Western populations. As noted above, we have some idea of the main reason for this increase. Our point, though, is that it seems quite unlikely to be able to discriminate between additive and multiplicative effects of radiation and lifestyle/environmental risk factors other than smoking, where the RRs for the other factors as well as those for radiation are usually modest.

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Lung Cancer after Hodgkin Lymphoma: The Roles of Chemotherapy, Radiotherapy and Tobacco Use

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Lung cancer is the most common tumor after Hodgkin lymphoma, with the results of cohort studies showing risks elevated as early as 1 to 4 years after treatment and persistent for several decades. Although ionizing radiation is an established lung carcinogen, the effect of chemotherapy is controversial (*1–3*). Several analytical investigations have aimed to clarify the relative importance of chemotherapy and radiotherapy for Hodgkin lymphoma in the development of lung cancer, but most results to date have been discrepant, based on sparse numbers, and inadequately adjusted for smoking habits (*1, 3*). No study has been able to address the relationship between radiation and chemotherapy in the production of excess lung cancer risks. Together with population-based cancer registries in Iowa, Denmark, Finland, Sweden, Ontario, New Jersey and The Netherlands, the NCI conducted a large case–control study of lung cancer among 19,046 Hodgkin lymphoma patients (1965–1994) (*4, 5*). Radiation dose to the specific area in the lung where cancer occurred, cumulative amount of cytotoxic drugs, and tobacco use were evaluated for 222 Hodgkin lymphoma patients in whom lung cancer developed (cases) and 444 matched Hodgkin lymphoma controls without lung cancer. The first paper (*4*) describing this study was primarily addressed to clinicians, and the second paper (*5*) was targeted to an audience mainly interested in radiation risk assessment. The major findings in the first paper (*4*) follow. A radiation dose of 5 Gy or more was followed by significantly increased sixfold risks of lung cancer and increased with doses of up to 40 Gy or more (P trend < 0.001). Alkylating agent chemotherapy, administered without radiation, was also related to significantly increased fourfold risks of lung cancer, which increased with increasing number of cycles (P trend < 0.001). Lung cancer excesses also increased with cumulative dose of the alkylating agents mechlorethamine and procarbazine ($P < 0.001$) when analyzed separately. Treatment with other alkylating agents was also followed by significant lung cancer excesses (RR = 6.3). Tobacco use was associated with a 20-fold elevated risk of lung cancer and multiplied risks associated with both radiation and chemotherapy. Significantly increased risks of lung cancer occurred as early as 1–4 years after alkylating agent therapy and remained elevated for the 5–9- and 10–14-year periods, whereas excesses after radiotherapy began after 5 years and persisted for over two decades. The difference in temporal trends is noteworthy.

In the second paper (*5*) based on the international study, more detailed attention was given to the radiation dose–response relationship, to interactions of tobacco use, radiotherapy and chemotherapy, and to the modifying effects of gender, age at exposure, time since exposure, and attained age. The estimated excess relative risk (ERR) per gray was 0.15 (95% CI: 0.06–0.39), and a linear function fitted the data well over the full range of doses even though the majority of Hodgkin lymphoma patients treated with radiotherapy received lung doses that exceeded 30 Gy. Based on an analysis in which radiation dose and the number of cycles of alkylating agent therapy were treated as continuous linear variables, the interaction of radiation and alkylating agent exposure was found to be almost exactly additive, and a multiplicative relationship could be rejected. By contrast, the interaction of radiation and smoking was consistent with a multiplicative relationship but not with an additive one. In fact, the ERR per gray increased with smoking categories defined by pack-years, although this variation could be explained by chance.

At least three previous analytical studies have assessed the influence of radiotherapy and chemotherapy for Hodgkin lymphoma on the subsequent risk of lung cancer (*1–3*). In the earliest endeavor (*1*), Kaldor and colleagues showed that the risk of lung cancer ($n = 98$ cases) after chemotherapy for Hodgkin lymphoma was twofold compared with patients who received either radiotherapy alone or chemotherapy together with radiotherapy. This group concluded that chemotherapy for Hodgkin lymphoma could be at least as carcinogenic to the lung as radiotherapy, even though no dose response with the number of chemotherapy cycles was observed. Average radiation dose to the entire lung in which cancer occurred was used in the analysis and expressed in terms of three groups (<1, 1–2.5 and >2.5 Gy); significant differences between these categories in terms of lung cancer risk for patients treated with radiotherapy alone were not observed (P trend = 0.48), although risk for those receiving >2.5 Gy was 1.6 (95% CI 0.7–4.1). Interactions between radiotherapy, chemotherapy and tobacco use were not addressed. A Dutch study of lung cancer ($n = 30$ cases) after Hodgkin lymphoma used estimates of radiation dose (<1, 1–5, 5–8 and 9 Gy or more) to the lobe of the lung in which cancer occurred and reported an increased 8.9-fold risk (95% CI 0.9–91) at the largest doses (P trend 0.02) (*2*). Positive interaction on a multiplicative scale was noted between smoking and radiotherapy, but no evidence of a relationship between chemotherapy and lung cancer was detected. Patients in the Dutch study (*2*) were included in analyses described by Gilbert *et al.* (*5*). In a recent study of British Hodgkin lymphoma patients, a risk of 1.7 (95% CI 1.0–2.8) for lung cancer ($n = 88$ cases) was observed after therapy with MOPP (*3*); however, no difference in lung cancer risk after one to six cycles (RR = 1.6) compared with seven or more cycles (RR = 1.8) was apparent, and information on cumulative dose was not collected. Radiation dose to the lung was not estimated, but was characterized as overall volume of radiotherapy (none/small/medium or large) received by the entire lung. Among Hodgkin lymphoma patients who did not receive chemotherapy, a 1.9-fold risk of lung cancer was apparent for those who received a large volume of radiation to the lung compared with those whose radiation exposure was characterized as none/small/medium. An analysis of interactions between risk factors was not presented. Of these investigations (*1–3*), only the study by van Leeuwen and colleagues (*2*) included detailed data on smoking habits. Information on tobacco use was available for 39% of patients in the British study (*3*) and 59% of patients in the early investigation by Kaldor *et al.* (*1*). In both studies, data on tobacco use was available in terms of never smokers compared to ever smokers.

The molecular pathways that might link alkylating agent chemotherapy in Hodgkin lymphoma patients with subsequently increased risks of lung cancer are not entirely defined. Mechlorethamine, procarbazine and chlorambucil cause lung cancer in laboratory animals (*6*), and mechlorethamine is similar in chemical structure to sulfur mustard, a human lung carcinogen (*7*). Alkylating agents achieve their anti-tumor effects by direct reactions with DNA bases. Methylating agents, such as procarbazine, can form the same DNA adduct (O^6-methylguanine) that is produced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). O^6-methylguanine is mutagenic and carcinogenic (*8*), with concentrations of this DNA adduct linearly correlated ($P < 0.01$) with cumulative amount of procarbazine in lymphoma patients (*9*). NNK is also a tobacco metabolite that is a potent lung-specific carcinogen in laboratory animals (*10*). Future

investigations should clarify the carcinogenic pathways underlying the elevated risks of lung cancer after both alkylating agent therapy and radiation for Hodgkin lymphoma. It is not clear, however, whether results in Hodgkin lymphoma patients can be generalized to patients with other cancers, given the immune defects inherent to this lymphoma and the high prevalence of tobacco use among case patients and control subjects in the international investigation (*4, 5*). Similarly, whether the radiation dose–response relationship observed for lung cancer among Hodgkin lymphoma patients, with risk persisting at doses as large as 40 Gy or more (*4, 5*), can be extrapolated to other populations who receive substantially lower radiation doses is unclear.

From a clinical perspective, the large excesses of lung cancer after Hodgkin lymphoma should underscore efforts aimed at smoking cessation, especially in view of the multiplicative relationship between tobacco use and either radiotherapy or alkylating agent chemotherapy. Although alkylating agents for Hodgkin lymphoma, in particular, MOPP-based regimens, appear to increase the risk of lung cancer, it will be important to determine whether this classic treatment, as well as more modern chemotherapy regimens, might also increase the risk of other types of solid malignancies, and if so, the nature of any interactions with radiotherapy.

Cancer treatment represents a double-edged sword, but it should be kept in mind that it is advances in therapy that have been responsible for the marked gains in survival experienced by Hodgkin lymphoma patients. Thus the benefits of many cancer therapies for Hodgkin lymphoma far outweigh the risk of late effects, including lung cancer. The international study, with its large size, quantitative data on radiation dose, chemotherapy and smoking, and the large relative risks associated with all three variables, provided an unusual opportunity to obtain information on interactions (*4, 5*). However, because of the very high radiation doses received by Hodgkin lymphoma patients, there are questions regarding the applicability of these findings to populations exposed to much lower doses. The immunodeficiency inherent to this lymphoma and that associated with chemotherapy are additional reasons for caution in generalizing these findings to other groups.

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Residential Radon, Smoking and Lung Cancer

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Radon is by far the largest source of radiation exposure to the general population, contributing about 40% of the total annual exposure worldwide (*1*). Most radon exposure occurs indoors, especially in houses and apartments, where the principal source is usually the subsoil under the building or, occasionally, the building material. Residential radon concentrations are very variable and, in many countries, range over several orders of magnitude. Studies of underground miners have consistently demonstrated associations between radon concentrations and lung cancer risk in both smokers and nonsmokers (*2*). Associations have also been demonstrated experimentally in rats and dogs, and radon has been classified as a human carcinogen by the International Agency for Research on Cancer (*3*). Calculations based on risk estimates derived by extrapolation from studies of miners suggest that in many countries residential radon may cause a substantial number of lung cancers and may be the second most important cause of lung cancer after tobacco. Direct evidence is now emerging to support this view. In most buildings, radon can be reduced for a moderate cost, and low concentrations often can be achieved in new buildings at minimal cost; thus the amount of lung cancer caused by indoor radon has potential public health relevance.

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Lung Cancer and Plutonium Exposure in Rocky Flats Workers

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We conducted a nested case–control study of the association between lung cancer mortality and cumulative internal lung doses among a cohort of plutonium workers employed at the Rocky Flats Plant in Colorado from 1951 to 1989 (*1, 2*). We used incidence density sampling to select controls from the set of subjects at risk at the time of death of each case. Four controls for each of the 180 cases were randomly selected from risk sets defined by sex, date of birth, and employment for at least 6 months.

Annual equivalent doses to the lung—primarily from plutonium, but also from americium and uranium isotopes—were calculated for each subject with an internal dosimetry model based on International Com-

mission for Radiological Protection (ICRP) Publication 30 (*3*). Other exposure variables included cumulative external penetrating radiation dose, cigarette smoking frequency, cumulative exposure to chemical carcinogens (asbestos, beryllium, hexavalent chromium and nickel, as determined by a job exposure matrix), which were lagged by 5-, 10- and 15-year periods. Age at first internal lung dose, calendar period of first hire, and duration of employment were also assessed as confounding variables.

We were unable to obtain data on smoking frequency for 31.7% of cases and 15.7% of controls, and excluding subjects who lacked these data removed proportionately more cases than controls with low cumulative penetrating and internal lung doses. Models with data for smoking histories were analyzed with three different approaches: (1) maintaining matching between cases and controls, thus excluding any matched group of one case and four controls that had one or more subjects who lacked data on smoking; (2) including 730 subjects for whom smoking data were collected by breaking the 1:4 matching and adjusting analyses for birth year in ordinary logistic regression models; and (3) maintained matching by using a missing-indicator variable to adjust for differences between those with and those without smoking histories.

Results

The odds ratios (ORs) for lung cancer increased with pack-year categories for cigarette smoking for all lag periods. In some models there was an interaction of borderline statistical significance between smoking frequency and cumulative internal lung doses greater than 0.4 Sv. In all multiple variable models, smoking did not confound the relationship between cumulative internal lung dose and risk for lung cancer mortality. Because smoking was not a confounder of dose–response relationships and to reduce selection bias, we developed final models with matched analyses for all subjects, without smoking data.

Lung cancer risk was elevated among subjects with cumulative lung doses >0.4 Sv lagged by 10 years (OR = 2.20, 95% CI: 1.13, 4.26). The odds ratios did not, however, increase monotonically with doses >0.644 Sv in most models. Generally, the ORs for cumulative lung dose categories were slightly higher when lagged by 10 years, compared to 5- and 10-year lag periods. The period of first hire between 1960 and 1967 was statistically significantly associated with elevated lung cancer risk (OR = 1.79, 95% CI: 1.02, 3.13) compared with hire between 1968–1989. Employment duration in years (OR = 0.96, 95% CI: 0.94, 0.99) and number of years with above-zero lung doses (OR = 0.97, 95% CI: 0.94, 0.99) had statistically significant protective effects.

The ORs for all cumulative lung dose categories were substantially lower for subjects employed for more than 25 years and somewhat lower for those employed for 10 years or fewer. Restricting analysis to those employed for 15–25 years produced a statistically significant linear trend with cumulative dose, suggesting a strong healthy worker survivor effect. Adjustment for the number of years with above-zero doses produced a substantial increase in the ORs over all cumulative lung dose categories, suggesting errors in dose estimates for subjects with long periods of plutonium deposition in the lung. The association between age (in years) at first internal lung dose and lung cancer mortality was statistically significant (OR = 1.05, 95% CI: 1.01, 1.10). No associations were found between lung cancer mortality and either cumulative external penetrating radiation dose or cumulative exposures to asbestos, beryllium, hexavalent chromium, or nickel.

Elevated risks for lung cancer in Mayak plutonium workers have been detected at unlagged doses above 1.8 Sv, but the risks (as estimated with standardized mortality ratios stratified by dose) were not statistically significant at unlagged doses lower than 7 Sv (*4*). We detected statistically significant risks at doses above 0.4 Sv, lagged by 10 and 15 years. A recent dose–response model (with doses lagged by 5 years) for Mayak workers estimated the excess risk per unit dose to be about 0.23 Sv^{-1} (*5*). This estimate is similar to the risks estimated for radon doses received by uranium miners (*6*) and is lower than our estimates of risk by a factor of ten. Our estimates of risk are about two times higher than those summarized for solid tumors in atomic bomb survivors exposed to doses lower than 0.5 Sv (*7*).

Our findings suggest the risks for lung cancer from Rocky Flats plutonium exposures are much greater than those estimated with the Mayak data. Future analyses of the Rocky Flats cohort with improved dosimetry and exploration of the impact of the healthy worker survivor effect and other confounding variables are important for determining whether plutonium workers are adequately protected by current regulations, and whether decisions based on current risk estimates are fair for compensating plutonium workers with lung cancer under the Energy Employees Occupational Illness Compensation Program Act of 2000.

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INTERACTIONS OF RADIATION WITH GENETIC FACTORS: ATM

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Multiple Gene Effects in Radiation Oncogenesis

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In several different walks of life, the implicit assumption is made that the human population is homogeneous in its response to radiation, except for a small number of individuals, such as ATM homozygotes, who are easily identified by their clinical symptoms. Examples where this assumption is made include (a) radiation protection limits for occupational exposure, (b) radiation limits for astronauts on missions in space, and (c) radiotherapy protocols, where little effort can be made in practice to individualize therapeutic doses, or to assess individual risks of induced second malignancies.

There is some evidence that this basic assumption is not correct, namely that the human population is not homogeneous in radiosensitivity, but rather includes a number of sensitive subgroups. Such individuals would suffer an increased incidence of detrimental effects when exposed to radiation and would also distort the shape of the dose–response relationship used to estimate risks.

In the long run it may prove possible to use global gene expression arrays to identify genes, or families of genes, that control radiosensitivity. This approach has not, to date, proven to be productive. The alternative strategy is to investigate genes that are already known, or suspected, of influencing radiosensitivity, and to test their effect on both deterministic and stochastic end points using knockout mice. Obvious candidates include *ATM*, *BRCA1* and *BRCA2*, and *RAD9*. There is reasonably good evidence that haploinsufficiency for all of these genes confers radiosensitivity for deterministic end points, including cell lethality and ocular cataracts. To date, only for *Atm* has radiosensitivity been demonstrated for a stochastic end point, namely oncogenic transformation in mouse embryo fibroblasts as a surrogate for carcinogenesis. Of particular interest are combinations of genes that operate in the same signal transduction pathway. *RAD9*, for example, functions downstream of *ATM*.

We have developed an assay for apoptosis in mouse thymocytes that allows rapid screening of multiple genes. Apoptosis is a characteristic cellular response to DNA damage. The proportion of cells that undergo apoptosis after exposure to radiation depends on the levels of the Atm and Rad9 proteins. The expression levels of these proteins is halved in heterozygous cells, and undetectable in the Atm knockout cells. Only heterozygous animals are available for the *Rad9* gene, since the knockout is embryonically lethal. Cells in which Atm is knocked out show a greatly reduced level of apoptosis; Atm heterozygous cells show a reduced intermediate level. It is of interest to note that the effect of a low Atm protein level on apoptosis is the opposite of its effect on oncogenic transformation: decreased apoptosis and elevated transformation. The logical explanation of these results is related to the Atm activity. When we investigated apoptosis in cells where two interacting proteins in the DNA repair pathway—Atm and Rad9—were haploinsufficient as a result of heterozygocity, we obtained a striking picture—a very low number of cells underwent apoptosis after DNA damage (close to the Atm-deficient phenotype). Interestingly, the haploinsufficiency for Rad9 had little if any effect on apoptosis.

These results led us to the conclusion that haploinsufficiency for specific signaling proteins leads to partial loss of control of apoptosis. The lost apoptotic control in Atm-deficient cells leads to high degree of transformation. The same could be true for the Atm/Rad9 haploinsufficient cells and for cells haploinsufficient for other interacting proteins in the DNA repair pathway. Atm is a protein linked to several targets. Haploinsufficiency for Atm and any of its targets may lead to cancer predisposition and progression and in principle could be linked to any tumor.

Therefore, we raise the hypothesis that haploinsufficiency for two related proteins in DNA repair cell signaling pathways leads to partial loss of control of apoptosis. Cells haploinsufficient for these proteins have a greater predisposition for accumulation of mutations than normal cells and respectively increased predisposition to transformation. As a result, the real predisposition to cancer could be linked to heterozygocity and most important to a combination of heterozygocity for multiple genes in the same cell or organism.

Multi-center Screening of Mutations in the ATM Gene among Women with Breast Cancer—The WECARE Study

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Deficiencies in cellular responses to DNA damage can predispose an individual to cancer. Ionizing radiation can cause clustered damage and double-strand breaks (DSBs) that pose problems for cellular repair processes (*1, 2*). ATM, the product of the *ATM* gene mutated in the autosomal recessive disorder ataxia telangiectasia, has a critical function in signaling the presence of DSBs that are induced by ionizing radiation, radiomimetic chemicals, and developmental DNA rearrangement events (*3*). Upon activation by ionizing radiation or other DSB-inducing agents, ATM phosphorylates a large number of downstream targets that control pathways whose activation can result in cell cycle checkpoint controls, DNA repair, and apoptosis (*4*). These targets include the products of several genes implicated in breast cancer susceptibility, including *BRCA1*/2 and *CHEK2* (*5–9*). There is evidence from epidemiological studies of ataxia telangiectasia families, mutation screening of ATM in breast cancer cohorts, and experimental animal model systems that carrier status for at least a subset of ATM mutations is associated with an increased risk for breast cancer (*10*). However, the mechanism mediating this increased risk and the potential involvement of radiation exposure have yet to be elucidated.

To examine the joint roles of radiation exposure and genetic susceptibility in the etiology of breast cancer, we designed the WECARE Study (for Women's Environmental, Cancer, and Radiation Epidemiology), a case–control study nested within five population-based cancer registries (*11*). We hypothesized that a woman carrying a mutant *ATM* allele is more susceptible to radiation-induced breast cancer than is a noncarrier. In our study, 700 women with asynchronous bilateral breast cancer were individually matched to 1400 controls with unilateral breast cancer on date and age at diagnosis of the first breast cancer, race, registry region, and survival to the age at diagnosis of the case, and countermatched on radiation therapy. We chose to study second primary breast cancer because the prevalence of causal genetic variants would be enriched in the at-risk population (women who had survived a first breast cancer) and because they were very likely to have been exposed to high-dose radiation. Each triplet comprised two women who received radiation therapy and one woman who did not. Radiation absorbed dose to the contralateral breast after initial treatment was estimated with a comprehensive dose reconstruction approach that included experimental measurements in anthropomorphic and water phantoms applying patient treatment parameters. Blood samples were collected from all participants for genetic analyses. All study participants were screened for *ATM* gene mutation carrier status using a staged approach, DHPLC followed by direct sequencing, appropriate for analysis of this complex gene (*12*).

We will discuss the importance of the counter-matched design and analysis incorporating sampling weights in improving the power for detecting the main effect of ionizing radiation and its interaction with *ATM* genotype, and the modest price to be paid for this gain on the power for the main effect of *ATM* genotype (*13–15*). Simulation studies show that a 2:1 countermatched design (with two patients receiving radiation therapy and one not in each matched triplet) to be the most efficient design. The repository of data and biological specimens developed for this study serves as a resource for investigating the etiological role of other genes, particularly genes involved in DSB damage and repair pathways. Since it may not be cost effective to genotype all subjects for all possible polymorphisms in every gene within a targeted pathway, we discuss subsampling designs and analytical approaches that combine information from both the parent study and substudy in a manner that maximizes the efficiency for estimating the joint effects of these genes (*16*). We will also discuss dose–response modeling accounting for dose uncertainties (*17, 18*) and for the differential distribution of radiation doses across quadrants of the contralateral breast. We plan to use genomic approaches to characterize haplotype variation in candidate genes (*19, 20*), and Bayesian modeling of risks for highly polymorphic genes (*21*) incorporating functional and *in silico* predictors (*22, 23*) as ''prior covariates''. Finally, we discuss the use of physiologically based pharmacokinetic models (*24*) and Bayesian hierarchical models (*25*) for synthesizing the effects of ionizing radiation and the various genes (e.g. *ATM, BRCA1/2, CHEK2, RAD51* and others) involved in the DSB repair pathway.

This study raises a number of challenging design and analysis issues. Our focus on the study of bilateral breast cancer improves the potential for detecting gene–environment interactions when both gene mutations

and the environmental exposures of interest are rare in the general population. This is particularly applicable to the study of radiation dose and genetic susceptibility because both have important etiological roles, possibly by interactive mechanisms. By using countermatching, we further optimized the informativeness of the collected dosimetry data by increasing the variability of radiation dose within the case–control sets and enhanced our ability to detect radiation–genotype interactions. This methodology, along with key elements of the WECARE Study design, will be presented.

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RADIATION, GENETICS, AND EPIGENETICS

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The Roles of BRCA1 and BRCA2 in the Cellular Response to Ionizing Radiation

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The BRCA2 protein is now regarded as a protein required for the assembly of RAD51 filaments at the sites of DNA damage or stalled DNA replication. Consequently, BRCA2 facilitates the process of homologous recombination (HR). We have provided direct evidence that the interaction of BRCA2 and RAD51, via the BRC repeat motifs of BRCA2, is critical for its function in HR. Furthermore, BRCA2's role in facilitating HR is dependent on having a replicating DNA template, closely linking the process of HR to DNA replication. Recent structural information has supported the importance of the BRC repeats in binding RAD51 and has also identified single-strand DNA binding motifs (RPA-like OB folds) in the C-terminal region of the protein. To date, no other role for BRCA2 has been elucidated *in vivo.*

BRCA1, by contrast, has a complex series of functions, including a supportive role in HR, a possible role in non-homologous recombination

(NHR), a transcriptional co-activator function, and E3 ubiquitin ligase activity. The protein undergoes extensive post-translational modification, principally by phosphorylation, both in S phase and in response to DNA damage. We have shown that ATM-dependent modifications of BRCA1 are important for S and G_2/M checkpoints but have no direct impact on DNA repair. However, a CHK2-dependent modification of BRCA1 at serine-988 appears critical for the promotion of RAD51-dependent HR and the inhibition of MRE11/RAD50/NBS1 (MRN)-dependent repair. MRN-dependent repair can be observed by assaying DNA integration or by measuring end joining after end modification: Intermolecular joining between two separate DNA duplexes is the common factor and error-prone repair is the result. Simple re-ligation after double-strand cleavage appears to be independent of MRN and dependent on KU/DNA-PK. Direct modification of CHK2 kinase activity, by overexpression of a kinase-dead CHK2, results in the same phenotype as seen with the S988A mutation of BRCA1. Taken together, these results suggest that a CHK2-BRCA1-BRCA2-dependent pathway promotes error-free HR, suppresses error-prone NHR, and thereby maintains genomic stability. The implication is that any one member of this ''pathway'' may be disrupted in generating a subset of breast cancers, and we are developing functional assays of this pathway to be used in human breast cancer samples (tissues and cytology).

The Genetic Basis for Variation in Radiation Sensitivity in the General Population

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This discussion focuses on current understanding of the genetic basis for variation in the consequences of radiation exposure. The emphasis is on the ''general population'', meaning everyone. Rare individuals with chromosomal instability syndromes such as ataxia telangiectasia, Bloom syndrome, Fanconi anemia and Nijmegen breakage syndrome help to define extreme radiation sensitivity but are not the focus. ''Radiation sensitivity'' can be viewed broadly, including not only cell survival but also other primary and secondary outcomes, such as somatic mutation, inflammation and replacement proliferation of stem cells that puts such cells at increased risk of mutation. These events may contribute to increased risk of cancer, cardiovascular disease, and mutations in offspring, to name a few health outcomes. The examples presented relate to variation in genes critical for repair of damaged DNA. The principles are expected to apply to many domains of health.

Unexplained variation among individuals in biomarkers of genetic damage stimulated our interest in this issue. Biodosimetry is an important aspect of radiation research. The frequencies of chromosome aberrations, either dicentrics soon after exposure or translocations at later times, are the best validated biodosimeters. Biodosimeters necessarily reflect both exposure and the individual's response to the exposure. In the absence of pre-exposure data, variation among individuals in baseline biodosimeter values determines the ability to estimate dose after exposure for an individual. We have studied biomarkers in Russians who either did or did not participate in the cleanup after the Chernobyl nuclear power reactor accident. Cytogenetic analyses led to an estimated exposure of 9.5 cGy for the group of cleanup workers studied. We were struck by the observation that roughly 75% of the interindividual variation in both the frequency of stable chromosome aberrations and the frequency of HPRT mutants in peripheral blood lymphocytes was not explained by known exposures or age (*1*). We recognized that incomplete ascertainment of exposures was part of the story. However, it seemed that the time was right to begin developing the knowledge needed to learn how much of this variation was due to genetic differences among people. The sequencing of the human genome was well along and knowledge of the genes required for repair of radiation-induced damage was well developed. We had the conviction that, if we looked, we would find a large amount of sequence variation in genes related to radiation sensitivity, that much of it would have potential to affect the consequences of radiation exposure, and that this variation not only would help explain variation in biodosimetry but also would provide the basis for testing the hypothesis that variation in the health consequences of radiation exposure has a genetic component. It was further hypothesized that this same genetic variation has health consequences independent of radiation.

There is variation in the inherited sequences of DNA repair genes, much of which is predicted to affect function. Finding genetic variation in the general population is straightforward. One simply sequences each gene of interest in a number of people, a process called resequencing. We have resequenced almost 40 DNA repair genes in DNA from 90 people (*2*). Our focus was on the exon sequences, reasoning that we could best predict the functional significance of sequence variants that affected the protein encoded. Most of the sequence differences identified are single nucleotide substitutions, called SNPs (single nucleotide polymorphisms); the convention is that alleles with frequencies $\geq$1% are considered polymorphisms. Additional lower-frequency sequence variation can be identified if one resequences genes of more people.

Multiple SNPs encoding amino acid substitutions were identified in most genes. The few non-variant proteins may highlight proteins whose function is both key to life and very delicately built. Among 37 repair genes there were 127 amino acid substitution SNPs with an average allele frequency of 0.047 (*2*). Repair genes are not exceptional; other families of genes have been resequenced by others with similar results. For repair of DNA damage induced by radiation, genes (proteins) in base excision repair, nonhomologous end joining, recombinational repair, mismatch repair and transcription-coupled repair are relevant, as are the damage recognition and cell cycle control proteins and proteins with antioxidant properties. Because hundreds of genes are involved overall, the number of different inherited combinations of variant genes (SNPs) related to the DNA repair aspects of radiation sensitivity is very large.

To obtain insight into the potential of repair gene polymorphisms to affect function, particularly given the large numbers of polymorphisms, we have employed published algorithms that predict functional impact based on evolutionary conservation of protein sequence and the biochemical nature of observed amino acid substitutions. Concordance of predictions of two algorithms suggests that ~20–30% of the missense polymorphisms are likely to have an impact on protein function (*3*).

We began another approach to assessing the impact of SNPs by analyzing the relationship between cellular phenotypes and comprehensive pathway genotypes. We measured the level of single-strand DNA breaks in cell lines derived from healthy people and related variation in this phenotype to a large number of SNPs identified in base excision repair (BER) genes by the resequencing. We started with this example because of the predominance of oxidative DNA damage among the substrates for the BER pathway (making it relevant to damage induced by both radiation exposures and normal oxidative metabolism) and the depth of knowledge about the BER pathway. We hypothesized that the steady-state level of a key pathway intermediate, single-strand breaks, would be affected by SNPs that affect BER capacity. The preliminary studies have measured single-strand breaks in cells from 80 individuals. The genotypes included 99 SNPs (89 in BER genes and 10 in antioxidant genes) present in two or more individuals. The results of cross-validated random forests regression (RFR) indicate that a genotype of six SNPs explains a substantial proportion of the interindividual variation in the level of endogenous single-strand breaks and is the optimal predictor of this phenotype for the set of SNPs analyzed and the population sampled. Some SNPs were associated with increases, others with decreases of single-strand breaks. It is notable that three of the six influential SNPs have allele frequencies of 0.03 and are present in our small population only in the heterozygous state. Larger data sets are needed to detect which of the many even lower-frequency SNPs affect function. Simulation studies suggest that the effectiveness of RFR in detecting the impact of an SNP on phenotype is determined, to first order, by the magnitude of the impact, not by the

number of SNPs being examined. The large number of low-frequency alleles presents another challenge to the ability of exploratory methods such as RFR to discover more subtle relationships. Many more trees need to be examined than are typically used in RFR analyses, thus considerably increasing the required computational load. In addition to using methods like RFR to discover potentially important genotypes, tree-based methods can also be used to discover instances in which the presence of two or more specific SNPs has more than additive impact on phenotype (gene–gene interactions).[1]

Evidence is growing that polymorphisms in DNA repair genes affect human health. Knowledge of repair gene polymorphisms, and the relative ease of obtaining genomic DNA and genotyping, has led to a flurry of first-generation molecular cancer epidemiology studies. Many of these studies suggest that individual polymorphisms may have modest effects on health. Some hint that the impact of an SNP may be evident only in the face of low levels of carcinogen exposure. Others indicate that there can be additive effects of multiple SNPs (*4*). Most studies have assayed only small numbers of high-frequency SNPs in relatively small populations, with the consequence that results among studies are some times contradictory. Studies of populations with occupational radiation exposures are just beginning (*5*).

Development of analytical methods and epidemiological resources is critical to identifying genotypes that affect the consequences of radiation exposure. Identification of genotypes that affect the risks from radiation exposure (population studies) and understanding the underlying mechanisms (cell-based studies) will require research and the development of statistical/analytical methods that conquer the high dimensionality of genotype data. Methods should be robust enough to take on comprehensive genotypes, so that results are not limited by gaps in our understanding of which genes and SNPs are relevant. There is much yet to be discovered. Methods for relating genotype to continuous variables such as cellular phenotypes and some health outcomes will be needed, as well as outcomes that deal with categorical health outcomes such as having a disease or not. Methods that identify independent gene effects, gene–gene interactions, and gene–environment interactions are needed. Ultimately predictive models should incorporate all three.

Developing and testing such models requires large epidemiological studies that endeavor to define both genotype and environment. Defining genotype will be far simpler than defining ''environment''. Key elements will be documentation/assessment of exposures (not only radiation and traditional environment factors that an individual cannot control, but also potentially controllable factors such as smoking, diet, exercise and therapies) and a broad range of health outcomes (not just cancer), and also the establishment of repositories of biological specimens for genotyping. Because most complex diseases are the result of decades of events and the impact and interaction of endogenous and exogenous exposures and genetic variables in tissue-specific and developmental stage-specific ways, viewing health as the product of a life span will help put all the elements in perspective.

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Gene Environment Interactions in a Cohort of Irradiated Retinoblastoma Patients

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Survivors of hereditary retinoblastoma (RB), a rare childhood cancer of the eye caused by germline mutations of the *RB1* tumor suppressor gene, have an elevated risk of developing sarcomas, brain cancer or melanoma, whereas survivors with non-hereditary RB, caused by somatic mutations in the *RB1* gene, do not appear to be prone to secondary cancer (*1, 2*). Since it is likely that the subsequent cancer risk in hereditary RB continues throughout adult life, we have been studying a large cohort of RB survivors to determine their risk of subsequent cancer and to evaluate the relationship between radiation, environmental and genetic factors that may modify this risk (*1–3*).

The cohort consists of 1,601 RB survivors who were diagnosed 1914–1984 at two medical centers in the United States. The cohort includes 963 (60%) hereditary patients with either bilateral RB or a unilateral tumor associated with RB in a family member, and 638 (40%) non-hereditary patients with sporadic unilateral RB. Accrual of person years of follow-up began 1 year after diagnosis of RB and ended at date last known alive, death or December 31, 2000, whichever occurred earlier. Follow-up since RB averaged 25.2 years for hereditary RB and 29.5 years for non-hereditary RB patients. The expected number of cancers was estimated from age-, sex- and calendar year-specific cancer incidence rates from the Connecticut Tumor Registry. We calculated the standardized incidence ratio (SIR) as the observed (O) number of confirmed, invasive cancers compared to the expected number of cancers, with exact 95% confidence intervals based on the Poisson distribution. We also calculated the cumulative incidence of a second cancer with adjustment for competing risks.

Consistent with earlier findings in this cohort, the risk of subsequent cancers in 963 hereditary patients (SIR = 19, O = 260) greatly exceeded the risk in 638 sporadic patients (SIR = 1.2, O = 17). Among hereditary RB patients, the risks were highest (SIR $>$ 100) for cancers of the bone, connective tissue, eye and orbit, and nasal cavities. Risks were substantially elevated (SIR $>$ 10) for pinealoblastoma, melanoma, and cancers of the brain and CNS, buccal cavity and corpus uteri. In addition, significantly elevated risks (SIR $<$ 10) were noted for cancers of the lung, female breast and colon.

RB patients treated with radiotherapy, primarily external beam, had tumor doses that ranged from 15–115 Gy (average 48 Gy) to the entire retina delivered in 15 fractions over several weeks. The risk of subsequent

cancer was elevated almost sevenfold in nonirradiated hereditary RB patients, while radiotherapy further increased this risk by 3.1-fold (95% CI = 2.0–5.3). Among the non-hereditary cases, only breast cancer was significantly increased in females (SIR = 2.8), especially in those who received radiotherapy (SIR = 10, O = 3).

At 50 years after diagnosis of hereditary RB, the cumulative incidence of a second cancer was 36% (95% CI = 30.8%–41.1%) compared with 5.7% (95% CI = 1.4%–10%) for non-hereditary RB.

Increased risks likely related to radiation were observed for cancers of the bone, soft tissue, brain, nasal cavities, and eye and orbit. A radiation dose response for sarcomas, predominantly in the head and neck, after hereditary RB has been convincingly demonstrated (*2*). Significant risks possibly linked to radiation in the hereditary patients included cancers of the salivary gland, tongue and nasopharynx based on small numbers and cancer of the breast.

Excess risks were also noted for melanoma, and cancers of the lung, colon and uterus but they are unlikely to be associated with radiation in this cohort. As we reported previously (*3*) and Fletcher *et al.* recently confirmed (*4*), hereditary RB patients have an increased risk of lung cancer, suggesting a genetic susceptibility to the carcinogenic effects of smoking.

At the time of last follow-up, the excess risk of breast cancer appeared limited to non-hereditary RB (*2*). With additional follow-up, risks were similarly increased for irradiated patients with hereditary and non-hereditary RB. Doses to the breast after treatment for RB ranged from 0.3 to 0.9 Gy. The risk of radiation-related breast cancer is known to be heightened when the exposure occurs at very young ages, as observed after irradiation for enlarged thymus glands or hemangioma and after exposure to the atomic blasts in Japan.

Increased risks were apparent for cancers of the salivary gland, tongue and nasopharynx, based on small numbers. Doses to the salivary glands after treatment for RB ranged from 1.6 to 4.3 Gy. Cancer of the salivary gland has been linked previously to radiotherapy during childhood for tinea capitis, enlarged tonsils and bone marrow transplantation.

We also noted excess risks of cancers of the colon and corpus uteri among the hereditary patients. Five of the seven uterine cancers and one of the three colon cancers were leiomyosarcomas, consistent with genetic susceptibility to a variety of sarcomas (*5*). In another series of long-term RB survivors (*4*), bladder cancer was significantly elevated, while the risk of this tumor was nonsignificantly increased in our study of hereditary RB patients.

In our extended follow-up, the cumulative incidence for developing a second cancer 50 years after diagnosis of hereditary RB was 36%, in contrast to 51% that we reported previously, whereas the cumulative incidence remained the same for non-hereditary RB (*2*). The lower cumulative risk through 2000 compared to 1993 is encouraging, since it probably reflects the lower doses of radiation received by patients after 1960. However, the continuing elevated cancer risk in hereditary but not non-hereditary RB points to the role of germline *RB1* mutations in a variety of subsequent cancers, especially those treated with radiation.

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Second Cancers after Radiotherapy: Any Evidence for Radiation-Induced Genomic Instability?

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The long-held belief that a radiation effect required the traversal of the nucleus is challenged by the observations of radiation-induced genomic instability. The ideas (1) that irradiated cells transfer genomic instability to unirradiated adjacent cells (bystander effects), (2) that apparently normal irradiated cells transmit instability after multiple cell divisions to their progeny, (3) that traversal of the cell's cytoplasm confers genomic instability, or (4) that clastogenic plasma factors induced after radiation exposure perennially increase free radical production, creating a ''stressed'' cellular environment, have not been completely recognized in radiobiological models of radiation carcinogenesis [reviewed in ref. (*1*)]. The extent to which genomic instability, whether innate or induced, plays a role in the development of a second malignancy within, near or far outside the margins of tissue irradiated for a first cancer is not known. We will address first primary cancers that arise after radiation exposure and how these may provide clues about the influence of genomic instability, summarize the epidemiology of radiotherapy-related second cancers, evaluate studies of second tumors arising in irradiated tissues for hallmarks of induced somatic genomic instability, and describe possible future studies.

Radiation-Induced Genomic Instability and Cancer

Documenting that radiation induces events that lead to genomic instability, which in turn contributes to progression toward cancer, is a thorny problem. There is ample experimental evidence that genomic instability, manifested as increased frequencies of chromosome aberrations, gene mutations or microsatellite sequence instability, can occur after radiation exposure. It may not be possible to definitively distinguish cancers associated with radiation-induced genomic instability from cancers that develop in radiation-exposed cells with a pre-existing genomic instability. One approach, however, might be to identify signatures of genomic instability in tumor cells that are associated with specific, genetically defined genomic instability syndromes (and hence specific repair genes and pathways) and then determine whether tumors developing after radiotherapy have mutations with a radiation mutation spectrum involved in any of the genes associated with inducing that signature of instability. For example, genes involved in mismatch repair would be assessed in tumors displaying microsatellite instability (MIN), and genes involved in double-strand break repair would be evaluated in tumors with chromosome instability. To add to the complexity of this approach, one must be mindful that a given tumor may display multiple types of genomic instability, yet radiation may have been directly responsible for the induction of only one type.

Second Cancer Risk after Radiotherapy Treatment

Several studies have investigated second cancer risk after radiotherapy for a first primary cancer. These studies clearly indicate increased risk of subsequent cancers with increasing dose to the surrounding tissues, including the bone marrow, and also indicate that not all tissues exhibit the same uniform risk per unit dose and that age at the time of treatment also influences risk estimates. Presumably, if radiation-induced genomic instability is transferred to nearby cells (bystander effects) and contributes to second cancer risk, this phenomenon should be evident in the low-

dose portion of the dose–response curve (*2*). Although large epidemiological studies can distinguish between a linear and curvilinear dose response in second cancer risk, detecting more subtle alterations convincingly supportive of a bystander effect at low doses (say under 100 mGy) would be exceedingly difficult. So, although it is presently impossible to ascribe the shape of a dose–response curve or any proportion of second cancers arising within a cohort of cancer survivors to late effects of radiation-induced genomic instability, the observational risk estimates for humans must include the effect, if it exists.

Tumor Tissue Analysis of Second Primary Cancers

Second primary human tumors arising in an irradiated field for a first cancer are intuitively attractive for study in attempting to discern a signature for radiation causation or, in this context, induced somatic genomic instability. On the other hand, tumors are notoriously complicated, displaying a wide variety of aberrant conditions such as karyotypic abnormalities, proliferative signaling, TP53 mutations, gene amplification, loss of heterozygosity, multinucleation, gene expression changes, micro- and minisatellite instability, etc. It is hard to define what events pre- or postdate tumor formation. There is some evidence suggesting that the spectrum of mutations expected after radiation-induced instability relative to those induced directly by radiation would be small-scale or point mutations (rather than large deletions) [reviewed in ref. (*3*)]. To determine whether the published literature might provide some evidence for radiation-induced genomic instability, we examined the results from 11 second cancer studies in which the tumor arose within a field irradiated for the first cancer [reviewed in ref. (*4*)].

We found few unifying threads of commonality among these studies. Several included patients treated with both radiation and chemotherapy (rather than radiation alone), and several found evidence of genomic instability existing in the patient before occurrence of the second cancer. Although radiation-related second tumors possessed unique karyotypic patterns distinct from sporadic cancers, it was difficult to categorize these as early events. Also, about an equal number of studies found that the type or frequency of TP53 mutations was either elevated or not different from sporadic tumors. We conclude that the studies published to date do not provide sufficient evidence to determine whether radiation-induced genomic instability contributes to secondary malignancies in humans after radiotherapy.

Future Studies and Recommendations for Research

Given that genomic instability is a hallmark of cancer in general, finding genomic instability in a tumor that occurs after radiotherapy is not sufficient to prove that radiation was causative. More convincing would be identification of mutations in the second tumor in genes that can lead to the specific types of genomic instability that are documented in that tumor, and determination that the mutations have a spectrum consistent with radiation. It is necessary to determine that the mutations do not appear in normal tissues that were not in the radiation field; they might be present in normal tissue adjacent to the tumor of an individual, however. The mutations should not be present in the first, radiation-independent tumor. The tools to perform such analyses are presently under development. Highly sensitive methods are needed to screen small tissue samples for all types of genomic instability to classify a tumor with respect to the pathways and hence genes that are candidates for the radiation-induced somatic mutations that started the progression of instability. One can envision a suite of assays that apply next-generation methods related to comparative genomic hybridization and gene expression arrays to detect chromosomal alterations and related expression phenotypes and the detection of microsatellite mutations. As knowledge of the genes responsible for DNA repair and different types of genomic instability becomes ever more complete, it will be relatively easy to define the sets of genes to screen for mutations in each individual. Oligonucleotide arrays could be used to exhaustively search for mutations in each gene [as recently done for ATM mutations in lymphomas (*5*)]. Finding somatic mutations in candidate genes associated with genomic instability is the first step. Then it is necessary to compare the spectra of mutations to determine whether there is a mutation signature in the tumors after radiotherapy that distinguishes (some of) them from the mutation signatures in unexposed tissues and tumors from subjects with no radiotherapy.

Summary

Do second primary cancers in humans arise from radiation-induced somatic genomic instability after radiotherapy for the first malignancy? The amount of truly pertinent human information on this issue is sparse, leading to the conclusion that we cannot confirm or refute the hypothesis that induction of instability by radiation is involved. However, the *in vitro* findings of radiation-induced genomic instability induced through bystander effects or increased mutation rates in cell progeny of apparently normal but irradiated cells are provocative, and their transferability to human *in vivo* biology deserves further investigation. We are reminded that cells *in vitro* are not cells *in vivo*; certainly the tissue structure and epigenetic signals a cell experiences also influence tumor formation (*6*). Because the occurrence of a first and then a second cancer in the same individual is unpredictable, the study of these malignancies will require the collaborative commitment of multiple large institutions to tumor tissue procurement and retrieval. In addition, detecting the temporal progression of genomic instability and identifying the salient genetic events as being radiation-induced will be pivotal. Execution of some of the studies needed is not possible now, but applying next-generation methods could bring the concepts to fruition. Because nearly one in ten cancer diagnoses are second (or higher) malignancies, it is important to understand the contribution of radiotherapy to second cancer induction and to pursue well coordinated efforts to determine the role of induced genomic instability.

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BANQUET AND KEYNOTE TALK

How important is ATM?

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To maintain the integrity of the genome, cells have evolved several mechanisms that recognize DNA damage and signal this to the DNA repair machinery, to cell cycle checkpoints, and to transcriptional control. Although there have been exhaustive reports on the nature of the lesions arising in DNA in response to a variety of damaging agents and on the mechanisms of repair, the ability of the cell to recognize these lesions and signal the appropriate cellular machinery has only recently begun to be unraveled. The description of a number of human genetic disorders characterized by chromosomal instability and cancer predisposition has accelerated our understanding of the process of DNA damage recognition. One such syndrome, ataxia telangiectasia (AT), has been a focal point because of the universal sensitivity to ionizing radiation and because of the central role the gene product involved plays in radiation signal transduction.

The AT locus was mapped by Gatti and colleagues (*1*) to 11q 22–23. Combined genetic and molecular analyses provided detailed physical maps of the region and a high-density array of genetic markers, leading to the identification of the ataxia telangiectasia mutated gene, *ATM* (*2*).

The *ATM* gene is large, occupying 150 kb of genomic DNA, containing 66 exons, and encoding a 13-kb transcript. The open reading frame [complementary DNA (cDNA), 9.168 kb] predicts a 350-kDa protein composed of 3056 amino acids, but the actual size is closer to 370 kDa, at least in part due to phosphorylation of the protein (*3*).

ATM is a member of a family of proteins that share a phosphatidylinositol 3-kinase (P13K) domain. This group includes the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), AT and RAD3-related protein (ATR), and proteins in other organisms responsible for DNA damage recognition or cell cycle control. ATM kinase is rapidly activated by ionizing radiation to phosphorylate a series of substrates involved in radiation signaling. However, evidence also exists to show that ATM can be regulated at both the transcriptional and translational levels. A more widespread role for ATM in events other than DNA damage recognition exists including receptor signaling, cellular proliferation, K^+ channel activity, and insulin signaling pathways. It is possible that ATM plays a direct role in these processes, or that in its absence cellular homeostasis is altered by oxidative stress or some other form of perturbation, leading to the myriad of defects described in AT cells.

It is evident that a basal level of ATM kinase activity exists in untreated cell extracts, and this increases twofold to fourfold when the cells are exposed to radiation or radiomimetic agents (*4*, *5*). It appears likely that ATM recognizes double-strand breaks in DNA and is activated as a consequence to phosphorylate a number of key substrates associated with DNA damage recognition and cell cycle control. The exact mechanism of activation of ATM kinase by DNA damage remains undescribed but appears to involve phosphorylation and dissociation of a dimeric form into an active monomer.

ATM plays an important role in activating cell cycle checkpoints at G_1/S, S and G_2/M phase. Defective control in the absence of ATM contributes to the genomic instability and cancer predisposition that is characteristic of this disease. This presentation is designed to examine the importance of ATM in a range of cellular processes in response to DNA damage.

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RADIOTHERAPEUTIC INTERACTIONS

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Radioprotectors: Current Status and New Directions

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With the advent of the nuclear age came the realization that people would require protection against the toxic effects of radiation that could occur from nuclear accidents, medical exposures, and nuclear war, with the latter risk being the primary driving force for government research and development of radioprotectors during the Cold War. With the collapse of the Soviet Union and the lessening of tensions along with the inherent toxicity problems of many of the radioprotector drugs developed during this period, their use became focused to address clinical problems of normal tissue toxicity induced by radiation therapy (*1*). The only radioprotective drug that has been approved by the FDA for use in radiation therapy is the radioprotector amifostine, which is sold under the trade name Ethyol® for use in the prevention of xerostomia in patients treated for head and neck cancer. However, the clinical use of radioprotectors in radiation therapy continues to be plagued by issues relating to possible tumor protection and diminution of therapeutic gain (*2*). After the disaster of the destruction of the World Trade Center on September 11, 2001 and the rise of a nuclear terrorism threat, there has been a new interest focused on the development of novel effective and nontoxic radioprotectors for potential use in homeland defense as well as in selected medical applications. Radioprotector development for non-oncology-related uses has the advantage of not having to deal with tumor protection issues. Four general approaches to radioprotection will be discussed regarding the use of drugs to reduce the risk of mutations and cancers, induce transcription of endogenous antioxidant genes, inhibit apoptosis, and stimulate progenitor cell growth.

Prevention of Radiation-Induced Mutagenesis and Carcinogenesis

Phosphorothioate radioprotective drugs as exemplified by amifostine exhibit the ability to protect against radiation-induced mutations at the hypoxanthine-quanine phosphoribosyl transferase (*Hprt*) locus even when administered to mice up to 3 h after exposure to radiation (*3*). While a dose of 400 mg/kg is required to demonstrate optimum cytoprotection by amifostine, its antimutagenic effect persists at a dose as low as 25 mg/kg. Furthermore, this class of drugs has been demonstrated not only to inhibit radiation-induced mutagenesis and cellular transformation but also to protect against radiation-induced carcinogenesis (*3*). While the induction of all classes of tumors was reduced, amifostine treatment prior to

irradiation virtually eliminated the risk of lymphoreticular tumors developing in mice exposed to 2 Gy of low-LET γ rays. The proposed underlying mechanism of action leading to the antimutagenic effectiveness of these drugs is not explained by their antioxidant properties since this effect occurs even when cells are exposed up to 3 h after irradiation. Rather, it has been proposed that the polyamine-like properties of the phosphorothioates may result in a stabilization of DNA damaged sites facilitating a slower and more error-free repair of damage (*3*).

Delayed Radioprotective Effect: Transcription of Antioxidant Genes

The clinically approved drugs captopril, mesna, *N*-acetyl-L-cysteine and amifostine each exhibit antioxidant properties because of their respective free thiol groups. As such they each have been demonstrated to activate the nuclear transcription factor NFKB, which results in the enhanced expression of the antioxidant gene manganese superoxide dismutase (*SOD2*). Correlating with enhanced gene expression and elevated endogenous levels of SOD2 protein is an increased resistance to ionizing radiation that has been described as a delayed radioprotective effect (*4*). This effect can be abrogated through the use of *SOD2* antisense or NFKB inhibitors. It is possible that chronic exposure to low doses of thiol-containing drugs may lead to an enhanced radiation resistance in cells and tissue systems due to this effect.

Anti-apoptosis Agents

While apoptosis is a naturally occurring process that maintains the homeostasis of proliferating cell systems, it is also an inducible cell death process brought on by deleterious agents such as radiation. While there is concern that interfering with this process can lead to enhanced mutagenesis and carcinogenic risk as a tradeoff for enhanced survival, it is also conceivable that interference with radiation-induced apoptosis by certain agents will merely reflect the repair of radiation-induced mitochondrial lesions so as to prevent initiation of the associated signaling pathways leading to apoptosis. Under this scenario inhibition of apoptosis will not reflect the salvaging of high-risk cells carrying forward incorrectly repaired DNA damage but rather will represent a real radioprotective effect.

Growth Factors

Radiation-induced hematopoietic and gastrointestinal syndromes are major contributors to mortality and morbidity as a result of whole-body radiation exposure. To address these radiation protection issues, considerable effort has been directed to the development of growth factors to specifically expand particular stem and progenitor cell populations to repopulate these critical normal tissue systems and thereby lead to increased survival and radioprotection (*5*). Both cytokines and hormones have been investigated using cultured cells and animal model systems, with several of these agents being shown to be effective in subsequent clinical use. Some of the more successful approaches have used G-CSF and GM-CSF, recombinant stem cell factor, IL11, megakaryocyte growth and development factor, erythropoietin and steroids such as androstenediol.

Examples from each of these four categories of radiation protectors will be presented and discussed. While each of these categories are somewhat arbitrary and represent distinct applications and differing underlying mechanisms of action, it is anticipated that an integrated approach to radiation protection will be the most effective strategy and that agents developed in each of these categories will contribute to this goal.

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Radiation Interactions with Taxanes, Old and New

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Combination therapy with two or more agents or with multiple modalities has become common practice in cancer treatment. The objective of using drugs in combination is to increase antitumor efficacy, decrease toxicity to normal tissues, and reduce or postpone development of drug resistance. Combination of chemotherapeutic drugs and radiotherapy is a particularly appealing approach to improve the results of cancer treatment. By their independent cytotoxic action, chemotherapy drugs reduce cell burden in tumors undergoing radiotherapy. Some drugs, in addition, sensitize tumor cells to radiation, which together with their independent cytotoxic action increase the probability of local tumor control. Moreover, chemotherapy drugs spatially cooperate with radiotherapy through their systemic action on metastatic disease. Taxanes meet all these requirements and thus have high potential to be effective when combined with radiotherapy. Many clinical trials have now proven that chemotherapy administered during the course of radiotherapy (concurrent treatment) is superior to radiotherapy alone in controlling locoregional disease and in improving patient survival (*1*). These improvements to therapy have, however, been gained at the expense of increased normal tissue toxicity.

Potential strategies to improve chemoradiotherapy efficacy and reduce toxicity are being actively explored. One such strategy is to combine taxanes with radiotherapy because of the ability of the drugs to arrest cells in the radiosensitive G_2/M phases of the cell cycle. Many *in vitro* studies have shown that the radiation response of cells pretreated with taxanes can be enhanced by factors ranging from 1.1 to more than 3 (*2*). *In vivo* studies have identified two major mechanisms of tumor radioenhancement: reoxygenation of radioresistant hypoxic cells and G_2/M cell cycle arrest. Both of these mechanisms are operative in tumors that respond to taxanes by mitotic arrest and apoptosis. In tumor cells that do not die by apoptosis (and necrosis) in response to taxane exposure, only the mechanism of G_2/M cell cycle arrest is operative. Additionally, specific differences in mechanisms of action have been identified for paclitaxel and docetaxel. For example, docetaxel has been shown to be toxic to cells in S phase, a phase of the cell cycle known to be resistant to the effects of ionizing radiation [reviewed in ref. (*2*)]. In preclinical studies using fractionated irradiation, it was demonstrated that therapeutic gain was diminished with each additional dose of docetaxel given during a 5-days-per-week radiation schedule (*3*). Advances are now being made in methods to improve the efficacy of taxanes while simultaneously decreasing their toxicity to normal tissues.

Conjugating drugs with polymeric carriers is one such way to improve the selective delivery of taxanes to tumors (*4*). Due to the enhanced permeability and retention effect, PG-taxol is selectively concentrated in the tumor and released over a prolonged period. PG-taxol was shown to dramatically enhance tumor radiocurability after both single-dose and fractionated irradiation by factors of the order of 7–8

and did not affect normal tissue radioresponse (*5*). Therefore, therapeutic gain was markedly better than when the parent drug was used in combination with radiation.

Overall, preclinical studies showed that taxanes, as a class of drugs, can enhance radiation sensitivity of tumor cells, potentiate tumor response, and increase the therapeutic ratio of radiotherapy. Clinical trials exploiting the information gained from preclinical studies combining taxanes and radiotherapy are ongoing. Clinical trials of taxane chemoradiotherapy include non-small cell lung cancer, cancers of the head and neck, and esophageal, gastric, pancreatic, brain and breast cancer (*1*).

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The Proteasome and Radiation

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Introduction

A cell needs to produce and degrade proteins constantly, and the level of expression of a protein is governed by the balance of these two processes. There are two major reasons why timely removal of proteins is important. One is that excess, misfolded, effete and damaged proteins are toxic to the cell. Second, the half-life of activated proteins must be kept short so as to maintain control of normal physiological cellular processes. Activation involves phosphorylation, acetylation, methylation, prenylation, glycosylation or other modifications such as addition or removal of chemical modifying groups guides the involvement of molecules in functional pathways. These modifications and primary protein structure have co-evolved with the ubiquitylation (Ub) system, which controls protein half-life and function by facilitating their recognition and destruction. By ultimately determining the rate of protein degradation, the proteasome assumes a role as ''master controller'' over responses of the cell to most signals and insults, including the challenge presented by exposure to ionizing radiation (*1, 2*).

Proteasome Structure and Function

The proteasome is a large, cylindrical, multicatalytic complex that accounts for approximately 1% of total cellular proteins. It is ubiquitous in all eukaryotes, and its components are well conserved, although they exist in several forms (*3*). All contain a common 28-subunit core that has chymotrypsin-like, trypsin-like and peptidylglutamyl-hydrolizing activities sequestered within the central core. Proteins have to be unfolded and fed into this central chamber to be degraded, and this requires the presence of a 19S regulatory complex or an 11S activator at either or both ends of the barrel-shaped core structure.

19S multi-subunit structures form ATPase-dependent 26S proteasomes that are responsible for degradation of polyubiquitinylated proteins. This involves the action of E1 and E2 enzymes to form polyubiquitin chains that are added to protein by substrate-specific E3 ligases. A protein marked for degradation by polyubiquitinylation can be rescued by the action of deubiquitinating enzymes (DUBs), but once the chain is bound to the lid of the 26S proteasome, the protein substrate is irreversibly committed to degradation. Through this pathway, the 26S proteasome post-transcriptionally regulates expression of a large number of short-lived proteins that mediate basic physiological processes such as cell cycle progression [cyclins A, D and E, TP53, CDKN1A (p21), p27, MDM2, HIF1A], DNA transcription (IKB/NFKB, MYC, JUN, FOS, AP1, STAT1), DNA repair [DNA-PKcs (PRKDC), RAD23], apoptosis (TP53, p21, MDM2, BCL2, BAX, caspase 3), inflammation and immunity (IKB/NFKB, TNFR1), and cell growth (EGFR, IGFR, PDGFR).

In contrast, addition of the 11S activator complex results in a non-ATP-dependent proteasome that has a proclivity to degrade damaged proteins, with recognition being through exposure of hydrophobic residues. In addition to existing in a constitutive form, the 20S core can exist in an IFNG inducible form known as an ''immunoproteasome''. This structure is thought to be particularly important in antigen presentation and immune responses.

Proteasomes, Stress and Irradiation

Signaling proteins involved in cell cycle arrest, DNA repair and cell death/survival are key players in radiation response—processes that are closely controlled by the Ub-proteasome system. This suggested to us that the proteasome could play the role of a sensor and coordinator of cellular response to stress, including radiation. In recent years evidence has accumulated that ionizing and UV radiation, heat shock, and oxidative stress all inhibit proteasome activity [reviewed in ref. (*2*)].

We have shown that exposure of cells to radiation doses ranging from as low as 20 cGy to as high as 20 Gy inhibited the proteasome's ability to degrade small fluorogenic peptides (*2, 4*). Inhibition was independent of dose and was only partial. Unlike proteasome-specific drug inhibitors that are able to completely abolish proteasome activity, radiation inhibited activity by 30–50%, irrespective of the dose used. Inhibition occurs very rapidly, maybe almost immediately, and activity recovers over a 24-h period. Interestingly, direct irradiation of partially purified proteasomes causes inhibition of activity with the same kinetics and radiosensitivity as that of whole cells. The 26S, but not the 20S, proteasome is affected, suggesting that sites of proteolysis are not affected, but rather the 19S ''lid''. Free radical scavengers can prevent the inhibitory effect of radiation, and there is accumulating evidence from a variety of sources that the 26S proteasome is a redox-sensing structure that responds to multiple stresses in a similar way. The consequences of proteasome inhibition are accumulation of ubiquitinylated proteins and altered levels of various signaling proteins, including NFKB. Indeed, radiation-induced proteasome inhibition may be responsible for early alterations in protein expression that signal cell cycle arrest, DNA repair, and cell death.

It is worth speculating on what appears to be a ''switch'' mechanism within the proteasome system that allows it to work at about 50% of full function after stress. The degradation rate of proteins is under complex control. Full, 100%, loss of function is lethal. To exist in any state, multiple proteins must have evolved in a coordinated fashion to allow a dynamic equilibrium to exist. The finding of 50% residual activity after irradiation presumably represents a change by the cell from ''unstressed'' to ''stressed'' stable states. This is presumably an adaptive response to environmental change that will be characterized by a characteristic alteration in protein expression profile that will involve a very large number of proteins. These findings have implications for protein expression profiling after exposure to environmental challenges in general and after radiation in particular, including after low doses.

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Radiation-Induced Gene Therapy

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Improved targeting of radiation to solid tumors and innovations including conformal radiotherapy and intensity-modulated radiation therapy have enabled more precise dose delivery to the tumor volume while limiting exposure to surrounding normal tissues. Nevertheless, the treatment of certain tumor types remains problematic. These include the many solid tumor types where local control is the predominant issue and where hypoxia is a prognostic factor predicting poor outcome (such as brain, prostate, cervix, rectum, and head and neck). Any strategy that can enhance local control of tumor growth by addressing the problem of radioresistance and/or hypoxia has great potential to improve treatment outcome. A gene therapy system that is controlled by radiation and/or hypoxia and can in turn kill or radiosensitize tumor cells is an especially promising way of increasing the efficacy of radiotherapy and thus patient survival.

Radiation-Induced Gene Therapy

For gene therapy to be optimally effective and safe, expression of the therapeutic gene would ideally be limited to within the tumor volume. Systemic gene delivery vectors that specifically target tumor cells are not yet available; thus direct intratumoral vector administration is still the primary approach. After delivery, transgene expression can be regulated at the level of gene transcription; radiotherapy can fulfill this role by regulating expression of therapeutic genes by radiation-responsive gene promoters. Since precision radiation treatment is used to treat the majority of solid tumors, this approach has inherently safe and effective tumor specificity of transgene expression built in and has the potential for widespread use in different cancer types.

The native early growth response 1 (*EGR1*) gene promoter has been adopted for the majority of the radiation-mediated cancer gene therapy studies (*1, 2*). The activation of *EGR1* is predominantly independent of TP53 and has been demonstrated in a number of tumor cell lines. This makes the *EGR1* promoter a good choice for cancer gene therapy vectors since TP53 mutations are frequently seen in malignant tumors. The *EGR1* promoter was found to be activated by reactive oxygen species produced after ionizing radiation exposure (*3*). We have constructed synthetic promoters derived from the radiation-responsive sequences (known as CArG elements) of the *EGR1* gene promoter (*4*). These synthesis promoters are free from other transcription factor binding sites present in the native *EGR1* promoter, are more radioresponsive, and exhibit lower levels of basal (i.e. nonirradiated) activity than the native *EGR1* promoter (*4, 5*). Furthermore, we have demonstrated that altering the number and core sequences of the CArG elements in synthetic promoters can significantly affect radioinducibility (*5*). These CArG promoters have been used successfully to drive expression of reporter (green fluorescent protein; GFP) and suicide gene (herpes simplex virus type 1 thymidine kinase/ganciclovir; HSVtk/GCV) systems in tumor cell cultures and animal models after clinically relevant single and fractionated radiation doses.

Combined Radiation- and Hypoxia-Induced Gene Therapy

The physiological condition of hypoxia is associated with radioresistance, poor clinical outcome, and disease progression. However, hypoxia induces the increased expression of a number of genes that can also be exploited for gene therapy. For most of these genes, the transcription factor hypoxia-inducible factor (HIF) is known to bind to hypoxia-responsive elements (HREs) within the promoter, causing subsequent gene expression. Hypoxia-induced increases in HIF levels have been noted in many tumor types. HRE-containing gene promoters have been used by several research groups, ourselves included, to drive experimental gene therapy under hypoxic conditions. Our group has produced synthetic gene promoters that can be triggered by radiation and/or hypoxia (*6*). These chimeric HRE/CArG promoters were able to control suicide gene therapy in tumor cells after irradiation, hypoxia or concurrent treatments and thus should be activated whether they are present in fully oxygenated or hypoxic regions of an irradiated solid tumor. This approach could be used to eliminate hypoxic tumor cells that often lead to poor radiotherapy outcome.

Molecular Switch Vector

To amplify and sustain levels of therapeutic gene expression induced by radiation or hypoxia, we have produced an innovative ''molecular switch'' vector system (*5*). All the components of this system have now been engineered into a single vector. The system is based on a two-step scheme whereby a condition-specific promoter, responding to radiation and/or hypoxia in our case, directly controls expression of Cre recombinase (derived from the P1 bacteriophage). This enzyme is able to recognize specific sequences (loxP sites) that have been inserted in vector DNA, causing intramolecular recombination. A unidirectional loxP site is located either side of a transcriptional 'Stop' cassette, thus flanking or ''floxing'' it. The cassette prevents expression of a downstream transgene (i.e. HSVtk) from an upstream ''secondary'' promoter. However, after irradiation or a reduction in oxygen tension, vector-transfected tumor cells are stimulated to produce Cre. This leads to excision of the Stop cassette by loxP-mediated recombination, leading to the juxtaposition of the strong, constitutive cytomegalovirus immediate early (CMV IE) gene promoter with the suicide gene and consequent high-level expression. Another advantage of this switch system is that alternative secondary promoters (e.g. condition- or tumor-specific) could be employed. This may be particularly useful where continuous and high-level expression of a suicide gene is deemed to be a safety issue, for example.

In summary, the combination of radiation- and hypoxia-inducible gene promoters and the molecular switch amplification system allows both spatial control of vector activation combined with substantial levels of therapeutic gene expression. Such innovations allow promising new strategies to improve radiation treatment outcome, particularly where tumor hypoxia is a predominant issue.

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