Modulated Gap Junctional Intercellular Communication as a Biomarker of PAH Epigenetic Toxicity: Structure–Function Relationship

Brad L. Upham, Liliane M. Weis, and James E. Trosko
Department of Pediatrics and Human Development, Michigan State University, East Lansing, Michigan

Cancer is a multistage multimechanism process involving gene and/or chromosomal mutations (genotoxic events), altered gene expression at the transcriptional, translational, and post-translational levels (epigenetic events), and altered cell survival (proliferation and apoptosis or necrosis), resulting in an imbalance of the organism's homeostasis. Maintenance of the organism's homeostasis depends on the intricate coordination of genetic and metabolic events between cells via extracellular and intercellular communication mechanisms. The release of a quiescent cell, whether normal or preneoplastic, from the suppressing effects of communicating neighbors requires the downregulation of intercellular communication via gap junctions, thereby allowing factors that control intracellular events to exceed a critical mass necessary for the cell to either proliferate or undergo apoptosis. Therefore, determining the role an environmental pollutant must play in the multistage carcinogenic process includes mechanisms of epigenetic toxicity such as the effects of a compound on gap junctional intercellular communication (GJIC). A classic example of a class of compounds in which determination of carcinogenicity focused on genotoxic events and ignored epigenetic events is polycyclic aromatic hydrocarbons (PAHs). The study of structure–activity relationships of PAHs has focused exclusively on the genotoxic and tumorigen- cinitating properties of the compound. We report on the structure–activity relationships of two- to four-ringed PAHs on GJIC in a rat liver epithelial cell line. PAHs containing a bay or baylike region were more potent inhibitors of GJIC than the linear PAHs that do not contain these regions. These are some of the first studies to determine the epigenetic toxicity of PAHs at the epigenetic level.

— Environ Health Perspect 106(4):975–981 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl4/975-981upham/abstract.html

Key words: polycyclic aromatic hydrocarbons, gap junctional intercellular communication, epigenetic toxicity, tumor promotion, tumor initiation

Introduction

Importance of Understanding the Mechanisms of Toxicities in Risk Assessment after Human Exposure to Chemicals

The fundamental challenge to risk assessors exists in understanding if exposure to a given chemical or a mixture of chemicals will lead to acute lethality of an individual or to the long-term development of a number of chronic diseases such as birth defects, cancer, diabetes, reproductive dysfunction, neurologic disorders, or hereditary diseases. What is needed is a mechanistic understanding of how the chemical, by interacting with complex genetic and biologic processes and developmental and sex-related factors, contributes to multistep processes involved in the initiation and progression of these diseases. Epidemiologic associations can give clues but cannot determine the underlying mechanisms by which the chemical might have been linked to the disease end point. Experimental animal studies, for a wide variety of reasons, have additional limitations and they also cannot provide underlying mechanisms by which a given chemical might or might not lead to human diseases. Molecular, biologic, and cellular studies, which can provide potential mechanistic insights as to how chemicals might be toxic, also suffer from limitations of all sorts (1).

To put chemicals in animals and check to see if disease can be induced, to check humans known to be exposed to a given chemical for the appearance of disease, and to measure DNA lesions, mutations, and cell death in vitro will not be sufficient to link a chemical as causing a disease via this or that mechanism. The reason is fundamental; all observations, epidemiologic or experimental bioassays, or in vitro assays are interpreted within accepted paradigms. The design and interpretation of the experiments and results presume some understanding of the disease processes and the techniques used to determine mechanisms. Although the field of toxicology existed long before Carson's Silent Spring (2) and societies have been concerned about exposure to certain chemicals since the dawn of time, heightened concern in recent decades has caused a rush to test chemicals already in use and known or suspected of contributing to human disease and to test chemicals in development. Understandably, testing protocols, guidelines for use and exposure, and decision-making interpretations for evaluation of the results must involve state-of-the-art information in our understanding of the causes of disease and the meaning of various test results.

Although this analysis must be limited to one disease, namely cancer, the implications probably hold true for most, if not all, human diseases. A major problem in the task of identifying the risk of cancer induction after exposure to a given toxicant is understanding how cancer is produced in a human being and how a toxicant might contribute to that process. One can get some idea of the origin of some of our present-day problems when encountering the word carcinogen—the very word we use to identify a toxicant known or suspected of contributing to a cancer.

Given that our current understanding of the carcinogenic process is of a multistage multimechanism process involving gene and/or chromosomal mutations...
(genotoxic events), altered gene expression at the transcriptional, translational, and post-translational levels (epigenetic events), and altered cell survival (necrosis or apoptosis), it should be clear that the word carcinogen would imply that a toxicant could bring about each and every mechanism in the multistep process of carcinogenesis. Even more succinctly, when the paradigm “carcinogen as mutagen” was created (3), it paralyzed the thinking of far too many basic scientists, industrial scientists, epidemiologists, and policy regulators into believing that one thing could cause cancer, that the one thing had to be a mutagen, and that assays to detect mutagens, when positive, actually did detect mutagens (4).

Unfortunately, this whole approach was incorrect. Carcinogenesis is not just mutagenesis (5). Therefore, to reject, modify, or refine our fundamental understanding of carcinogenesis, we must create a new paradigm. Currently, new knowledge of the genetic bases of various cancers (i.e., oncopoiesis, tumor suppressor genes), the roles of mutations, epigenetic mechanisms, and cell death (necrosis or apoptosis) during the carcinogenic process, the limitations of all bioassays and in vitro assays to detect genotoxicants, and the distinctive differences of the initiation, promotion, and progression steps of the carcinogenic process force us to generate a new paradigm.

Cancer as a Disease of Homeostasis

The human being is a collection of about 100 trillion cells, consisting of stem cells, committed progenitor cells, and terminally differentiated cells. These cells are all communicating with each other via extracellular, intracellular, and intercellular communication mechanisms (6). This integrated complex of communicating mechanisms regulates the major options of cell behavior, namely their ability to proliferate, differentiate, display adaptive responses if they are differentiated, and undergo apoptosis (Figure 1). In effect, homeostasis is the end result of these three major communicating mechanisms interacting with each other. From the onset of fertilization of the egg by the sperm, through growth and development of the embryo and fetus, to the expression of specific genes in the neonate, adolescent, and aging adult, health of the individual depends on this delicate coordination of the regulation of cell proliferation for growth and tissue repair and regeneration, for proper differentiation of cells at appropriate times, for adaptive responses of cells in tissues when exposed to endogenous or exogenous extracellular communicating signals, and for the timely regulation of apoptosis or programmed cell death.

Hormones, growth factors, and neurotransmitters produced by specific cells traverse over a distance to cells having specific receptors to bind to these extracellular signals. These cells that receive the extracellular signals transduce the molecular information into changes of various second messages that trigger a finite number of distinct signal-transconducting intracellular communicating mechanisms. In turn, these intracellular communicating mechanisms perform two major functions: they modulate gap junctional intercellular communication (GJIC) between contiguous cells and epigenetically alter the expression of genes or gene products of the cell. By means of the interlocking sequence of communicating mechanisms, a quiescent cell that is contact inhibited can escape the suppressing effect of communicating neighbors by having gap junctional communication blocked and thereby allowing the quiescent cell to accumulate the necessary factors needed to exceed a critical mass for the cell to divide. On the other hand, extracellular communicating signals such as hormones could modulate intracellular communication within certain cells to increase GJIC to allow differentiation (7), adaptive responses such as contraction of tissues (8,9) or modulation of endocrine function, (10,11) or apoptosis (12) to occur.

Cancer cells have long been characterized as cells with the inability to regulate their growth [loss of contact inhibition (13)] and the inability to terminally differentiate [oncogeny as partially blocked ontogeny (14)]. Cancer as a disease of differentiation or as a stem cell disease (15) suggests that during the carcinogenic process these cells lost their ability for homeostatic control. This implies a breakdown in one of the three communicating mechanisms. Experimental evidence seems consistent with the hypothesis that cancer is a breakdown in cell–cell communication (16). Most, if not all, cancer cells have defective homologous and/or heterologous GJIC (17). Chemical tumor promoters inhibit GJIC, whereas chemical antipromoters enhance GJIC (18). Oncogenes downregulate GJIC, whereas tumor suppressor genes upregulate GJIC (16). Antisense gap junction genes in normal cells render them cancerlike (19,20), whereas sense gap junction genes transfected into cancer cells restore their normal growth regulation (16).

Carcinogenesis as a Multistage Multimechanism Process Involves Both Mutagenic and Epigenetic Events

The observations that carcinogenesis consists of distinct operational phases during the evolution of a normal growth-controlled cell to an invasive and metastatic neoplastic growth disregulated and

Figure 1. Gap junctions in cellular homeostasis. This diagram illustrates the integration of intercellular, intracellular, and gap junctional intercellular communication mechanisms in the homeostatic control of the four primary functions of cells in a multicellular organism, namely, cell proliferation, cell differentiation, adaptive responses of differentiated cells, and programmed cell death or apoptosis. Cell adhesion and cell–matrix interactions are classified as subclasses of intercellular communication.
nonterminally differentiated cell led to the concepts of initiation, promotion, and progression (21). The concepts were derived in vivo after it was observed that subcarcino- genic doses of a carcinogen induced an irreversible event in a cell of the mice skin (initiation), which could be manifested as a visible papilloma, after repeated chronic exposure to a noncarcinogenic compound such as phorbol ester (promotion) (22,23). Further irreversible changes occurring in these initiated cells that have been clonally amplified by the promotion process then leads to a malignant tumor cell (24).

Although the underlying mechanisms for these distinct phases of carcinogenesis are not yet known, it has been hypothesized that because initiation is an irreversible event, mutagenesis could be the mechanism by which a normal stem cell could be irreversibly altered to prevent terminal differentiation (21). Furthermore, when these single initiated stem cells are exposed to agents or conditions that stimulate them to proliferate (promotion), the mechanism that brings about this clonal expansion of initiated stem cells that cannot terminally differentiate would be a mitogenic or epigenetic mechanism. Because the promotion phase of carcinogenesis can be interrupted, if not reversed, when exposure to the promoting condition or agent is stopped, it is clear that mutagenesis cannot explain this phase of carcinogenesis. Moreover, chemical promoting agents such as phorbol esters, phenobarbital, saccharin, hormones, polybrominated biphenyls, peroxisome proliferators, and wound healing are not mutagenic. These promoting agents and conditions all reversibly modulate GJIC (25).

Chemicals as Carcinogens or Carcinogens as Mutagens

Chemicals are labeled carcinogens when a population of humans with known exposure to a chemical has a greater frequency of tumors than those who were not exposed or when a tumor appears in an animal that has been given a chemical. Until now, this carcinogen was labeled as a mutagen for a number of extrapolated reasons, such as the cancer cell had mutations in various genes or the chemical induced a positive effect in an in vitro assay designed to detect mutations. This led to the paradigm "carcinogen as mutagen" (1).

However, mutagenicity cannot always be equated with carcinogenicity. This is particularly evident from the study of Ashby and Tennant (26) in which 301 chemicals were assessed for mutagenicity and carcinogenicity. Although this study indicated that structurally alerting/muta- genic agents had a high probability of being carcinogenic (84% mutagens were carcinogens), this correlative advantage was offset by an unacceptable high incidence of false-positive predictions (66% noncar- cinogens were mutagens) (26). Also, many nonmutagenic/nonalerting chemicals were carcinogenic (26). This was especially true for male rat kidney carcinogens, rodent leukemogens and thyroid carcinogens, and mouse liver carcinogens (26).

This unfortunately led to the negative term nongenotoxic carcinogens. The term does not indicate the underlying mechanisms of carcinogenicity but refers only to what the compound cannot do, i.e., the compound cannot cause a permanent genetic mutation. To the contrary, the term epigenetic toxicity implies a mechanism of reversibly altering the genetic phenotype of a cell through biochemical pathways that ultimately turn genes on and off via intracellular pathways, a process under the homeostatic control of neighboring cells via intercellular communication through gap junctions (15,16). Because intracellular pathways often converge onto intercellular events (15), the development of in vitro assay systems that measure GJIC has been successfully used to detect many epigenetic carcinogens (27,28). Rosenkranz et al. (28) used a structure–activity relationship model that utilized a multivariate algorithm correlating 11 different biophores of 251 chemicals with GJIC activity to show a high concor- dence between experimental and predicted results (74%).

PAHs as a Test of the Hypothesis That Carcinogens Are Mutagens

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds strongly sus- pected of being carcinogenic to humans (29). The potential human carcinogenicity of PAHs is based on many years of observation in animal and in vitro bioassays, epidemiology, and occupational medicine (29). Actually, the earliest association of an envi- ronmental agent linked to human cancer was made by Pott (30), who reported that prolonged contact of coal soot with skin resulted in increased scrotal cancer in chimney sweeps. The cancer-causing agent of coal soot was attributed to PAHs containing a dibenz[a]anthracene nucleus (31). Subsequent to this discovery, Kennaway and Hieger (32) directly tested the carcinogenic activity of dibenz[a,h]anthracene, resulting in the first demonstration of the carcinogenicity of a chemical with a defined structure. Since these discoveries were made a large-scale program to define structural features of chemical compounds necessary for carcinogenic activity was begun, in which a large number of compounds syn- thesized and screened for carcinogenicity were PAHs and heterocyclics (33). The car- cinogenic studies of structure–activity relationships of PAHs reported in the literature focused almost exclusively on either the complete carcinogenicity or tumor-initiating activity of PAHs (33,34). However, reports in the literature of the tumor-promoting properties of PAHs are sparse. This lack of data on structure–activity relationships of PAHs pertaining to tumor promotion is unfortunate considering that human risk from a carcinogen does not rest solely on the initiating properties of a chemical, particularly because many suspected car- cinogens are not genotoxic (35) and some genotoxic agents are noncarcinogenic and lack initiating activity (12).

For example, fluoranthene intraperitoneally injected into mouse neonates showed increased lung and liver tumors in a dose-dependent manner (35,36), but did not show any evidence of tumor-initiating activity in experimental animals (29) or in in vivo genotoxicity, as assessed using the mouse bone marrow micronucleus and rat liver unscheduled DNA synthesis assays (37). The tumor-promoting activity of fluo- ranthene has not been determined; however, fluoranthene downregulates GJIC in a rat liver epithelial cell line (38), which suggests that fluoranthene may be a tumor promoter because of its epigenetic properties.

Understanding the molecular basis of the carcinogenicity of PAHs must include a study of nongenotoxic series of epige- netic events such as those that affect GJIC, for reasons outlined in our discussion of GJIC biology.

Some structure–activity relationships of PAHs and GJIC have been reported by Upham et al. (39), who showed that monomethyl isomers of anthracene that possessed a baylike region (Figure 2) were more inhibitory than the parent compound or the monomethyl isomer containing no baylike region. A PAH containing a bay region, such as phenanthrene (Figure 2), was also more inhibitory than anthracene (38). To fur- ther explore the possibility that PAHs contain- ing bay or baylike regions are more inhibitory to GJIC, we compared the effects of naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, anthracene,
1-methylanthracene, 2-methylanthracene, 9-methylanthracene, phenanthrene, fluorene, 1-methylfluorene, and fluoranthen on GJIC in rat liver epithelial cells. The basic structures are shown in Figure 2.

**Results and Discussion**

Methylnaphthalene, which contains a baylike region, inhibited GJIC at a lower dose than the linear naphthalenes (Table 1). The increased inhibitory effect of PAHs containing bay or baylike regions was even more pronounced with the three-ring PAHs (Table 2). The linear PAHs did not inhibit GJIC even at the highest doses tested, but the PAHs containing either a bay or baylike region inhibited GJIC at a dose of 50 μM (Table 2). There was very little difference in the potency of PAHs containing multiple baylike regions (i.e., 9-methylanthracene and 9,10-dimethylanthracene) versus a PAH containing a single baylike region (1-methylanthracene) (Table 2).

PAHs containing bay regions such as phenanthrene and fluoranthen were equally as inhibitory to their counterparts containing baylike regions, such as 1-methylanthracene and 1-methylfluorene (Tables 2 and 3). However, fluorene, which contains no bay or baylike region, did inhibit GJIC but at a higher dose than fluoranthen (Table 3). Apparently the pentyl ring, in addition to bay regions, also increased the toxic effects of a PAH on GJIC.

Inhibition of GJIC by all of the PAHs tested was also a reversible process. Complete recovery occurred within 2 hr and 50% recovery occurred within 1 hr after replacing the PAH-containing medium with that of fresh growth medium. These results are consistent with the reversible nature of tumor promotion in vivo (16). Inhibition occurred in a short time period (<5 min) for all of the PAHs (Table 4), indicating that the gap junction proteins are being modified at the post-translational level. The more water-soluble GJIC-inhibiting PAHs, which have lower log octanol/water partition coefficients (log \( K_{ow} \)), downregulated GJIC in a shorter time period than the less water-soluble PAHs (Table 4). This relationship of log \( K_{ow} \) versus inhibition of a PAH was linear with an \( r^2 \) of 0.69 and could indicate that the cell receptor for the PAHs is more accessible in an aqueous environment.

The tumor-promoting activity of the PAHs used in our study has not been determined. However, the cocarcinogenic effect of benz[a]anthracene, phenanthrene, fluoranthen, and 3-methylcholanthrene, all of which possess bay or baylike regions,

### Table 1. Effect of naphthalene-type polycyclic aromatic hydrocarbons on gap junctional intercellular communication.

| PAH                | Structural determinant | Concentration, μM at 90% inhibition |
|--------------------|------------------------|-------------------------------------|
| Naphthalene        | Linear                 | >350 \(^b\)                          |
| 2-Methylnaphthalene| Linear                 | 350                                  |
| 1-Methylnaphthalene| Baylike                | 225                                  |

*GJIC was measured by the scrape-load/dye transfer technique according to the method of Upham et al. (64) using WB rat liver epithelial cells obtained from J.W. Grisham and M.S. Tsao (University of North Carolina, Chapel Hill, NC). The incubation time of the indicated chemical with the cells was 10 min. At the maximum soluble concentration of 350 μM, naphthalene inhibited GJIC approximately 50%.

### Table 2. Effect of the three-benzene-ringed polycyclic aromatic hydrocarbons.

| PAH                   | Structural determinant | Concentration, μM at 90% inhibition |
|-----------------------|------------------------|-------------------------------------|
| Anthracene            | Linear                 | >350 \(^b\)                          |
| 2-Methylnaphthalene   | Linear                 | >350 \(^b\)                          |
| 1-Methylnaphthalene   | Baylike                | 50                                   |
| 9-Methylnaphthalene   | Baylike                | 50                                   |
| 9,10-Dimethylnaphthalene| Baylike              | 50                                   |
| Phenanthrene          | Bay                    | 50                                   |

*GJIC was measured by the scrape-load/dye transfer technique according to the method of Upham et al. (64) using WB rat liver epithelial cells. The incubation time of the indicated chemical with the cells was 10 min.

### Table 3. Effect of the fluorene-type polycyclic aromatic hydrocarbons.

| PAH                | Structural determinant | Concentration, μM at 90% inhibition |
|--------------------|------------------------|-------------------------------------|
| Fluorene           | Linear                 | 75                                   |
| 1-Methylfluorene    | Baylike                | 30                                   |
| Fluoranthen        | Bay                    | 30                                   |

*GJIC was measured by the scrape-load/dye transfer technique according to the method of Upham et al. (64) using WB rat liver epithelial cells. The incubation time of the indicated chemical with the cells was 10 min.

### Table 4. Linear relationship of the water/octanol partition coefficient versus the time required for a polycyclic aromatic hydrocarbon to inhibit gap junctional intercellular communication at 0.5% fraction of the control.

| PAH                      | \( \log K_{ow} \) | \( \text{Time of inhibition, min} \)^a |
|--------------------------|------------------|------------------------------------------|
| 1-Methylnaphthalene, 200 μM | 3.87            | 0.45                                     |
| 2-Methylnaphthalene, 320 μM | 3.86            | 0.89                                     |
| Fluorene, 125 μM         | 4.18            | 1.59                                     |
| 1-Methylfluorene, 80 μM  | 4.50            | 1.74                                     |
| Phenanthrene, 50 μM      | 4.57            | 2.81                                     |
| 9-Methylanthracene, 50 μM| 5.07            | 2.02                                     |
| 1-Methylanthracene, 50 μM| 5.2             | 2.41                                     |
| Fluoranthen, 80 μM       | 5.22            | 2.84                                     |
| 9,10-Dimethylnaphthalene, 50 μM | 5.25    | 2.29                                     |

*The \( \log K_{ow} \) values were obtained from Mackay et al. (65). ^aValues were obtained by fitting a sigmoidal curve to data plotted as a function of GJIC expressed as a fraction of the control vs time using least-squares curve-fitting procedures, and the numerical parameters obtained from these curves were used to calculate the time in which inhibition occurred at a value 0.5 of the fraction of the control.
increased the initiating and promoting activity of benzo[a]pyrene (B[a]P) (40–44). These cocarcinogenic PAHs are not complete carcinogens (33) in which phenanthrene and fluoranthene lack tumor-initiating activity (37,41,45,46). The concept of bay or baylike regions possessing tumor-promoting activity certainly warrants further investigation.

The methylated PAHs are more potent carcinogens than their unmethylated parent structure (45–53). Bioalkylation of PAHs converts noncarcinogenic PAHs to carcinogenic PAHs (54,55) and the methyl substitution occurs in vivo at the methoxyanthracenic positions (53–56), which creates a baylike region. To be complete carcinogens, the methylated PAHs must undergo metabolic activation into a strong electrophile capable of reacting with DNA. Micosomal enzymes hydroxylate methyl groups (56–58), and hepatic sulfotransferases catalyze the formation of sulfate esters with the hydroxymethyl groups (58–60). Unfortunately, experiments were not conducted to determine whether enhanced carcinogenicity was a consequence of increased tumor initiation activity by the more electrophilic metabolites or due to greater promotonal activity, which could be a consequence of the less electrophilic methyl PAHs as well as the hydroxy methylsulfonates. Another classic example of a methylated PAH being more carcinogenic than the unmethylated counterpart is chrysene. However, the higher carcinogenic potential of 5-methylchrysene and its metabolites could not be related to its mutagenic potential (61). Thus, the formation of more electrophilic metabolites and stronger mutagens is not always essential in determining the carcinogenic potential of a PAH but can still exert a tumorigenic effect at the epigenetic stage of cancer by promoting preexisting spontaneously initiated cells.

In fact, carcinogenic PAHs are also effective modulators of signal transduction pathways involved in cell proliferative processes. B[a]P and 7,12-dimethylbenz[a]anthracene increase intracellular Ca2+ and cell proliferation in primary human mammary epithelial cells (62). Another example is the induction of proliferation of vascular smooth cells by B[a]P that did not involve mutational activation of c-Ha, c-Ki, or N-ras genes (63). Clearly, a better understanding of the cell proliferative roles of PAHs in tumor promotion will need further research in the effects of PAHs on mitogenic signal transduction pathways and the release of a transformed cell from normal homeostatic control mechanisms maintained by GJIC. Determining structure–activity relationships relative to intra- and intercellular communication should help in predicting the carcinogenic potential of the many different PAHs found in our environment.

Useful information can be obtained from in vitro experiments even though in vitro results are difficult to extrapolate to in vivo situations pertaining to the carcinogenic risk of a chemical. In vitro assays are usually better suited for studying structure–activity relationships at a more mechanistic level, which can increase our ability to predict the potency of tumor promoters based on chemical structure. Also, in vitro studies can ultimately reduce the extent of whole-animal testing. For example, our results showed that the effects of dose, time response, and time of recovery of the three- and fluorene-type PAHs on GJIC were similar (Tables 1–4). These results suggest a similar mechanism of action. Therefore, randomly selecting one or two of these chemicals rather than testing all of them could minimize in vivo experiments. Minimizing in vivo experiments is a particularly important motivation factor in reducing the expense and use of live animals when assessing the risk of chemicals before and after environmental remediation. To test every subtle change in existing environmental remediation strategies using in vivo assays would certainly be prohibitively costly and inhumane, and to limit in vitro assays to the measurement of genotoxicity would exclude the accurate assessment of risks to human health considering that many chemicals, including many carcinogens, are epigenetically toxic (16,17). Furthermore, monitoring the levels of known toxicants using analytical chemical techniques is not an acceptable alternative to eliminating in vivo experiments because simply removing the parent compound does not always ensure a safe environment: Toxic by-products could result from the chemical or biologic transformation of the parent compound (64). An example of such a situation was demonstrated by Upham et al. (38,64), in which selected PAHs were oxidatively removed using ozone but the resulting mixture became more epigenetically toxic than the parent compound.

In conclusion, the evaluation of epigenetic toxicity using in vitro assays should provide invaluable information in assessing the toxic risk of pollutants on human health before and after environmental remediation.

REFERENCES AND NOTES

1. Trosko JE. Challenge to the simple paradigm that "carcinogens" are "mutagens" and to the in vitro assays used to test the paradigm. Mutat Res 373:245–249 (1997).
2. Carson R. Silent Spring. Boston:Houghton Mifflin, 1962.
3. Ames BN, Durston WE, Yamasaki E, Lee FD. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc Natl Acad Sci USA 70:2281–2285 (1973).
4. Trosko JE. Radiation-induced carcinogenesis: paradigm considerations. In: Biological Effects of Low Level Exposures (Calabrese EJ, ed.). Boca Raton, FL:Lewis Publishers, 1994;205–241.
5. Trosko JE, Chang CC. Nongenotoxic mechanisms in carcinogenesis: role of inhibited intercellular communication. In: Banbury Report 31: Carcinogen Risk Assessment: New Directions in the Qualitative and Quantitative Aspects (Hart RW, Hoerger FD, eds). Cold Spring Harbor, NY:Cold Spring Harbor Laboratory Press, 1988;139–170.
6. Trosko JE, Chang CC, Madhukar BV. In vitro analysis of modulators of intercellular communication: implications for biologically based risk assessment models for chemical exposure. Toxicol In Vitro 4:635–643 (1990).
7. Chronier L, Bastide B, Herve JC, Deleze J, Malassine A. Gap junctional communication during human trophoblast differentiation: influence of human chorionic gonadotropin. Endocrinology 135:402–408 (1994).
8. De Mello WC. Gap junctional communication in excitable tissues: the heart as a paradigm. Prog Biophys Mol Biol 61:1–35 (1994).
9. Christ J, Spray DC, El-Sabban M, Moore LK, Brink PR. Gap junctions in vascular tissues: evaluating the role of intercellular communication in the modulation of vasomotor tone. Circ Res 135:251–264 (1996).
10. Munari-Silem Y, Rouset B. Gap junction-mediated cell-to-cell communication in endocrine glands: molecular and functional aspects: a review. Eur J Endocrinol 79:631–646 (1996).
11. Stagg RB, Fletcher WH. The hormone-induced regulation of contact dependent cell-cell communication by phosphorylation. Endocrinol Rev 11:302–325 (1990).

12. Trosko JE, Goodman JI. Intercellular communication may facilitate apoptosis: implications for tumor promotion. Mol Carcinog 11:8–12 (1994).

13. Barbe C, Sachs L. The difference in contact inhibition of cell replication between normal cells and cells transformed by different carcinogens. Proc Natl Acad Sci USA 56:1705–1711 (1966).

14. Potter VR. Phenotypic diversity in experimental hepatomas: the concept of partially blocked oncogeny. Br J Cancer 38:1–23 (1978).

15. Markert C. Neoplasia: a disease of differentiation. Cancer Res 28:1908–1914 (1968).

16. Trosko JE, Madhukar BV, Chang CC. Endogenous and exogenous modulation of gap junctional intercellular communication: toxicological and pharmacological implications. Life Sci 53:1–19 (1993).

17. Yamashita H, Naus CCG. Role of connexin genes in growth control. J Cell Biol 119:119–123 (1992).

18. Trosko JE, Chang CC, Madhukar BV, Dupont E. Oncogenes, tumor suppressor genes, and intercellular communication in the "Oncogeny as Partially Blocked Oncogeny" hypothesis. In: New Frontiers in Cancer Causation (Iverson OH, ed). Washington: Taylor & Francis Publishers, 1993;181–197.

19. Goldberg GS, Martyn KD, Lau AF. A connexin 43 antisense vector reduces the ability of normal cells to inhibit the foci formation of transformed cells. Mol Carcinog 11:106–114 (1994).

20. Ruch RJ, Guan X, Sigler K. Inhibition of gap junctional intercellular communication and enhancement of growth in BALB/c 3T3 cells treated with connexin 43 antisense oligonucleotides. Mol Carcinog 14:269–274 (1995).

21. Trosko JE, Chang CC, Madhukar BV, Dupont E. Intercellular communication: a paradigm for the interpretation of the initiation/promotion/progression model of carcinogenesis. In: Chemical Induction of Cancer: Modulation and Combination Effects (Arcos JC, Argus MF, Woo YT, eds). Boston:Birkhauser Publishers, 1996;205–225.

22. Bouwerr RK. The function and mechanisms of promoters of carcinogenesis. CRC Crit Rev Toxicol 2:419–443 (1974).

23. Potter VR, Hecht SS. Carcinogenesis: implications of experimental carcinogenesis in the genesis of human cancer. J Supramol Struct Cell Biochem 17:133–146 (1981).

24. Potter VR. Use of two sequential applications of initiators in the production of hepatomas in the rat: an examination of the Solt-Farber Protocol. Cancer Res 44:2733–2736 (1984).

25. Trosko JE, Chang CC, Medcalf A. Mechanisms of tumor promotion: potential role of intercellular communication. Cancer Invest 1:511–526 (1983).

26. Ashby J, Tennant RW. Definitive relationships among chemical structure, carcinogeticity and mutagenicity for 301 chemicals tested by the U.S. NTP. Mutat Res 257:229–306 (1991).

27. Budunova Iv, Williams GM. Cell culture assays for chemicals with tumour-promoting activity. In: The modulatory role of intercellular communication. Cell Biol Toxicol 10:71–116 (1994).

28. Rosenkranz M, Rosenkrantz HS, Kleopman G. Intercellular communication, tumor promotion and non-genotoxic carcinogenesis: relationships based upon structural considerations. Mutat Res 381:171–188 (1997).

29. IARC, IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol 32: Polynuclear Aromatic Compounds, Part 1: Chemical, Environmental and Experimental Data. Lyon:International Agency for Research on Cancer, 1985:95–447.

30. Port P. Cancer scrot. In: The Chirurgical Works of Percivall Pott. Philadelphia:James Webster, 1819:291–295.

31. Hieger I. The spectra of cancer-producing tars and oils of related substances. Biochem J 24:505–511 (1930).

32. Kennaway EL, Hieger I. Carcinogenic substances and their fluorescence spectra. Br Med J 1:1044–1046 (1930).

33. Dipple A, Moschel RC. Bigger CAH. Polynuclear aromatic carcinogens. In: Chemical Carcinogens, 2nd ed. Vol 1 (Searle CE, ed). ACS Monogr 182:41–163 (1984).

34. Dipple A. Polynuclear aromatic carcinogens. In: Chemical Carcinogens (Searle CE, ed). ACS Monogr 173:245–314 (1976).

35. Busby WF Jr, Goldman ME, Newberne PM, Wogan GN. Tumorigenicity of fluoranthene in a newborn mouse lung adenoma bioassay. Carcinogenesis 5:1311–1316 (1984).

36. Wang J-S, Busby WF Jr. Induction of lung and liver tumors by fluoranthene in a preweaning CD-1 mouse bioassay. Carcinogenesis 14:877–874 (1993).

37. Stocker KJ, Howard WR, Stathas J, Proudluck RJ. Assessment of the potential in vivo genotoxicity of fluoranthene. Mutagenesis 11:493–496 (1996).

38. Upham BL, Masten SJ, Lockwood BR, Trosko JE. Nongenotoxic effects of polycyclic aromatic hydrocarbons and their ozonation by-products on the intercellular communication of rat liver epithelial cells. Fundam Appl Toxicol 4:279–479 (1984).

39. Upham BL, Weis LM, Rummell AM, Masten SJ, Trosko JE. The effects of anthracene and methylated anthracenes on gap junctional intercellular communication in rat liver epithelial cells. Fundam Appl Toxicol 34:260–264 (1996).

40. Rusch HP, Kline BE, Baumann CA. The nonadditive effect of ultraviolet light and other carcinogenic procedures. Cancer Res 2:183–188 (1942).

41. Roe FJ. Effect of phenanthrene on tumor-initiation by 3,4-benzpyrene. Cancer 16:503–506 (1963).

42. van Duuren BL, Goldschmidt BM. Cocarcinogenic and tumor-promoting agents in tobacco carcinogenesis. J Natl Cancer Inst 56:1237–1242 (1976).

43. Steiner PE, Falk HL. Summation and inhibition effects of weak and strong carcinogenic hydrocarbons: 1:2-benzanthracene, benzo[a]pyrene, dibenz(a,h)anthracene, and 20-methylcholangthrene. Cancer 11:56–63 (1951).

44. Falk L, Kotin P, Thompson S. Inhibition of carcinogenesis. Arch Environ Health 9:169–179 (1964).

45. Hoffman D, Lavoie EJ, Hecht SS. Polynuclear aromatic hydrocarbons: effects of chemical structure on tumorigenicity. In: Polynuclear Aromatic Hydrocarbons: Physical and Biological Chemistry (Cookison R, Goldsmith T, Modan M, S. The natural history of carcinogenesis: implications of experimental carcinogenesis in the genesis of human cancer. J Supramol Struct Cell Biochem 17:133–146 (1981).

46. Poter VR. Use of two sequential applications of initiators in the production of hepatomas in the rat: an examination of the Solt-Farber Protocol. Cancer Res 44:2733–2736 (1984).

47. Trosko JE, Chang CC, Medcalf A. Mechanisms of tumor promotion: potential role of intercellular communication. Cancer Invest 1:511–526 (1983).

48. Ashby J, Tennant RW. Definitive relationships among chemical structure, carcinogeticity and mutagenicity for 301 chemicals tested by the U.S. NTP. Mutat Res 257:229–306 (1991).

49. Budunova IV, Williams GM. Cell culture assays for chemicals with tumour-promoting activity. In: The modulatory role of intercellular communication. Cell Biol Toxicol 10:71–116 (1994).

50. Rosenkranz M, Rosenkrantz HS, Kleopman G. Intercellular communication, tumor promotion and non-genotoxic carcinogenesis: relationships based upon structural considerations. Mutat Res 381:171–188 (1997).

51. IARC, IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol 32: Polynuclear Aromatic Compounds, Part 1: Chemical, Environmental and Experimental Data. Lyon:International Agency for Research on Cancer, 1985:95–447.

52. Port P. Cancer scrot. In: The Chirurgical Works of Percivall Pott. Philadelphia:James Webster, 1819:291–295.

53. Hieger I. The spectra of cancer-producing tars and oils of related substances. Biochem J 24:505–511 (1930).
methyl-substituted benz(a)acridines, benzo[a]phenothiazines and chrysenes, and their carcinogenic or antitumor activities. Anticancer Res 16:2757–2766 (1996).

53. Flesher JW, Myers SR, Stansbury KH. The site of substitution of the methyl group in the bioalkylation of benzo[a]pyrene. Carcinogenesis 11:493–496 (1990).

54. Flesher JW, Myers SR. Bioalkylation of benz[a]anthracene as a biochemical probe for carcinogenic activity; lack of bioalkylation in a series of six noncarcinogenic polynuclear aromatic hydrocarbons. Drug Metab Dispos 18:163–167 (1991).

55. Flesher JW, Myers SR. Rules of molecular geometry for predicting carcinogenic activity of unsubstituted polynuclear aromatic hydrocarbons. Teratog Carcinog Mutagen 11:41–54 (1991).

56. Myers SR, Blake JW, Flesher JW. Bioalkylation and biooxidation of anthracene, in vitro and in vivo. Biochem Biophys Res Commun 151(3):1441–1445 (1988).

57. Yang SK, Chu MW, Fu PP. Microsomal oxidations of methyl-substituted and unsubstituted aromatic hydrocarbons of monomethylbenz[a]anthracene. In: Polynuclear Aromatic Hydrocarbons: Chemical Analysis and Biological Fate (Cooke M, Dennis A), eds. Columbus, OH:Battelle Press, 1980:253–264.

58. Watabe T, Ishizuka T, Isobe M, Ozawa N. A 7-hydroxymethyl sulfate ester as an active metabolite of 7,12-dimethylbenz[a]anthracene. Science 215:403–405 (1982).

59. Surh YJ, Liem A, Miller EC, Miller JA. Metabolic activation of the carcinogen 6-hydroxymethylbenzo[a]pyrene: formation of an electrophilic sulfuric acid ester and benzylc DNA adducts in rat liver in vivo and in reactions in vitro. Carcinogenesis 10:1519–1528 (1989).

60. Surh YJ, Liem A, Miller EC, Miller, JA. 7-Sulfoxymethyl-12-methylbenz[a]anthracene is an electrophilic mutagen, but does not appear to play a role in carcinogenesis by 7,12-dimethylbenz[a]anthracene or 7-hydroxymethyl-12-methylbenz[a]-anthracene. Carcinogenesis 12:339–348 (1991).

61. Cheung YL, Gray TJ, Ioannides C. Mutagenicity of chrysene, its methyl and benzo derivatives, and their interactions with cytochromes P-450 and the Ah-receptor: relevance to their carcinogenic potency. Toxicology 81(1):69–86 (1993).

62. Tanheimer SL, Barton SL, Ethier SP, Burchiel SW. Carcinogenic polycyclic aromatic hydrocarbons increase intracellular Ca2+ and cell proliferation in primary human mammary epithelial cells. Carcinogenesis 18:1177–1182 (1997).

63. Zhang Y, Ramos KS. The induction of proliferative vascular smooth muscle cell phenotypes by benzo[a]pyrene does not involve mutational activation of ras genes. Mutat Res 373:285–292 (1997).

64. Upham BL, Jehng YJ, Trosko, JE, Masten SJ. Determination of the efficacy of ozone treatment systems using a gap junction intercellular communication bioassay. Environ Sci Technol 29:2923–2928 (1995).

65. Mackay D, Shu WY, Ma KC, eds. Polynuclear aromatic hydrocarbons, polychlorinated dioxins, dibenzo-p-dioxins. In: Illustrated Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals. Vol VII. Chelsea, MI:Lewis Publishers, 1992;597.