Molecular Tools and the Biology of Low-dose Effects

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Most environmental protection issues concern the often chronic exposure of large populations to low doses of chemical toxins and ionizing radiation. However, measuring the effects of low doses on populations exposed over long time periods is highly problematic. Politically driven opinions often tend to take the place of science. Part of the problem is that epidemiology is a weak tool when the level of exposure is low. High background levels of exposure, genetic diversity, and exposure uncertainties all contribute to "noise" and make dose-response relationships difficult to define. Uncertainty feeds anxiety, leading to polarized politics. This review looks at the promise of molecular technologies for identifying the effects of low doses of radiation and identifies some of the issues involved in defining risk after low-dose exposures. While the main pollutant discussed in this article is ionizing radiation, the analysis could apply equally well to other toxic exposures or to combined radiation and chemical pollutants.

Keywords: low-dose radiation effects, "omics" technologies, proteomics, molecular imaging, multiple stressors

The effects of environmental toxins such as ionizing radiation are clear when something dies or is born with two heads, but most other effects are subtle, requiring sophisticated statistics to detect. Often such effects are merely a higher-than-normal incidence of a common event, or evidence that a response pathway has been activated, though it is not actually harmful. The biggest challenge in low-dose radiation biology is determining the relationships between dose and effect, effect and harm, and harm and risk. At present, dose cannot simply be related to risk because we do not have adequate markers of low-dose risk, and because we tend to equate "effect" with "risk." Most effects, however, are due to the normal response of the body or ecosystem to change, and using an effect to claim harm or risk leads to confusion and unnecessary alarm. This is not to dismiss concerns about the effects of low doses of radiation or chemicals. Rather, it is to point out the need for caution in interpreting data and to stimulate research to identify useful markers that can address the relationships between dose and effect and between harm and risk.

Why extrapolations from high-dose effects don't work

Most environmental protection legislation is based on models or data sets requiring that data on high doses be extrapolated to low doses to derive predictions of potential health effects. This is particularly true in the radiation protection field, where regulation is based on the extrapolation of cancer incidence rates after the atomic bombing of Hiroshima and Nagasaki to arrive at predicted cancer incidences for populations exposed to very low or chronic doses of radiation (Brenner and Sachs 2006, Prise 2006, Tubiana et al. 2006).

This stochastic approach—the so-called collective dose concept (Cardis 2007, Vrijheid et al. 2007)—predicts that there is no safe dose of radiation, and that in a large population exposed to a very low dose, a certain number of people will contract cancer no matter how the dose is distributed. A recent example of this idea is the report that CAT (computed axial tomography) scans will account for 2% of cancers in North America (Brenner and Hall 2007). Such linear risk modeling is also used in chemical protection, but the concept of a no-effect level and the idea that there is a threshold at which a response changes abruptly from tolerable to toxic also hold sway (Bréchignac 2003, Holm 2004, Smith 2005).

Nonetheless, the problem is that this approach denies the possibility of beneficial effects—all effects are either neutral or harmful, and protection is driven to eliminate the pollutant whatever the cost. For example, the International Commission on Radiological Protection summarizes the radiation-induced effects in nonhuman species as early mortality, reduced reproductive success, and detectable DNA damage (Holm 2004). None of these categories recognizes the concept that the same radiation dose that results in genomic instability, which leads to tumorogenesis, may in a different cellular context also promote the beneficial removal of damaged cells (Feinendegen et al. 2007).

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The reality is that organisms show a great ability to adapt, as demonstrated by many instances of induced resistance, whereby exposure to low doses of toxins leads to resistance when high doses are encountered (Mothersill et al. 2005, Feinendegen et al. 2007, Matsumoto et al. 2007); for example, in the presence of low-dose radiation, levels of reactive oxygen species (ROS, DNA-damaging free radicals) are low (Feinendegen et al. 2007). In the 16th century, Paracelsus (figure 1) made the point that the dose makes the poison. That is to say, substances considered toxic can be benign or beneficial in small doses, and, conversely, an ordinarily benign substance can be deadly if overconsumed.

***Figure 1. Paracelsus's grave in Salzberg, Austria. Paracelsus, sometimes called the father of toxicology, wrote* "Alle Ding sind Gift, und nichts ohn Gift; allein die Dosis macht, daß ein Ding kein Gift ist" [*All things are poison and nothing is without poison, only the dose permits something not to be poisonous*].**

The challenge is to develop reliable markers of the dose at which the poison is made. Also, there is great confusion between the effect, toxic or otherwise, of a dose and the biological response to that effect. One is likely to be harmful (e.g., membrane leakiness due to a biological stress; Harper et al. 2004, Araki et al. 2005), but the other (e.g., ion channel fluxes aimed at initiating kinase pathways; Rosen and Cohen 2006, Abele et al. 2007) is a reflection of the cell's ability to stabilize the membrane or redress the harm. Both may be measured as effects of the dose.

The promise of "omics" in the field of low-dose effects

The "omics" technologies—genomics, proteomics, and metabolomics—would seem to have considerable potential to be ideal tools for resolving low-dose effects at the molecular level. Moreover, these technologies not only enable low-dose effects to be studied in detail but also conform to the following definition of biomarker: "any biochemical, histological and/or physiological alterations or manifestations of stress" (Holdway et al. 1995). Thus molecular biology is likely to continue to provide powerful techniques for the analysis of DNA and transcription and translation products.

Genome sequencing in particular has yielded a wealth of information on predicted gene products. Although this is not yet fully reflected in protein analysis, progress is made almost daily with newer proteomics techniques such as MALDI (matrix-assisted laser desorption/ionization) time of flight (figure 2) and SELDI (surface-enhanced laser desorption/ionization) time of flight mass spectrometry (MS). Both techniques are similar inasmuch as fragmented and ionized proteins are sorted according to the mass-to-charge ratio. With MALDI MS, the protein sample is mixed with a matrix and then allowed to dry and adhere to the target surface. Surface-enhanced laser desorption/ionization MS is a modification of MALDI, but the target surface itself is modified to promote the selective binding of certain proteins; in other words, protein binding acts as a separation step. Both methods can be combined with two-dimensional gel electrophoresis (figure 3) to identify proteomic changes implicated in low-dose effects, with the aim of defining mechanisms involved (Chaudhry 2006, Ménard et al. 2006, Schrock et al. 2006, Albrethsen 2007).

Investigations involving these techniques could be particularly important in understanding the biochemical mechanism of cancer treatment by profiling the proteome before and after radiotherapy, and appear to be applicable to a variety of tumors (Ménard et al. 2006). These techniques also provide potentially valuable biological indicators of radiation exposure. Stress response and death pathways in particular show very sensitive responses, and the added value is that the response can often be linked to the genotype, and thus the issue of genetic predisposition can be addressed (e.g., using defined mouse or other model organism strains or knockouts with specific genetic differences; Colucci et al. 1997, Mothersill et al. 1999, 2005, Gilmore et al. 2003, Lindsay et al. 2007). This is especially true if genomic analysis incorporates both global

gene expression profiles and the more subtle supervised classification of small groups of genes, as has been done to assess the incidence of papillary thyroid cancer arising from the Chernobyl explosion (Detours et al. 2007).

Applications of gene arrays include messenger RNA (mRNA) or gene expression profiling, in which expression levels for thousands of genes are measured simultaneously to study the effects of pollutants on gene expression. For example, microarray-based gene-expression profiling can be used to identify disease genes by comparing gene expression in diseased and normal cells, as was shown by Detours and colleagues (2007) and also by Lin and colleagues (2007). Comparative genomic hybridization—that is, assessing genome content in different cells or closely related organisms—is also commonly done using gene arrays (reviewed by Hahn et al. 2000) and can be applied to the diagnosis of cancers as diverse as thyroid (Finn et al. 2004) and breast (Varma et al. 2005) induced by low-dose radiation. This approach can be used to determine the relative sensitivity of different populations or species to pollutants by identifying genetic closeness within and between related species or races of specific organisms. The relative sensitivity can then be correlated with the genetic changes occurring in different population groups.

Detection arrays employing sodium nitroprusside, a nitric oxide donor, identify single nucleotide polymorphisms among alleles within or between populations (Mosquera et al. 2005). This is another popular application of particular value in low-dose exposure studies, especially when the action of ROS may be contributing significantly to the overall effect. The technique enables researchers to look at genomic instability induction, and it is most often used to investigate accelerated genetic drift in the progeny of exposed organisms (Gu CC and Rao 2003, Polanska and Kimmel 2005). Chromatin immunoprecipitation studies, which determine protein binding site occupancy throughout the genome, are a useful bridge linking proteomics and genomics approaches to the low-dose issue because they enable the genomic and proteomic differences between samples, individuals, or test versus a control to be evaluated as "defects" or as potentially responsive "effects" (Gu MB et al. 2004). Meta-analysis approaches are then applied to try to make sense of the data mountain.

Proteomics is an important new field in the study of protein properties (expression levels, interactions, posttranslational modifications, etc.). Extensive and variable posttranslational modification generally means that more proteins can be expressed than indicated by the genome (Pandey and Mann 2000). Thus, because proteomics goes beyond simply visualizing the genome, in theory it could offer more potential than mRNA as a biomarker of radiation exposure—indeed, mRNA and protein expression are poorly related (Anderson and Seilhamer 1997, Gygi et al. 1999). Proteomics deals with actual expres-

Figure 2. Micromass matrix-assisted laser desorption/ionization mass spectrometer.

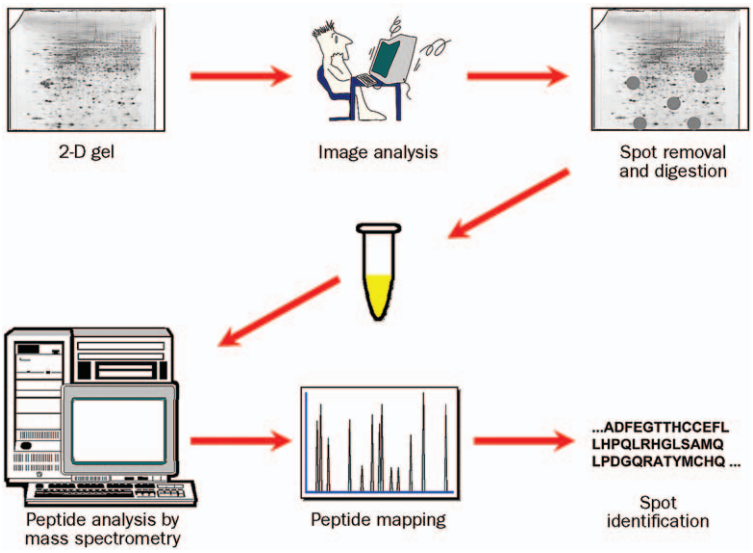

Figure 3. Determining proteomic changes by two-dimensional gel electrophoresis, trypsin digestion, and peptide analysis.

sion, which confirms the overall integrity (or otherwise) of the underlying genes, whereas genomic analysis does not indicate whether the gene is ultimately translated.

However, there is little doubt that studies involving parallel genomic and proteomic aspects could be even more powerful than proteomic or genomic investigations alone, particularly for understanding the precise mechanism behind molecular responses to low-dose radiation. This technology has been used recently for looking at low-dose radiation effects, with some success (An and Seong 2006, Orre et al. 2007, Smith et al. 2007, Mezhoud et al. 2008). Certainly, proteomic analysis of the rainbow trout gill appears to be able to separate the effects of direct radiation from those of the radiation-induced bystander effect (figure 4), and also provides additional evidence that the bystander signal may include ROS (Smith et al. 2007). Moreover, the emerging picture—although it must be conceded that only two studies have thus far been carried out—points to a degree of similarity in the protective nature of the proteomic response to the bystander effect in both mammalian and fish species (Gerashchenko et al. 2007, Smith et al. 2007).

How this technology should be used is another question. In terms of biomarker application, there is little doubt that whole animal or human studies are much more likely to yield data that can be used either to directly monitor at-risk individuals or to legislate exposure in a more general sense, particularly if proteomics, for example, is applied to blood serum (Ménard et al. 2006). This application has the distinct advantage of being able to offer the possibility for repeat and nonterminal analysis. In a mechanistic application, cultured cells have the obvious attraction of being able to isolate the effects of a radiation dose precisely at the cellular level (e.g., Finn et al. 2004). However, even here one must be aware of the hierarchical nature of radiological responses, which makes extrapolation from the cellular to the whole-organism level important (Feinendegan et al. 2007) and challenging (Begent 2007).

The bottom line with these genomics and proteomics studies, however, is that lots of genes are up-regulated, others are down-regulated, and others are induced *de novo*. Cytokine, stress, and apoptosis pathways are often affected (or often studied). Genetic background appears to be the dominant factor determining low-dose response and final outcome, although the distinction between damage caused by the toxin and response to that damage is difficult to resolve without studies aimed at detecting actual mutations in key genes known to be important in the process being investigated. Thus a chicken-and-egg situation often exists.

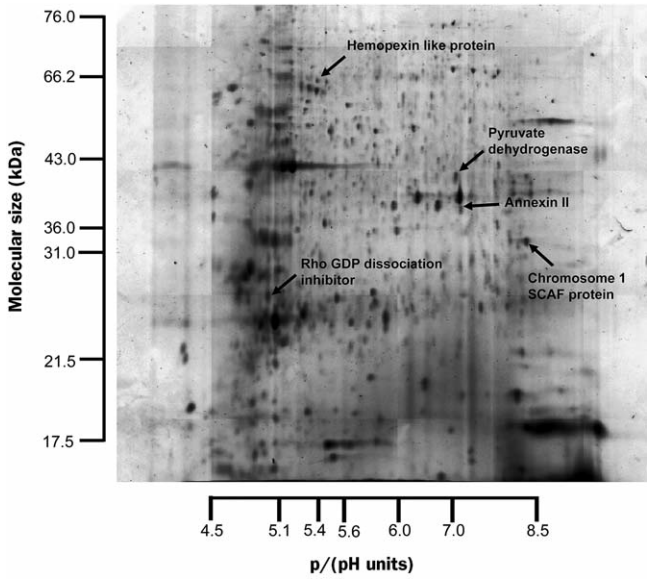

Figure 4. Protein profile of a rainbow trout gill, illustrating the two-dimensional gel location of annexin II, the tumorogenic protein up-regulated by direct exposure to 0.5 Gy (gray) X-radiation, and the protective proteins up-regulated by an X-ray-induced bystander effect (Smith et al. 2007). Abbreviations: SCAF, SR-like CTD-associated factor (SR, serine-arginine rich; CTD, carboxyl-terminal domain).

Cellular imaging techniques

Most of the above techniques rely on producing a gel or a blot, which is then further extracted to identify the products. This means that the actual cells are destroyed and much of the dynamic information is lost, as is the spatial relationship of cells in the tissue. But the reality is that cells usually function in communities, and the tissue or organism has overall control of function. The death of a cell therefore needs to be seen in the context of what is happening in the tissue or organism as a whole. One dead cell is not a problem. Figure 5 shows an image of a urothelial explant outgrowth showing the differentiation achievable in culture. We are only beginning to develop techniques capable of looking at functional activity in the context of individual cells in a tissue. Such information

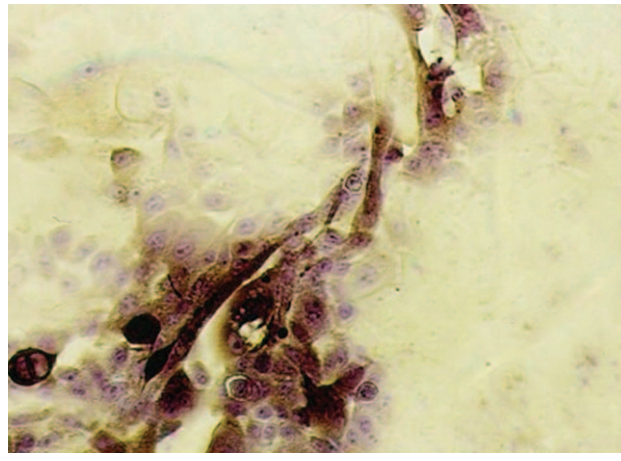

Figure 5. A culture of normal human urothelium derived from an explant. The lightly stained areas are covered with hyaluronic acid mucopolysaccharide produced by differentiated urothelium. Darker (growing) areas are stained for cytokeratin to show that the cells are epithelial.

is highly important following low-dose exposures because at these doses, the level of damage even to the cell is unlikely to be lethal. The outcome will be determined by metabolic parameters such as energy sufficiency, repair, ion-channel stability, cellular fitness, and cell activity (cycling or metabolizing) at the time of the exposure. Decisions on what response to mount are likely to be taken at the higher hierarchical levels of tissue or organism rather than at the individual cell level.

The growing field of metabolomics attempts to detect and quantify the low-molecular-weight molecules, known as metabolites, produced by active, living cells under different conditions and times in their life cycles. Nuclear magnetic resonance (NMR) is playing an important role in metabolomics because of its ability to observe mixtures of small molecules in living cells or in cell extracts (Lukas et al. 2005, Thorn and Mehra 2006, Barrett et al. 2007). However, it is not yet possible to use NMR to observe spatial interactions in intact tissues. A fusion of confocal microscopy and NMR technologies is needed to enable progress to this level. The goals of metabolomics are to catalog the small molecules concerned with function in the cell and to quantify them. The related field of metabonomics is the study of how the metabolic profile of a complex biological system changes in response to stresses such as disease, toxic exposure, or dietary change. This field is now at the cutting edge of attempts to understand low-dose responses.

Apart from NMR, confocal microscopy and live-cell fluorescence imaging techniques are the most valuable in this field. Advances in microscopy have made it possible to follow complex reactions in living cells. Two-photon optics, green fluorescent protein, photoelectronic detectors, and image deconvolution are also powerful recent additions to the arsenal of tools that allow visualization of processes occurring in living cells, and most important, processes in the context of cells within a tissue (Yamamoto and Shinohara 2002). Some examples of the application of these techniques in the low-dose exposure field include visualization of ion fluxes, metabolic reactions, membrane depolarizations, and receptor-ligand binding *in vivo* (Daly and McGrath 2003, Golden and O'Connell 2007, Müller-Taubenberger and Anderson 2007). The future promises exciting new developments, such as visualizing single molecules; monitoring, following, and modulating molecular interactions; mining information in images; and imaging complex tissues.

Pitfalls and false interpretations, Schrödinger's cat problems

The new techniques described above are of great power in the study of disease processes, where frank changes in tissues have occurred and where normal tissue samples are available for comparative purposes. All omics studies rely on comparison of normal patterns against altered patterns, preferably from the same patient or mouse strain, or at least from a large bank of normal or diseased tissues. The question posed in this review is not whether the techniques are useful but whether they can address the specific issue of low-dose exposure risks or assign causation of a cancer or birth defect, for example, to a particular toxin experienced at low doses. Several issues confound the answers.

The lack of validated markers—can omics help? Clearly, genomics and proteomics are all about identifying markers of change in tissues. So the answer should be yes, but the complexity and interrelationships between genes and proteins make it unlikely that we will be able to say whether a change at the genetic level or at the level of the proteome is affecting the risk of radiation causing a harmful effect. Validation of the usefulness of specific biomarkers is a major challenge, but it is really the only way forward. Such validation will most likely involve carefully controlled and interpreted animal work with more than one animal model.

How can omics help define which of the myriad effects are actually gatekeepers? This is the crux of the matter. It is very easy with modern molecular tools to identify *change* in response to a stressor. These changes can be quantified and statistically associated with the stressor exposure, and in most cases the metabolic pathways can be defined as well. The question is, Where does that get you? Some gatekeepers have been identified, such as p53 or voltage-gated sodium channels, at which mutations or posttranslational perturbations are very clearly associated with cancers. But coming from the regulatory end, it is essential to identify the markers in normal tissue that predict risk and not late-occurring mutations or misfunctions that occur along the road to cancer. In the case of noncancer diseases or risks of environmental harm in other species, gatekeepers predictive of low-dose exposure risk are largely unknown.

Multiple stressor or mixed-contaminant issue. A further complicating issue is that pollutants do not occur as single contaminants, and most environments on the planet now contain a cocktail of foreign substances, all of which are present at low doses. This makes it almost impossible to ascribe causation to any one agent in the mix. The great hope for omics technologies is that they will allow us to identify molecular signatures for different contaminants. Although this cannot determine whether the contaminant caused the harm, it will at least confirm whether it was "seen" by the tissue.

Are before-and-after tissue samples or a single genotype needed to verify and assess change? Most experiments with low doses of radiation or chemicals look at cells or animal tissues before and after experimental exposures. Very sophisticated live-cell imaging techniques that use NMR or confocal microscopy are leading to huge advances in this field. However, when trying to use these techniques to assess risk or even harm in humans, controls become a problem. Unless pre-exposure measurements are available, the variation in genotypes in the human population makes the signal-to-noise ratio too problematic when dealing with exposures to low

doses. However, the animal and cell models can reveal mechanisms and candidate genes, proteins, and pathways that could be important. It is likely that in the future, noninvasive live-imaging techniques will revolutionize the low-dose exposure field in this area and permit monitoring of molecular signatures in human populations.

Stress is the most common low-dose effect measured—but is it damage or response to damage, and does it matter? This is almost a philosophical point, but one that holds great hope for solutions from proteomics and metabolomics. The issue is that at low doses of stressors, stress responses get turned on, but what does that mean for risk assessment? Is a response necessarily a risk, or is it merely an indication that all is working well and the system is self-correcting any problems? Great care must be taken to distinguish damage from response to damage. Metabolomic techniques that look at energy budgets, ion-channel competency, or endoplasmic reticular stress are most likely to be useful, but, again, a systems biology approach is needed to model the interactions in an attempt to understand how complex interactions ultimately resolve. Rather than measure dose-response relationships, maybe we need to measure dose-response *failure* relationships as an end point.

Do the dyes used in imaging chemical "pollutants" cause a Schrödinger's cat paradox? Uncomfortable evidence in the literature suggests that ultraviolet fluorescence imaging, which is commonly used in low-dose radiation or chemical experiments using microbeams or confocal microscopy, can actually induce stress effects. In other words, interrogating the system to visualize what is happening may actually mask or exacerbate the measured effects (Fournier et al. 2007). Proponents of these techniques say that careful use of sham controls eliminates this problem, but this is not the case if the technique masks an effect, giving a positive result in both sham and actually treated samples. Also, if the imaging system and the pollutant produce a mixed-contaminant synergistic effect, that effect will not be detected as such. One solution to this problem is to exclude any up-regulations of proteins that occur in shams as well, or to include only those resulting exclusively from actual exposure, but in doing so, data can be lost.

Unanswered needs and the way forward

Given all these caveats, what does omics need to produce to remedy the situation? Probably the most useful thing would be a range of biomarkers operating at different hierarchical levels, which can predict low-dose effects in model systems that are positive for a desired disease end point. The important thing is to have a suite of markers and data for a range of susceptibility genotypes and phenotypes so that systems biology approaches can be applied. Huge advances in noninvasive live-cell imaging are forecast for the next 10 to 15 years. These techniques are unlikely to provide markers, but they will be extremely useful for evaluating potential biomarkers. Parallel advances in modeling will hopefully provide the means to interpret the data.

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