CELLULAR RESPONSES TO DNA DAMAGE: DNA REPAIR, p53 INDUCTION, CELL CYCLE ARREST AND APOPTOSIS UPON TREATMENT OF HUMAN CELLS WITH THE CARCINOGEN, anti-BENZO(a)PYRENE DIOL EPOXIDE

DISSERTATION

Presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of The Ohio State University

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To my mother and Edie
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ABBREVIATIONS

PAH, Polycyclic aromatic hydrocarbon; BPDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; (±)-anti-BPDE, racemic 7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; (+)-anti-BPDE, 7b,8a-dihydroxy-9a,10a-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (7R,8S,9S,10R steric configuration); (-)-anti-BPDE, 7a,8b-dihydroxy-9b,10b-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (7S,8R,9R,10S steric configuration); (±)-anti-BPDE-ssDNA, single-stranded DNA modified with racemic anti-BPDE; (+)- and (-)-anti-BPDE 11-mer, 11 base synthetic oligodeoxynucleotides containing a corresponding single (+)- or (-)-anti-BPDE adducted at N2-dG or N6-dA; ssDNA, single-stranded DNA.
Purpose

Cells have evolved a variety of sophisticated mechanisms to counteract the deleterious effects of continuous exposure to endogenous and exogenous agents that damage DNA. The efficient removal of lesions from the genome of the cell is necessary for the faithful propagation of genetic information during cell division. Among the various cellular defense mechanisms against DNA damage, the cytochrome P450 detoxification system and the DNA excision repair system have been well characterized (Conney, 1982; Sancar, 1995; Guengerich, 1992). In addition, the protective role of antioxidants and their potential use as chemopreventive agents against cancer has been an area of intensive study in recent years (Byers and Perry, 1992). Recent advances in molecular cellular biology have enabled us to understand other interrelated processes of cell cycle arrest and programmed cell death that function to prevent the adverse effects of genotoxins.
The purpose of this study was (i) to show that antibodies can distinguish the conformational differences between the enantiomeric forms of the carcinogen adducts formed by (±)-anti-BPDE, (ii) to demonstrate that the quantitative detection of (±)-anti-BPDE DNA lesions in non-competitive immunoassays was dependent on the mono-adduct specificities of antibodies, (iii) to confirm the role of post-translational protein modifications on the stabilization of the p53 tumor suppressor protein and (iv) to corroborate the presence of DNA damage response pathways in cell lines with defective p53.

The molecular basis of carcinogenesis

Epidemiological studies indicate that 80-90% of human cancers originate due to DNA damage (Doll and Peto, 1981; Vogelstein and Kinzler, 1993). In simplistic terms, cancer can be defined as a disease resulting from the improper control of cell division, cell differentiation and cell death. Cancer arises from the gradual deterioration of information in the genome leading to inappropriate regulation of cellular functions via the activation/inactivation of vital genes. It has been established that carcinogenesis is a multistage process resulting from genetic and epigenetic damage caused by genotoxins in susceptible cells. The process of carcinogenesis consists of an initiation step that involves the formation of genetic changes in cells that are exposed to chemical and physical carcinogens. Initiated cells exhibit an altered response to their micro-environment and also
attain a selective growth advantage in comparison to the normal cells. The second step involves the promotion step in which the initiated cells have an increased proliferative or survival potential than the uninitiated cells. The promotion step is usually expedited if additional genetic changes occur allowing the affected cells to progress towards a neoplastic endpoint. It is of importance to note that the process of carcinogenesis is a complex phenomenon that usually requires at least half a dozen alterations at the genetic level. The commonly altered genes in cancer are the proto-oncogenes and the tumor suppressor genes. Thus the accurate transmission of genetic information is of utmost importance for cellular homeostasis (Harris, 1991). However, recent studies have also shown that the inactivation of vital genes (e.g., tumor suppressor genes) can also occur via DNA methylation in the absence of genetic changes during tumorigenesis (Herman et al., 1994).

DNA damage and mutagenesis

Genotoxins give rise to mutations by virtue of their ability to interact with DNA and cause chemical alterations of the nucleotides. Physical agents (e.g., UV irradiation) and chemical agents (e.g., simple alkylators) can cause DNA damage directly while others require metabolic activation (Guengerich, 1992). Genotoxins that need metabolic activation include polycyclic aromatic hydrocarbons, aflatoxins and nitrosamines. In addition, endogenous damage to
the DNA can also occur within the cell leading to modified DNA bases or strand breakage. The endogenous processes that cause DNA damage are oxidation, methylation, deamination and depurination. It has been estimated that $10^4$ lesions per day are formed due to oxidative damage alone in the human cell (Ames et al., 1993). The formation of stable adducts by mutagens may interfere with the normal functionality of the cellular replication machinery which may lead to the permanent fixation of replication errors during cell division.

**Cellular Responses to DNA damage**

**The cytochrome P_{450} detoxification system:**

The major organ involved in the metabolism of foreign chemicals in mammals is the liver. The metabolism of a majority of the drugs and carcinogens like polycyclic aromatic hydrocarbons is carried out by a the cytochrome P_{450}-dependent monooxygenases (Parke, 1994). It has been well established that the cytochrome P_{450} enzymes are inducible via enhanced transcription of the respective genes. For example, the PAH metabolizing enzyme P4501A1 is induced by several PAHs leading to their increased metabolism (Whitlock et al, 1996). Besides the liver, this enzyme activity albeit at reduced levels, has been shown to be present in the kidney, adrenals, lung, testes, thyroid, skin, sebaceous gland and small intestine in a variety of species (Zedeck, 1980). Furthermore, multiple cytochrome p450s with a range of
specificities to various chemicals also exist (Conney, 1982; Guengerich, 1992). The primary function of the microsomal enzymes is to convert foreign lipophilic compounds into more hydrophilic forms that can be readily excreted. Usually carcinogens like polycyclic aromatic hydrocarbons are converted dihydrodiol derivatives, phenols and glutathione derivatives (Zedeck, 1980). However, these derivatives are formed via an epoxide intermediate capable of forming covalent adducts with cellular macromolecules.

**DNA repair mechanisms**

Mammalian cells defend against the deleterious effects of DNA damage at the cellular and genetic level using complex mechanisms that are beginning to be understood (Hanawalt, 1995). The cytochrome P450 enzyme system primarily functions in the detoxification and the excretion of xenobiotics that are ingested by organisms thereby preventing their interaction with the cellular genetic material. However, the inherent disadvantage of forming reactive metabolites that can either be converted to excretable intermediates or DNA adducts by this system serves as a double edged sword. Detoxification of reactive metabolites (e.g., reactive oxygen molecules and several epoxide metabolites) also involves the mineral-dependent enzyme, glutathione peroxidase and smaller molecules like vitamins E and C, glutathione and carotenoids (Byers and Perry, 1992). To counteract the effects of carcinogens after DNA damage, mammalian cells
possess a second line of defense, an array of sophisticated DNA repair systems. The study of DNA repair has emerged as a fruitful area of research during the past decade and has led to the identification of various important links between DNA repair and disease (Griffin, 1996). The predominant form of eukaryotic DNA repair mechanism is the excision repair system which is further classified as base-excision (BER), nucleotide excision (NER) and mismatch repair (Sancar, 1995). Besides nucleotide excision, other mechanisms namely direct reversal and recombination repair also exist in mammalian cells. The base excision repair mechanism is involved in the removal of subtle DNA alterations that do not generally alter the double helical structure of the DNA. The mismatch repair system ensures the removal of mis-incorporated bases during the replication of DNA. The nucleotide excision repair (NER) system usually removes bulky DNA adducts that distort the helical structure of DNA (Sancar, 1994).

The higher incidence of skin cancer in patients with the hereditary disorder Xeroderma pigmentosum was shown to be due to defects in nucleotide excision repair in 1968 (Cleaver, 1968). This initial observation linking DNA repair deficiency to cancer has led to an active research in the area of DNA repair and the cloning of a majority of the gene products involved in the process of NER via complementation analysis (XPA through XPG). Cells belonging to the various complementation groups exhibit defects in NER to varying degrees. The nucleotide excision repair system in humans consists of ~ 17 polypeptides.
and a majority of these have been cloned. The basic steps involved in NER are, (a) recognition of the damage; (b) incision of DNA on either side of the lesion and (c) gap filling and ligation of the excised region. In the mammalian NER system, a 29-mer oligonucleotide is excised at the site of the DNA damage leaving a repair patch that is filled by DNA polymerase (Sancar, 1995).

While the above mentioned repair mechanisms are quite efficient in the removal of various types of DNA damage, the continuous exposure of the cells to DNA damage would be a formidable task to overcome at the molecular level. The fact that only ~1% of the eukaryotic genome consists of coding regions would mean that the cellular repair processes might be involved in the futile restoration of useless DNA sequences. However, as expected with the fine tuning of cellular processing and perfection by evolution, the phenomena of transcription coupled repair and strand specific repair enables the cell to steer the repair machinery to the actively transcribed regions of the genome (Hanawalt, 1994).

**DNA damage and cell cycle arrest**

The presence of various detoxification systems that prevent DNA damage and DNA repair mechanisms that remove base alterations from the DNA are not sufficient to counteract the continuous exposure of the cells to genetic damage. Since DNA damage is an induced non-physiological event that can occur to any
type of cell and at any stage of development/growth, additional mechanisms like cell cycle arrest and apoptosis are necessary to ensure the prevention of heritable DNA damage.

The cell cycle can be defined as the ordered progression of specific events like DNA replication, nuclear envelope breakdown, spindle formation and chromosome segregation that ultimately lead to the genesis of two identical cells from a single cell (Grana and Reddy, 1995). The various phases of the cell cycle are tightly regulated by protein complexes called the cyclins and cyclin dependent kinases (CDKs) (Sherr and Roberts, 1995). Recent advances in molecular biology has led to the cloning of the gene products that are involved in the regulation of specific stages of the eukaryotic cell cycle (Sherr, 1993). The basic theme underlying the regulation of the cell cycle involves the activation/inactivation of specific nuclear proteins via phosphorylation/dephosphorylation by the cyclin-CDK complexes. However, only a few downstream targets of the cyclin-CDK complexes have been identified. The retinoblastoma tumor suppressor gene product (pRb) has been recognized as a target for various cyclin-CDKs and has been shown to be phosphorylated in a cell cycle dependent fashion. The role of pRb involves the positive transcriptional activation of genes whose protein products are necessary for S-phase progression. In addition to the positive regulators of the cell cycle (cyclins and CDKs), a new family of proteins that are involved in the inhibition of
various CDK activities have been identified (Grana and Reddy, 1995; El-Deiry et al. 1993; Sherr and Roberts, 1995). Apart from the cyclins, CDKs and CDKIs, one of the important regulators of the cell cycle is the p53 tumor suppressor protein.

The primary function of p53 is to bring about cell cycle arrest when cells encounter DNA damage. It has been shown that p53 functions primarily to cause the cells to arrest at the GI-S and the G2-M boundary of the cell cycle. The delay at specific stages of the cell cycle occurring after DNA damage, most likely favors the efficient and optimal removal of DNA lesions from the cellular genome before cell division (El-Deiry et al. 1993; Canman et al. 1994; Guengerich, 1988; Kastan et al. 1991).

Programmed cell death as a defense against DNA damage:

In addition to cell cycle arrest, the programmed death of cells may be yet another mode necessary for the elimination of potential precursor cells escaping DNA repair (Canman et al. 1994; Hoffman and Liebermann, 1994). In this cellular defense mechanism, eukaryotic cells activate a series of events that lead to the systematic destruction of the cell. Programmed cell death or apoptosis is distinct from necrotic cell death as the cell undergoes a typical pattern that involving cell vacuolization, chromosomal condensation, and DNA degradation resulting in the formation of oligonucleosome sized DNA fragments. While recent data suggest that programmed death of cells is evolutionarily conserved.
and may be an integral process necessary during the developmental stages of an organism, there is ample evidence indicating that apoptotic cell death can serve as a DNA damage response (Chinnaiyan and Dixit, 1996; Canman et al. 1994). The initiation of apoptosis after genotoxic damage has been shown to occur with agents that include topoisomerase inhibitors, ionizing radiation and DNA reactive agents (e.g. cisplatin). The mechanisms of programmed cell death have begun to emerge during the past few years. While several of the effector molecules responsible for the execution of programmed cell death have been identified, the process involves a cascade of events that result in the systematic destruction of the cellular blueprint for survival i.e. the DNA. The activation of several proteases and endonucleases are necessary for the process of apoptosis. Interestingly, the p53 tumor suppressor protein has been shown to play a necessary role in the apoptotic response triggered by DNA damage. However, apoptosis has been shown to also occur in a p53 independent fashion.

**p53- the mediator of cellular responses to DNA damage**

The p53 tumor suppressor protein was initially identified as an oncprotein bound to the SV40 large T antigen. However, further analysis of the gene indicated that the mutant form of p53 is capable of transforming cells and the wild type functioned as a tumor suppressor (Donehower and Bradley, 1993; Ko and Prives, 1996). Recent reports have demonstrated that the p53 tumor
suppressor protein plays an integral role in the cellular response pathways to DNA damage (Nelson and Kastan, 1994; Kastan et al. 1991; Kastan et al. 1992). The rapid accumulation of transcriptionally active p53 tumor suppressor protein upon genotoxic damage has been shown to be essential for G1-S cell cycle arrest. This occurs via the induction of p21, a potent inhibitor of cyclin dependent kinases regulating the cell cycle (El-Deiry et al. 1994; Dulic et al. 1994; Nelson and Kastan, 1994; Kastan et al. 1991; Kastan et al. 1992). While p53 is shown to play a definite role in triggering apoptosis following DNA damage (Kastan et al. 1992; Smith et al. 1994; El-Deiry et al. 1994; Price and Park, 1994; Lowe et al. 1993), cells deficient in p53, have also been shown to undergo apoptotic cell death indicating the presence of other p53 independent pathways (Nelson and Kastan, 1994; Venkatachalam et al. 1993). Besides this function, p53 can act as a transcriptional activator of repair related genes like gadd45 and very recent studies have implicated a direct role of p53 in the excision repair process (Ford and Hanawalt, 1995; Wang et al. 1995). In addition, p53 has also been shown to inhibit DNA replication (Miller et al. 1995) and exhibit 3’-5’ exonuclease activity (Mummenbrauer et al. 1996). Thus, the accumulation of p53 after DNA damage mediates a range of processes that ensure the faithful propagation of parental genomic sequences and the avoidance of mutations. In concordance with its role in guarding the genome, the p53 gene has been reported to be mutated in a majority of human cancers (Hollstein et al. 1991; Greenblatt et al. 1994).
Biochemical mediators of p53 stabilization:

The increase in p53 levels following DNA damage of cells has been shown to result from an increase in half-life of the protein (Liu and Pelling, 1995; Abrahams et al. 1995). While the biochemical and molecular modulators responsible for the stabilization of cellular p53 are yet to be discerned, some studies suggest that excision repair coupled formation of transitory DNA strand breaks is an important initial factor in the DNA damage response pathway (Nelson and Kastan, 1994). Furthermore, DNA strand breaks that induce p53 protein levels also are known to activate various damage dependent cellular enzymes like poly(ADP-ribose)polymerase and DNA-dependent protein kinases (Nelson and Kastan, 1994; Carson et al. 1986; Stierum et al. 1994a; Anderson, 1993). Indirect evidence from recent studies indicates that the post-translational protein modifications, namely poly ADP-ribosylation and phosphorylation may be involved in the increased half-life of the p53 protein (Whitacre et al. 1995; Khanna and Lavin, 1993). In addition, cellular p53 levels have been shown to rise even after treatment of cells to agents like heat thereby implicating it as a common stress response mediator.

The role of p53 mutations in cancer

The cellular functions of p53 range from transcriptional activation, DNA damage recognition, regulation of DNA replication and apoptosis (Ko and
Prives, 1996; Donehower and Bradley, 1993). Thus the intimate functional relationship between DNA damage and p53 indicate that the gene might be an appropriate target in the multistep process of carcinogenesis. In agreement with the essential role of p53 in safeguarding the genome, individuals with a germline mutation in the p53 gene develop cancers at an early age and are predisposed to genetic instability (Srivastava et al. 1992). In addition, p53 has been identified as one of the commonly mutated genes in a majority of the cancers (Hollstein et al. 1991). The recent crystallization of the p53 protein DNA binding domain has enabled the understanding of the basis of p53 mutations in tumorigenesis (Arrowsmith and Morin, 1996; Cho et al. 1994).

The tumor suppressor protein consists of three distinct functional domains. The amino terminal contains the activation domain, the central core consists of the DNA binding domain and the multi-functional carboxy-terminal domain is involved in the tetramerization of the protein and nonspecific binding to DNA (Ko and Prives, 1996). A majority of the mutations are concentrated in the DNA binding domain of the protein suggesting the importance of the DNA binding function of p53. Based on the crystal structure, p53 mutations have been classified as structural mutants and contact mutations. The most frequently mutated residues that almost always occur in the conserved sequences either destabilize the structure of the DNA binding surface or abolish the DNA contact points. For instance, the mutation hotspot site Arg248 that accounts for ~10% of
the total p53 mutation is necessary for p53-DNA interaction (Cho et al. 1994). Thus, the mutations that lead to a loss of DNA binding function of p53 seem to be necessary for the process of tumorigenesis. However, the effect of specific mutations on the various cellular functions of the protein awaits systematic analysis.

The cellular levels of p53 are also tightly regulated and the half-life of the wild type protein is ~15 minutes. The short half-life of the protein may prevent the inappropriate cell cycle arrest of dividing cells. In contrast to the normal cells, some tumor cell lines with mutant p53 exhibit an increase in stability of the tumor suppressor (Selivanova and Wiman, 1995). The mutant protein has been shown to have a dominant negative effect whereby it binds to the normal protein and interferes with its cellular functions (Hann and Lane, 1995; Srivastava et al. 1993). Thus the over expression of mutant p53 protein can also perturb the functions of the normal protein and lead to transformation. However, this dominant negative effect may occur in a cell type and p53 mutant dependent fashion as reported recently (Forrester et al. 1995).

Benzo(a)pyrene diol epoxide- an overview

The role of chemicals in the process of carcinogenesis has been known for more than two centuries (Miller and Miller, 1974). The higher incidence of scrotal cancers in chimney sweeps, noted by Percival Pott in 1775, is among one
of the earliest observations that pointed out the relationship between chemicals and cancer (Zedeck, 1980). The cancer causing agents in coal tar were identified as polycyclic aromatic hydrocarbons more than a century later in 1915 by Yamagiwa and Ichikawa. Polycyclic aromatic hydrocarbons (PAHs) contain three or more fused benzene rings arranged in linear and non-linear fashion. The early identification of PAHs as carcinogens has led to the accumulation of a great amount of detail regarding their biological effects.

Among the various PAHs, benzo(a)pyrene has been extensively studied as a model carcinogen during the past several decades (Phillips, 1983). The various sources of this ubiquitous carcinogen are forest fires, volcanic fumes, automobile exhaust, industrial wastes, thermal power production, refuse burning, cigarette smoke and various foods (Zedeck, 1980; Conney, 1982). In the United States, as much as 800 tons of benzo(a)pyrene have been estimated to be emitted in the air every year (Zedeck, 1980). Benzo(a)pyrene is metabolized by the mammalian cytochrome P<sub>450</sub> system involved in the detoxification of various PAHs. The ultimate carcinogenic form of benzo(a)pyrene is an epoxide intermediate capable of forming stable covalent interactions with DNA. The metabolic activation of benzo(a)pyrene that leads to the formation of anti-BPDE is shown in Figure 1.
Fig. 1. Metabolic activation of Benzo(a)pyrene (Zdeck, 1980)
At the cellular level the cytochrome P$_{450}$ enzyme system enables the cell to convert the polycyclic aromatic hydrocarbons (PAHs) to excretable forms via a series of oxidation and hydroxylation reactions. During the course of detoxification, benzo(a)pyrene is converted to several reactive intermediates that can be converted to excretable conjugates. These include quinones, phenols, dihydrodiols and glutathione conjugates (Zedeck, 1980). The metabolic activation of benzo(a)pyrene has been shown to occur in tissues and cells of diverse origin that include liver, kidney, lung, pancreatic ducts and fibroblast cells in culture (Milo et al. 1978; Guengerich, 1992; Sims and Grover, 1974).

The classification of benzo(a)pyrene as a carcinogen stems primarily from the ability of its diol-epoxide metabolites to form covalent DNA adducts thereby initiating the affected cells in the path of neoplastic transformation. (±)-anti-BPDE is the most potent and an ultimate carcinogenic metabolite of benzo(a)pyrene belonging to the group of bay region epoxides (Conney, 1982; Buening et al. 1978). (±)-anti-BPDE reacts at several nucleophilic sites in the DNA leading to the formation of various adducts. Among them, the N$^2$-deoxyguanosyl adduct comprises more than 90% of the total adduct population. In addition, several minor adducts are formed with N7 of guanine, N3 of cytosine and N6 of adenine (Straub et al. 1977). While the various biological effects of the different types of anti-BPDE DNA adducts are not completely understood, the carcinogenicity of anti-BPDE has been attributed to the major
\(N^2\)-guanosyl adduct. As shown in Figure 2, the metabolic activation of benzo(a)pyrene leads to the formation of four diol epoxides that can interact with DNA. However, the formation of (+)- and (-)-anti-BPDE predominate due to the preferential nature of the cytochrome P450 system.

![Diagram of benzo(a)pyrene metabolites](image)

Fig. 2. Formation of benzo(a)pyrene metabolites that are capable of causing DNA damage. Heavy arrows show the major metabolic pathways. (Conney, 1982)
Among the various diastereomeric enantiomers, the (+)-anti-BPDE enantiomer has been shown to be the most carcinogenic form (Buening et al. 1978). The differences in the biological effects of the two enantiomeric forms of anti-BPDE have been attributed to the differences in the conformation of the adducts formed in the DNA with the guanosyl base (Cosman et al. 1992; Santos et al. 1992). Spectroscopic analysis of the adducts formed by BPDE have been classified as site I and site II adducts depending on their conformational status in DNA. In the site I-type conformation, the pyrene moiety of the hydrocarbon interacts significantly with the base and is positioned in a "quasi-intercalative" fashion in the DNA. In the site II-type adducts, the pyrenyl residue is present external and solvent exposed sites of DNA (Jernström and Gräslund, 1994).

Comparison of the (+)- and (-)-anti-BPDE-N2-dG adducts have shown that they belong to the site II- and site I-type classifications, respectively. Besides these differences, solution conformation of the major adduct, (+)-anti-BPDE-dG indicate that the BP ring is positioned in the minor groove and directed towards the 5' end of the modified strand (Cosman et al. 1992). This conformation of the major adduct contrasts with that of the minor, (-)-anti-BPDE-N2-dG adduct which is directed towards the 3' end of the modified strand. These differences in the conformation of the major and minor adducts of anti-BPDE have raised the possibility of the differential interaction of the adducts with the cellular enzymatic repair systems leading to a varied response in their biological effects.
Analytical methods for detecting DNA damage:

The development of specific and sensitive methodologies to detect various types of DNA damage arising due to exposure to physical and chemical agents has become important to study the cause-effect relationship between DNA damage and cancer. Besides mechanistic studies aimed at elucidating the nature of culprit DNA adducts, DNA repair, cytotoxicity, mutagenesis, transformation and carcinogenesis, the quantitative measurement of (+)-anti-BPDE adducts in DNA allows the identification and categorization of the exposed human populations to disease susceptible risk groups (Santella et al. 1990b). Over the years, many different approaches ranging from physico-chemical measurements to more elaborate biochemical and molecular biological techniques have been described for monitoring (+)-anti-BPDE induced DNA damage (Phillips et al. 1988; Manchester et al. 1990; Weston, 1993; Stierum et al. 1994b). While protein-carcinogen adducts have been characterized for carcinogens (Skipper and Tannenbaum, 1990), the assessment of DNA adducts is considered to be more relevant in terms of exposure associated mutagenesis and carcinogenesis. Gas chromatography coupled with mass spectrometry (Manchester et al. 1988), 32P-postlabeling (Reddy et al. 1984) and immunological methods have been widely used for the estimation of DNA adducts (Zedeck, 1980; Poirier et al. 1977; Wani and D'Ambrosio, 1987; Wani et al. 1987; Santella et al. 1990a; Wani and Arezina, 1991). GC-MS while being extremely sensitive and specific, requires prior
derivatization of the modified adducts. $^{32}$P-postlabeling has been used for sensitivity and for detecting unknown adducts but has disadvantages like nonspecific labeling of contaminants, quantitation difficulties due to variable labeling characteristics and identification of adducts. Immunoassays have proven to be highly specific and sensitive with quantitation limits as low as 1 adduct/10$^8$ bases (Santella et al. 1990b). Antibody based quantitations can measure non-labeled DNA modifications and obviate the need for hazardous and often unavailable radiolabeled carcinogens.

Immunological approaches, based upon the exceptional ability of DNA damage specific antibodies to recognize individual DNA adducts have been of special interest (Santella et al. 1990a; Poirier, 1984; Poirier, 1993; Wani and D'Ambrosio, 1987; Wani et al. 1987; Wani and Arezina, 1991). Exploiting these features, (±)-anti-BPDE adduct specific antibodies have been applied for hazard evaluation in the monitoring of human populations exposed to varying degrees of environmental and occupational benzo[a]pyrene (Santella et al. 1990b; Perera et al. 1988; Perera et al. 1987). Generally, the assay used in these studies is a competitive ELISA which determines the inhibition of antibody binding to immobilized antigen by a test DNA sample containing relatively low levels of modified bases (Poirier et al. 1980; Santella et al. 1984). Alternatively, non-competitive immunoassays have proven to be very valuable in antibody characterization, adduct quantitation and spatial localization of lesions within specific target sequences (Venkatachalam and
Wani, 1994; Wani and Arezina, 1991; Denissenko et al. 1994). However, direct quantitation by these assays has been hampered by the amount of DNA that can be quantitatively immobilized to plastic surface in ELISA and due to concerns of variability in adduct detection. The higher antigen binding capacity of nitrocellulose membranes has in turn overcome these impediments (Wani et al. 1987). The fine mapping of DNA repair at the level of genes and specific nucleotides has been possible by the development of molecular approaches that include repair enzyme coupled southern hybridization and ligation mediated polymerase chain reaction (Bohr et al, 1985; Gao et al 1994).

Experimental Hypotheses:

(1). Several elaborate physico-chemical methods have demonstrated that the conformation of (+)- and (-)-anti-BPDE adducts with guanine vary significantly with respect to the DNA double helix. Thus, the conformationally different adducts may be subject to differential processing by cellular components like DNA repair enzymes. The specific question addressed was whether antibodies can differentiate the conformational changes between the (+) and (-)-anti-BPDE DNA adducts.

(2). Epidemiological studies indicate that an increased exposure to genotoxins results in a proportionate increase in the risk to develop cancer. This may be a
result of increased mutagenesis in cells with higher exposure to carcinogens. The inefficient removal of DNA damage from cells with higher levels of DNA lesions in comparison to cells with lower levels may be the reason behind the above-mentioned relationship. The measurement of the extent of DNA repair as a function of DNA damage level would allow us to identify the role of DNA repair in risk assessment and cancer development.

(3). The p53 tumor suppressor protein plays an important role in activating the cellular responses to DNA damage. The increase in the half-life of the protein after DNA damage occurs in a transcription independent manner. Thus, the post-translational modifications namely, phosphorylations and poly ADP-ribosylation, might be necessary for the stabilization of the protein after DNA damage.

(4). While the p53 gene has been found to be mutated in a majority of the cancers, the process of tumorigenesis requires the inactivation/activation of several other gene products. In addition, evolution requires cells to develop various fail-safe mechanisms that can in turn complement the multi-faceted role of p53 in maintaining the integrity of the genome. Therefore, several DNA damage response pathways that do not require functional p53 will be present in human cells.
CHAPTER II
MATERIALS AND METHODS

Reagents

(±)-anti-BPDE was purchased from the NCI repository (Midwest Research Institute, MO). Alkaline phosphatase conjugates of goat anti-rabbit IgG (GARI-AP), goat anti-mouse IgG (GAMI-AP) and horse radish peroxidase conjugates of goat anti-mouse IgG (GAMI-HRP) were from Boehringer Mannheim Biochemicals. Calf thymus DNA, alkaline phosphatase substrates nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), p-nitrophenyl phosphate (PNPP), calphostin-C, propidium iodide, Fast green, 3-aminobenzamide, RNAse A, Proteinase K and nocodazole were from Sigma (St. Louis, MO). Nitrocellulose (NC) membranes were from Schleicher & Schuell (Keene, NH). The p53 specific antibodies, p53 Ab-2 and p53 Ab-6 from hybridoma clones 1801 and DO-1 respectively were purchased from Neomarkers. Freemont, CA). The chemiluminescence substrates were obtained from Pierce (Rockford, IL) and the X-ray films were from Kodak (Rochester, NY). Dulbecco's Modified Eagle Medium was purchased from Life Technologies (Gaithersburg, MD).
Buffers

Phosphate buffered saline (PBS), 10 mM Na₂HPO₄, pH 7.4, 140 mM NaCl; PBS-Tween, PBS with 0.1% Tween-20; TE, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA; DEA, 1 M diethanolamine, pH 9.5, 1 mM MgCl₂; AP 9.5, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 2 mM MgCl₂; Blotto, 20 mM Tris-HCl, pH 8.0, 120 mM glycine, 5% dry milk powder, 0.5% gelatin, 0.01% thimerosol, 0.01% antifoam-A and 0.1% Tween-20. TBST buffer, 100 mM Tris, pH 7.4, 150 mM NaCl, 0.05 % Tween. Sample lysis buffer, 2% sodium dodecyl sulfate, 10% glycerol, 10 mM dithiothreitol in 62 mM Tris-HCl, pH 6.8, 10 μg/ml pepstatin and 10 μg/ml leupeptin. TAE buffer, 40 mM Tris-acetate, 1 mM EDTA; TE buffer, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

Establishment of human fibroblasts

The normal human fibroblast cell line (HF1) was established in culture by following the methodology as described earlier with minor modifications (Gibson-D'Ambrosio et al. 1986). Briefly, human foreskin tissue (obtained from the Ohio State University Tissue Procurement Services) was cut into pieces of ~1-2 mm² and disaggregated further in a stomacher bag containing 60 ml of trypsin-EGTA solution (0.1 mg/ml trypsin and 100 mM EGTA) for 20 minutes. To the cell suspension, 10 ml bovine calf serum was added and the cells were further diluted by the addition of 130 ml ice cold Hanks balanced salt solution, passed through a stainless sieve to
remove tissue fragments and single cells were collected by centrifugation. The cell pellets were then dissolved in DMEM containing 20% fetal calf serum and antibiotics, seeded in a tissue culture dish (100 cm²) and incubated in a humidified atmosphere (5% CO₂) at 37 °C for two weeks. After two weeks, the viable cells that had attached to the surface of the tissue culture dish were rinsed with phosphate buffered saline, trypsinized and seeded at density of ~10,000 cells/cm². Population doubling analysis was also performed to determine the growth characteristics of the cells. Population doubling was calculated using the following equation. PDL = 3.219x log number of cells seeded/ log number of cells harvested. As shown in Figure 3A, the cells exhibited a typical cellular growth pattern with an initial faster phase followed by a slower phase that showed a gradual plateau indicative of cellular senescence.

The second cell line, OSU-2, was established using a modified protocol that involved the use of collagenase to disaggregate the foreskin tissue. Human foreskin tissue (obtained from the Ohio State University Tissue Procurement Services) was disaggregated using scalpels and incubated for 4 h in collagenase medium (MEM containing 5% FCS and 0.25% collagenase) at 37 °C on a rocking platform. The tissue was then spun down, washed twice with serum free DMEM, seeded in a tissue culture flask containing culture medium (DMEM containing 20% FCS and penicillin-streptomycin) and incubated in a humidified atmosphere supplemented with 5% CO₂ at 37 °C for three days. The viable cells that had attached to the surface
of the tissue culture flask were rinsed with phosphate buffered saline to remove tissue debris and dead unattached cells, trypsinized and seeded at density of ~10,000 cells/cm². The cells were allowed to undergo at least three population doublings before use in various experiments. Population doubling analysis of the OSU-2 cells is shown in Figure 3B.

Preparation of (±)-anti-BPDE modified DNA standards

(±)-anti-BPDE was freshly dissolved in 100% ethanol and the integrity and concentration of the stock was determined from absorption spectra (ε₄₈₅=48,400) of an aliquot in 95% ethanol. For higher modifications (100 mM to 3 mM (±)-anti-BPDE), DNA (100 mg/ml in TE) was reacted in ethanol (33%) medium for 2 h at 37°C in dark. Control DNA was prepared by concurrent treatment with 33% ethanol. (±)-anti-BPDE modified DNA was extracted 9 times with water saturated ether, twice with water saturated isoamyl alcohol and thrice with water saturated ether. The modification levels of highly adducted DNA were quantitated according to the relationship described below (Pulkrabek et al. 1977):

\[
\text{% Modification} = \frac{[\text{anti-BPDE}]}{[\text{DNA}]} \times 100
\]

where \([\text{anti-BPDE}]=A_{350}/29,000\) and \([\text{DNA}]=A_{260}[0.18 \times A_{350}]/6650\).

For low modification of DNA \textit{in vitro} or \textit{in vivo}, (±)-anti-BPDE of appropriate concentration in 95% ethanol was directly added to the DNA in appropriate buffer or medium and incubated for desired time. The lower concentrations were generally achieved by serial dilutions of the stock with ethanol and immediate
addition to DNA samples or cell cultures. The concentration of ethanol in these reactions did not exceed 1%. Low modified calf thymus DNA (0.6 [3H]-BPDE adducts/10^6 nucleotides) was generously provided by Dr. Peter Shields (National Cancer Institute, NIH).

**Oligodeoxynucleotides**

The (+)- and (-)-trans-anti-BPDE-N^2-dG 11-mers (5'-C1-C2-A3-T4-C5-(BP)G6-C7-T8-A9-C10-C11-3') were generous gifts of Dr. N.E. Geacintov, Department of Chemistry, New York University. These anti-BPDE-N^2-dG 11-mers, prepared by HPLC separation of racemic (+)-anti-BPDE and oligonucleotide reaction mixtures, exhibit characteristic circular dichroism spectra and are reported to have a C/G/T/A/(BP)G ratio of 6.0:0.2:2.2:1.9:0.9 respectively for the modified strand (Cosman et al. 1992). The (+)- and (-)-trans-anti-BPDE-N^6-dA 11-mers (5'-C1-G2-G3-A4-C5-(BP)A6-A7-G8-A9-A10-G11-3') were generously provided by Dr. T. Harris, Department of Chemistry, Vanderbilt University. These HPLC purified 11-mers, contained (+)- or (-)-adducts at the adenine within the ras-61 sequence CAA. The duplexes of the modified (+)- and (-)-trans-anti-BPDE-N^2-dG 11-mers with the unmodified complementary strand (5'-GGTAGCGATGG-3') were made by incubating equimolar strand mixtures at 65°C for 30 min and slow cooling to room temperature. Secondary modification of (+)- and (-)-trans-anti-BPDE-N^2-dG 11-mers were done by exposure to 1 KJoule · m^2 u.v. (254 nm) radiation of the samples
on ice as described (Wani et al. 1984). The (+)- and (-)-trans-anti-BPDE-N6-dA 11-mers were secondarily treated with 2 mM (±)-anti-BPDE. The plasmid pUB3 modified with (+)-anti-BPDE (>35 adducts/molecule), provided by Dr. E. Loechler, Boston University, was prepared as described (Rodriguez and Loechler, 1993).
Fig. 3. Population doubling analysis of HF1 (A) and OSU-2 cells (B). Cells were seeded at \( \sim 10^4 \) cells per cm\(^2\) and grown until they reached \( \sim 80\% \) confluency. The cumulative population doubling vs. days in culture is plotted.
Preparation of antibodies

Polyclonal antibodies

Antibodies were developed in one month old female New Zealand white rabbits according to immunizations schemes established in our laboratory for obtaining high affinity and high titer antisera (Wani et al. 1984; Wani et al. 1989; Wani and D'Ambrosio, 1987; Wani et al. 1984). (+)-anti-BPDE modified calf thymus DNA (~2 adducts/ 10^2 nucleotides) was complexed with an equal amount of methylated BSA as described by Plescia (Plescia et al. 1964). (+)-anti-BPDE-DNA-protein complex (2 mg/ml in PBS) was emulsified with two volumes of complete Freund's adjuvant and injected i.m. in the hind quarters and 25-50 intradermal sites within the neck and back areas. Three weeks later, the animals were boosted by injecting 0.5 mg of (+)-anti-BPDE-DNA-protein complex emulsified with Freund's incomplete adjuvant. The rabbits were bled weekly, antisera monitored for titers and finally recovered 16 days after the booster injections. Antibodies were precipitated with 40% ammonium sulfate and the protein suspension stored at 4°C. For analysis, an appropriate aliquot of the suspension was centrifuged and the IgG protein pellet reconstituted in PBS to its original concentration. Among the antisera developed, antibody BP1 characterized to have the highest (+)-anti-BPDE-DNA titer and binding affinity, was used in the present study.
Standardization of antibody titers for immunoassays

The specificity and sensitivity of antibodies usually depend on the assay conditions. The signal to noise ratio in non-competitive immunoassays can be modulated by the concentration of primary antibody and the ionic strength of the incubation medium. Figure 4, shows the effect of primary antibody (BP1) dilution on the antigenicity of modified and unmodified DNA. An excess amount of antigen (10 ng of 1.3% anti-BPDE modified and unmodified ss calf thymus DNA) was used to determine the titers for optimal binding to the antigen. An antibody concentration dependent increase in the signal was noticed with both modified and unmodified DNA. At least a ten thousand-fold increase in antibody concentration was necessary to achieve a similar level of antibody binding to the unmodified control DNA (Figure 4). The binding of antibodies to unmodified DNA at higher concentrations of the antibody was due to the relative increase in the fraction of antibodies that bind to unmodified DNA in the polyclonal population. However, this non-specific binding to control unmodified DNA was easily abolished by either higher dilutions or by the addition of NaCl in the antibody incubation mixture without any loss of specific binding to the modified DNA samples.

Monoclonal antibodies

Hybridoma cells producing the antibody designated 5D2 were a generous gift from Dr. Regina Santella (Columbia University). These cells were initially
derived from spleens of mice immunized with (±)-anti-BPDE modified calf thymus DNA complexed with methylated BSA (Santella et al. 1985). For recovery of antibodies, the cells were grown in SFM medium (Gibco-BRL) for 7 days without any medium change. Figure 5 shows the increase in antibody titers as function of growth of the antibody producing hybridoma cells. Hybridoma cells were seeded at an initial density of 10⁶ cells/ml and grown in culture for seven days. An aliquot of the cell culture supernatant was removed everyday and used in ELISA at a dilution of 1/1000. Antibody titers were obtained with an excess of highly modified DNA (10 ng, 1.3% modified). After seven days antibody containing supernatant was recovered and IgG protein was further purified and concentrated by hydroxyapatite column chromatography (Harlow and Lane, 1988). Protein concentration of the antibodies was determined by absorbance at 280 nm (1 absorbance unit equal to 0.8 mg IgG protein/ml).

Repair analysis and treatment of cells

Normal human foreskin fibroblasts (HF1) established in culture as described earlier and XP12BE fibroblasts (GM05509A) obtained from NIGMS Human Genetic Mutant Cell Repository were grown in the presence of [³H]dThd (20 nCi/ml) in DMEM supplemented with 10% fetal calf serum and antibiotics penicillin and streptomycin. For genotoxin treatment, cells were grown in 60 cm² plastic tissue culture dishes and kept at confluence for two days prior to exposure.
Fig. 4. Standardization of polyclonal antibody titers. ELISA was performed with an equal amount of modified and unmodified DNA antigen adhered to the wells.
Fig. 5. Antibody titer vs. growth of antibody producing cells. Non-competitive ELISA was performed from cell culture supernatants recovered between days 1-7. Absorbance values (filled circles) are shown for a single dilution (1:2000) of the supernatant. The hatched bars indicate the increase in the number of cells during the period of analysis.
The culture medium was replaced with HBSS and cells were exposed to the carcinogen by addition of a 1/100 volume of freshly prepared (±)-anti-BPDE stock in 95% ethanol. After 0.5 h exposure at 37°C, cells were washed with PBS and either harvested immediately or maintained in culture medium without serum for varying post-treatment times. Detachment and loss of cells from the culture dishes was not apparent for 24 h after treatment with doses up to 4.0 mM of the carcinogen. At the indicated time points, cells were washed, trypsinized and collected by centrifugation. Genomic DNA was isolated after protein and RNA digestions in high EDTA-salt followed by repeated organic extractions with phenol-chloroform and chloroform-isoamyl alcohol (Maniatis et al. 1982). DNA was quantitated and the absence of any contaminating RNA established by monitoring of UV absorbance at 260 and 280 nm and by micro-diphenylamine (DPA) assay, a modification of the procedure described by Burton (Burton, 1956). Single-stranded DNA was prepared by heat denaturation of unmodified or in vitro and in vivo modified DNA at 100°C for 5 min followed by cooling on ice.

Microdiphenylamine assay

The microdiphenylamine assay which is essentially based on the original method of Burton was developed to quantitate DNA samples in the range of 0.1 to 1 μg. In the modified method, the volume was scaled down by a tenth and the boiling step was replaced with an overnight incubation step to minimize the
evaporation of the smaller volumes used. To 20 μl of the DNA sample in TE, 80 μl of freshly prepared DPA reagent (1 ml glacial acetic acid, 0.5 ml 20% perchloric acid and 0.1 ml 0.16% acetaldehyde) was added and incubated overnight at 37°C. Colorimetric estimation of the samples and standard DNA was performed by measuring absorbance at 595 nM and 700 nM. The interference at 700 nM was subtracted from the 595 nM values to give an accurate absorbance measurement of the samples.

Immunoassays

Enzyme-linked Immunosorbant Assay (ELISA)

Non-competitive ELISA was performed in 96-well polystyrene microtiter plates (Immulon II) essentially as described earlier (Wani et al. 1984; Wani et al. 1989; Wani and D’Ambrosio, 1987). Wells were coated in duplicate with concentration ranges of appropriate duplex or heat denatured (±)-anti-BPDE modified or unmodified DNA and oligodeoxynucleotides in 0.1 ml PBS by overnight drying at 37°C (Wani et al. 1984). Wells were blocked for non-specific binding and appropriate dilutions of polyclonal or monoclonal (0.5 mg/0.1 ml Blotto supplemented with 0.15 M NaCl unless indicated otherwise) antibody were allowed to bind at 37°C for 1 hour. Binding of antibody was ascertained upon subsequent treatment with corresponding secondary antibody reagent (0.1 ml of 1:1000 dilution of GARI-AP in Blotto for the bound primary PAb or 0.1 ml of 1/1000 dilution of
GAMI-AP for bound primary MAb antibody) and colorimetrically assaying the bound alkaline phosphatase activity against p-nitrophenyl phosphate (1 mg/ml in 1 M DEA buffer, pH 9.5) substrate (Wani et al. 1984).

**Immuno-slot blot assay (ISB)**

Different amounts of modified and unmodified single-stranded DNA in 0.2 ml of 1 M ammonium acetate were applied onto nitrocellulose (NC) filters under low vacuum in a BRL Convertible (48 slot, 1.0x7.5 mm configuration) manifold. Binding of antibodies to anti-BPDE adducts in DNA immobilized on NC filters was determined by ISB assays as described (Wani et al. 1989; Wani et al. 1987). Filters were blocked and incubated at 37°C with PAb BP1 or MAb 5D2 (5 mg/ml in Blotto supplemented with 0.15 M NaCl) antibodies for 1 hr and the corresponding secondary antibodies (GARI-AP or GAMI-AP at 1:1000 dilution in Blotto) for 1 hr. In between each treatment, filters were thoroughly washed with several changes of PBS-Tween. In the last step, filter bound enzymatic activity was determined by color development with a solution of 0.33 mg NBT and 0.17 mg BCIP/ml in AP 9.5 buffer as described earlier (Wani et al. 1984; Wani et al. 1989). The densitometric evaluation of the color intensity was performed on air dried filters using a LKB laser scanner. The data were transmitted to an interfaced computer and analyzed with LKBL gelscan XL software as described (Wani et al. 1989).
Treatment of human cells for p53 analysis

Normal human fibroblasts (OSU-2) established by us and Li-Fraumeni fibroblasts (087 and 041) obtained from Dr. M.A. Tainsky (MD Anderson Cancer Center, TX) were grown in DMEM supplemented with 10% fetal calf serum. Normal (GM02184C) and repair-deficient (GM02250E, XPA) lymphoblastoid cells were obtained from the NIGMS Human Genetic Cell Repository (Camden, NJ) and grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics at 37 °C in a humidified atmosphere of 5% CO₂. Exponentially growing cells were treated for 30 minutes with (±)-anti-BPDE (Midwest Research Institute, NCI Repository, Kansas City, MO) by the addition of 1/100 volume of freshly prepared carcinogen stock in 95% ethanol after replacing the growth medium with Hank’s balanced salt solution. The monolayers of fibroblast cells were washed with PBS and maintained in fresh culture medium for varying post-treatment times. The lymphoblastoid cells, grown as suspension cultures were centrifuged and cell pellets were washed once with PBS before resuspending in fresh culture medium at a density of 0.3 x 10⁶ cells/ml. Cells were harvested at various post-treatment times, lysed by boiling for 10 minutes in sample lysis buffer, snap frozen in liquid nitrogen and stored at -20 °C. For treatment with 3-aminobenzamide, cells were either pre-incubated for one hour with 5 mM 3-AB before genotoxin exposure or mock treated with PBS. Both the pre-incubated and mock treated cells were further resuspended in fresh medium.
containing 5 mM 3-AB. In experiments with calphostin-C, cells were pre-incubated for 1.5 h with 300 nM of the PKC inhibitor before carcinogen treatment.

**Immunoblot analysis of p53 protein**

Western blot analysis was performed with aliquots from a constant number of cells obtained from the estimation of DNA content in the cell lysates. For DNA content estimation, aliquots of the cell lysates were precipitated with 5% ice cold TCA in the presence of 0.5 μg tRNA and pelleted by centrifugation at 13,000 rpm. The precipitates were briefly rinsed with 70% ice cold ethanol, resuspended in TE and processed by microdiphenylamine assay as described (Venkatachalam et al. 1993). Protein extracts from equivalent number of cells (1.2 x 10^6 cells i.e. 875 ng DNA from TCA precipitates) were separated in 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by using a semi-dry electroblotter (Hoeffer, San Francisco, CA). Equal protein loading was confirmed from visualization of the membranes stained for 15 to 20 minutes with fast green (0.1% fast green, 5% glacial acetic acid and 20% methanol). For p53 protein detection, the nitrocellulose membranes were blocked with 5% milk powder in TBST buffer for 2 h and incubated overnight with a mixture of anti-p53 protein antibodies at 1:200 dilution at room temperature. The membranes were further incubated with the secondary antibody reagent (goat anti-mouse
horse radish peroxidase conjugate antibodies) at 1:2000 dilution for 2 hours at room temperature. Following each antibody incubation the membranes were thoroughly washed with TBST buffer. The p53 protein bands were detected from the peroxidase activity using the enhanced chemiluminescence substrate reaction essentially according to the manufacturer's instructions using Kodak X-OMAR AR film.

**Flow Cytometric analysis of cells**

Carcinogen treated and mock treated human cells in culture (1-2 x 10⁶) were rinsed with phosphate buffered saline, resuspended in fresh cell culture media containing 0.4 μg/ml nocodazole and incubated further for 24 hours (Fan et al. 1994). Cells were then collected, fixed in ice-cold ethanol (70%), centrifuged at 1500 rpm, washed with PBS and resuspended in 0.8 ml PBS to give a final concentration of 1-2 x 10⁶ cells per ml. To the resulting single cell suspensions, RNase-A (0.1 ml of 0.1 mg/ml) and propidium iodide (0.1 ml of 0.4 mg/ml) were added and cells were incubated at 37 °C for 30 minutes. Cell cycle analysis was performed using a Coulter EPICS Elite cytometer (Hialeah, Fl). At least 15,000 gated cells were analyzed for each time point with Multicycle software (Phoenix Flow Systems, San Deigo, CA) and the coefficient of variance (CV) values were below 5%.
DNA fragmentation analysis

DNA fragmentation analysis was performed essentially as described (Sellins and Cohen, 1987). Briefly, DNA from cells was isolated by cellular lysis and fragmented DNA was separated from native intact DNA by centrifugation. The 13,000 g supernatant was treated with RNAase-A for 30 minutes at 37°C followed by Proteinase K for one hour at 45°C. Aliquots of DNA corresponding to ~1x10⁶ cells were resolved by electrophoresis in 1.2% neutral agarose using TAE buffer and visualized by ethidium bromide staining.
CHAPTER III

RESULTS

DIFFERENTIAL ANTIBODY BINDING TO (+)- AND (-)-anti-BPDE-DNA ADDUCTS

Differential anti-BPDE enantiomer adduct specific antibody binding in ELISA

DNA treated in vitro with (±)-anti-BPDE contains a mixture of modified bases, albeit in varying proportions. The structures of the modified guanine base with the enantiomeric species of this compound are illustrated in Figure 6. The oligomers modified with (±)-anti-BPDE, were appropriately resolved to provide monopure samples with (+)- or (-)- single base adduct (Cosman et al. 1992). Non-competitive binding to immobilized antigens was assessed with an antibody excess concentration (≈5 μg/ml) determined by prior ELISA to give an optimum signal with no detectable noise (undetectable binding to an excess of control unmodified DNA). The observed binding response was limited only by the amount of adduct contained in the DNA antigen immobilized to the matrix. As shown in Figure 7, the absorbance increase with different antigen samples exhibited an adduct
concentration dependent response. Replicate ELISA analysis demonstrated less than 2% variation and correlation coefficients for the DNA concentration curves were generally in the 0.98 to 0.99 range. Highest binding was seen with PAb BP1 (1/slope= 8.4x10^{-16} mole) and modified ssDNA. The sensitivity of the assay (represented by the reliable, reproducible and quantitative detection limit in ELISA) was below 0.1 fmol of anti-BPDE adduct. Despite higher adduct frequency of the oligomers (adduct/deoxynucleotide molar ratio of 9x10^{-2}), the affinity of the polyclonal antibody for modified 11-mers, containing a single anti-BPDE adduct, was less than that for heavily in vitro modified DNA. While the frequency of adducts was identical for the (+)- and (-)- oligomeric antigens, the polyclonal antibody binding to (-)-anti-BPDE-dG 11-mer was approximately 35- to 40-fold lower than (+)-anti-BPDE-dG 11-mer and much lower than that of the modified ssDNA (Table 1). Conceivably the observed binding for the (+)- and (-)-anti-BPDE-dG 11-mers could be due to possible differences of antigen adsorption to the plastic surface of the microtiter wells. This was ruled out by 254 nm u.v. light induced dimerization of the two CC, one TC and one CT potential pyrimidine dimer sites in the (+)- and (-)- dG adduct containing oligomers (Wani et al. 1984). These irradiated oligomers, containing N²-dG adduct as well as pyrimidine dimers, revealed an identical binding affinity for dimer specific UV-3 polyclonal antibodies in ELISA (results not shown). This confirmed that the decreased binding of the (-)-anti-BPDE-dG 11-mer was due to a lower antibody affinity for the (-)-anti-BPDE enantiomer adduct.
Fig. 6. Structures of (+)- and (-)-anti-BPDE and the corresponding adducts with guanine bases.
Fig. 7. Binding of antibodies to anti-BPDE modified DNAs. Increasing concentrations of modified DNA antigens, (+)-anti-BPDE-ssDNA (○, ●), (+)-anti-BPDE-N²-dG 11-mer (▲) and (-)-anti-BPDE-N²-dG 11 mer (□, ■) were quantitatively immobilized to microtiter plates by overnight drying. The binding of PAb BP1 (A) and MAb 5D2 (B) antibodies was determined by ELISA as described in chapter II. The average of duplicate determinations for 1 hr incubation with substrate was plotted by linear regression (Sigmaplot).
Fig. 7.
Monoclonal antibody 5D2 showed a 90-fold lower binding with (±)-anti-BPDE-ssDNA than the polyclonal antibody (Table 1). In addition to decreased overall binding, the preference of monoclonal antibodies to various antigens in ELISA was also different (Figure 7B). The (−)-anti-BPDE-dG 11-mer gave highest binding (1/slope=2.6×10^{-14} mole), whereas, the (+)-anti-BPDE-dG 11-mer the lowest binding in ELISA (1/slope=1.3×10^{-12} mole). Neither antibody exhibited detectable binding with (+)- or (−)-anti-BPDE-N^6-dA 11-mers. This absolute lack of antibody recognition of N^6-dA adduct in the anti-BPDE modified DNA was confirmed by demonstrating the antigenicity of the secondarily in vitro (±)-anti-BPDE treated 11 base oligomers, already containing a single (+)- or (−)-anti-BPDE-N^6-dA (data not shown).

Antibody binding to single- and double-stranded DNA

Microtiter well bound modified DNA shows optimal antigenicity and accessibility to antibodies in single-stranded form (Wani et al. 1984). However, our data show that the modified oligomers, annealed with the complementary unmodified 11-mer strands, efficiently adsorbed to microtiter wells and the antibodies were able to recognize the anti-BPDE adducts in duplexed oligomers (Table 1). Nevertheless, affinity of both the polyclonal and monoclonal antibodies was reduced against the adducts in duplexed antigens. Reduction of binding was lowest (~40%) for polyclonal antibody and highest (~80%) for monoclonal
TABLE 1  Relative affinity of antibodies for single- and double stranged antigens.

<table>
<thead>
<tr>
<th>anti-BPDE modified Antigen</th>
<th>Binding* A(405 nm)</th>
<th>Relative affinity†</th>
<th>Binding* A(405 nm)</th>
<th>Relative affinity†</th>
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</thead>
<tbody>
<tr>
<td>(+)-11-mer, ss</td>
<td>115.59±1.90</td>
<td>100.0</td>
<td>0.44±0.007</td>
<td>0.38</td>
</tr>
<tr>
<td>(+)-11-mer, ds</td>
<td>71.78±1.90</td>
<td>62.0</td>
<td>0.06±0.001</td>
<td>0.05</td>
</tr>
<tr>
<td>(-)-11-mer, ss</td>
<td>2.84±0.05</td>
<td>2.5</td>
<td>26.65±0.740</td>
<td>23.17</td>
</tr>
<tr>
<td>(-)-11-mer, ds</td>
<td>0.97±0.03</td>
<td>0.8</td>
<td>9.15±0.280</td>
<td>7.95</td>
</tr>
<tr>
<td>(±)-DNA, ss</td>
<td>1137.00±6.53</td>
<td>980.0</td>
<td>12.40±0.090</td>
<td>10.78</td>
</tr>
</tbody>
</table>

Binding of antibodies, to a range of concentration of modified ssDNA and single- and double-stranded N²-dG 11-mers, was determined by ELISA as described in chapter II.

*Binding corresponds to an A(405 nm) obtained upon 1 hr incubation with the substrate. The values were calculated by linear regression of the response curve and extrapolation to 1 pmole of adduct in corresponding antigens.

†Affinity values are the antibody binding relative to PAb BP1 binding to single-stranded (+)-11-mer (arbitrarily chosen as 100%).
antibody, both with (+)-anti-BPDE 11-mer as the antigen. This reduced antigen-antibody interaction coincides with the strongest binding of polyclonal and weakest binding of monoclonal antibodies against the (+)-anti-BPDE 11-mer antigen. The binding with duplexed (-)-anti-BPDE 11-mer was decreased by approximately 3-fold with both polyclonal and monoclonal antibodies. Interestingly, the preferential order of binding to different adduct enantiomeric forms was maintained in the duplexed DNA antigens. The antibodies did not bind to control unmodified ssDNA, dsDNA and single and duplexed oligonucleotides at concentrations up to 100 ng per well in ELISA (data not shown).

Effect of ionic strength on antibody binding to enantiomeric DNA base adducts

The avidity of binding to various adduct forms was determined by the antigen-antibody interaction under varying ionic strength. Absence of NaCl in the incubation media, resulted in highest binding of polyclonal as well as monoclonal antibody with both (+)- and (-)-anti-BPDE enantiomer adducts (Figure 8). It may be noted that very low relative binding, for (-)-anti-BPDE-dG 11-mer antigen and with monoclonal antibody, seen in Figure 8 (top), was easily quantifiable in ELISA as absolute adduct specific binding. A small amount of nonspecific antibody reactivity to unmodified DNA was completely abolished by raising the ionic strength (results not shown). Inclusion of 150 mM NaCl in the incubation buffers and the washing
Fig. 8. Effect of NaCl concentration on antibody binding to modified oligomers. Varying amounts of (+)- and (-)-anti-BPDE-N²-dG 11-mer oligonucleotides within linear concentration response range were immobilized to microtiter wells. The ELISA was performed with polyclonal and monoclonal antibodies as in Figure 6, except that dilution and incubation with primary antibodies was done in Blotto containing 0.0, 0.15, 0.5 or 1.0 M NaCl. For the no salt wells subsequent exposure to secondary antibody and washings were also in buffers without NaCl. (Top), The relative binding was determined from the slopes of linear regression plots and are expressed as the absorbance at 405 nm for 1 hr incubation from antibody binding to 1 pmole adduct. (Bottom), Due to lower relative binding by monoclonal antibody and (-)-enantiomeric adduct, the relative binding values were normalized and expressed as the percent binding to that observed in the absence of NaCl. The bars represent the S.D. of the mean of replicate assays.
Fig. 8.
incubation buffers and wash buffers caused a reduction of approximately 50-60% of specific antibody binding. The rapid drop of binding to modified antigens in the presence of salt could be partly attributed to the elimination of the nonspecific binding of the antibodies to DNA. A further increase in ionic strength during incubation showed a gradual decrease in polyclonal antibody binding to (+)- and (-)-anti-BPDE-dG 11-mers. At 1.0 M NaCl about 20% of the initial no-salt binding was still retained. The extent of salt induced lowering of monoclonal antibody binding to (+)-anti-BPDE-dG 11-mer was similar to that of polyclonal antibody. However, the binding to (-)-anti-BPDE-dG 11-mer was not altered by high salt concentration beyond the initial drop observed with 150 mM NaCl. This indicated that the monoclonal antibody, 5D2, preferentially and avidly binds to the (-)-anti-BPDE-dG adduct in DNA. The overall results of salt modulation of antibody binding demonstrate the differential nature of interaction between the immunoglobulin molecules with distinct adduct conformations of the genotoxic lesions.

**ISB analysis of enantiomeric adducts**

The extent of quantitative binding of DNA to nitrocellulose filter matrix is several magnitudes higher than to plastic wells. Accordingly ISB provides higher sensitivity, since much greater amounts of moderately modified DNA can be utilized for analysis. The results of a representative immunodetection
experiment with *anti*-BPDE modified DNAs and oligomers, immobilized to nitrocellulose filters, is shown in Figure 9. A gradient of color intensity is seen with increasing concentrations of various modified nucleic acids. Polyclonal antibody clearly detected lower than 15 pg each of (±)-*anti*-BPDE modified calf thymus and plasmid DNA, (+)-*anti*-BPDE modified plasmid DNA and the (+)-*anti*-BPDE-N2-dG 11-mer. A very low binding of polyclonal antibody to (-)-*anti*-BPDE-N2-dG 11-mer was detectable only at relatively high antigen concentration (1 ng/slot). Monoclonal antibody binding occurred only with (±)-*anti*-BPDE modified calf thymus and plasmid DNA and (-)-*anti*-BPDE-N2-dG 11-mer, albeit at ng/slot concentrations. At these high concentrations binding could not be seen with plasmid DNA modified with (+)-*anti*-BPDE. Extremely low but quantifiable monoclonal antibody binding was seen with 100 ng of the (+)-*anti*-BPDE-N2-dG 11-mer. An excess amount of the unmodified DNAs or oligomers bound to the same filters did not show detectable slot intensity with polyclonal or monoclonal antibodies in ISB assays.

**Absence of antibody binding to *anti*-BPDE-N6-dA adducts**

Among the various adducts formed by (±)-*anti*-BPDE with DNA, the (±)-N6-deoxyadenine adducts form a minor species comprising about 6% of the total adduct population. To determine if the polyclonal and monoclonal antibodies were able to bind the minor adenine adduct, 11-mers containing the (+)- and (-)-*anti*-BPDE-N6-dA adducts were used as antigens in the ISB assay. Figure 10 shows the
spectrophotometric scan of purified 11-mers modified with the two enantiomers of anti-BPDE. As expected, the modified 11-mers exhibited a characteristic peak at 350 nM indicative of anti-BPDE adduction (Pulkrabek et al. 1977). In contrast the unmodified 11-mer did not show any absorbance at 350 nM.

ISB assays, though highly sensitive, did not reveal any detectable binding affinity of polyclonal and monoclonal antibody to (+)- and (-)-anti-BPDE-N⁶-dA adduct. A representative experiment (Figure 11) with polyclonal antibody shows the lack of binding to high concentrations (35 to 280 fmol adduct) of (+)- and (-)-anti-BPDE-N⁶-dA 11-mers. The same filter shows absence of binding to unmodified DNA (negative control) and specific binding to low levels of guanine base adducts (positive control). In contrast, the oligomers containing (+)- and (-)-anti-BPDE-N⁶-dA adducts, secondarily exposed to anti-BPDE, proved to be suitable antigens in ISB assay. A comparable level of concentration dependent binding was observed with both oligomers (Figure 11, lanes B5 to B8 and C5 to C8). Therefore, absence of antibody binding, seen in lanes B1-B4 and C1-C4 of the nitrocellulose filter, was not due to a lack of binding by the modified oligomers to the matrix.

Figure 12 provides a composite illustration of the results of specific polyclonal and monoclonal antibody binding to various control and modified DNA by two different assay systems. In non-competitive ELISA and ISB assay, both antibodies show a high degree of selectivity and do not exhibit any nonspecific binding to unmodified DNA or oligomers. Sensitivity of detection of anti-BPDE
adducts was greater with polyclonal than with the monoclonal antibody. Polyclonal antibody BP1 demonstrated a clear preference for the (+)-anti-BPDE-N²-dG adduct, whereas monoclonal antibody 5D2 showed lesser binding for this adduct and a high preference for the (-)-anti-BPDE-N²-dG conformation in DNA. Neither antibody showed an affinity for the adenine base adducts of anti-BPDE.
Fig. 9. ISB analysis of antibody binding to guanine base adducts with enantiomeric forms of anti-BPDE. Two-fold dilutions of various modified DNA and oligomers were applied to nitrocellulose filters and detected by ISB assay with an excess of PAb BP1 and MAb 5D2. Control (con) slots at the bottom of each lane contained 100 ng of unmodified nucleic acids. Plasmid DNAs were linearized with restriction endonuclease BamH1. All the DNA and oligomer samples were heat denatured before applying to filters for quantitative antigen immobilization.
Fig. 10. Spectrophotometric scan of 11-mers modified with (+)- and (-)-anti-BPDE at the adenine base. The purified 11-mers were diluted in TE buffer and scanned for absorption between 200-400 nM. The scans of unmodified (1), (+)-anti-BPDE modified (2) and (-)-anti-BPDE (3) 11-mers is indicated.
Fig. 11. Absence of antibody binding to anti-BPDE-N6-dA adduct. Modified and unmodified DNA and oligomers containing single anti-BPDE-N6-dA adduct were analyzed by ISB using PAb BP1. (Lanes A1-A4), 12, 25, 50 and 100 ng of unmodified ssDNA; (Lanes A5-A8), modified ssDNA containing 3.5, 7, 14 and 28 fmol (±)-anti-BPDE adducts; (lanes B1-B4), 11-mer containing 35, 70, 140 and 280 fmol (±)-anti-BPDE-N6-dA; (Lanes B5-B8), same as lanes B1-B4 except 11-mer secondarily modified with 2 mM (±)-anti-BPDE, (lanes C1-C4), 11-mer containing 35, 70, 140 and 280 fmol (−)-anti-BPDE-N6-dA; (Lanes C5-C8), same as lanes C1-C4 except 11-mer secondarily modified with 2 mM (±)-anti-BPDE.
Fig 12. Specificity of binding of polyclonal and monoclonal antibodies to enantiomeric anti-BPDE adducts. The immunoassays with various antigens were performed using PAb BP1 and MAb 5D2 antibodies essentially as described in Methods. The relative antibody binding for 1 pMol anti-BPDE adduct in different modified antigens was determined from the slope values derived from the linear regression of each DNA concentration vs. antibody binding plot. Control values were derived from binding obtained for 20 ng (ELISA) or 200 ng (ISB) of ssCT DNA or unmodified 11-mer oligonucleotides. The bars are S.D. values for the entire concentration plot.
Fig 12.
Binding of antibodies to (+)-anti-BPDE-DNA in non-competitive ELISA

Binding of polyclonal and monoclonal antibodies was measured by non-competitive ELISA. An excess of antibody concentration (~5 mg/ml) that gave optimal binding with modified DNA and undetectable non-specific binding to unmodified DNA was used against a series of in vitro modified (+)-anti-BPDE DNA samples. The binding of antibodies increased as a function of the immobilized DNA antigen. Smaller amounts (10-50 pg) of heavily modified DNA and larger amounts (10-20 ng) of sparsely modified DNA were required to attain the absorbance values within the quantifiable range of the assay. Thus, the antibody binding was proportional to the absolute adduct levels irrespective of the amount of DNA immobilized to the plates. However, the linear antibody binding relationship with anti-BPDE adducts was limited by the quantitative antigen binding capacity (~20-30 ng of DNA) of the polystyrene micro-titer plates, beyond which a non-linear response was observed. Figure 13 shows a representative binding response of polyclonal and monoclonal antibodies to DNA modified in vitro with 0 µM to 100 µM (+)-anti-BPDE. The monoclonal antibodies did not cross react with unmodified DNA while the polyclonal antibodies showed slight binding to high concentrations.
 (> 100 ng/well) of unmodified control DNA. This non-specific binding of polyclonal antibodies could be prevented by increasing the ionic strength of the incubation medium. In contrast, the binding of monoclonal antibodies to modified DNA was very sensitive to the ionic strength of the incubation medium and decreased several fold in the presence of NaCl. Despite the absence of NaCl during antibody incubation for obtaining maximum binding, the affinity of monoclonal antibodies was about 20-fold lower than the polyclonal antibodies. Binding comparison at identical ionic strength (0.5 M NaCl) showed more than 100 fold lower affinity of the monoclonal antibodies than that of the polyclonal antibodies (data not shown).

**Immunoanalytical and spectrophotometric quantitation of adduct induction**

In order to validate the use of non-competitive immunoassays for the quantitative assessment of (±)-anti-BPDE DNA adducts, the carcinogen dose dependent induction of adducts was measured by spectrophotometry (Pulkrabek et al. 1977) and correlated with immunological determinations using polyclonal (BP1) and monoclonal (5D2) antibodies. To establish a common standard, an in vitro modified DNA sample (calf thymus DNA reacted with 100 μM (±)-anti-BPDE) was used as the reference. Its modification level (0.45 %) was determined by the well established spectrophotometric method of quantitating heavily modified DNA.
Fig. 13. Binding of antibodies to (±)-anti-BPDE modified DNA. Increasing amounts of DNA (0.01-50 ng) modified with 100 μM ( ●, ●), 25 μM (●, ●), 6 μM ( o, o) and 0 μM (△, △) of (±)-anti-BPDE were immobilized to microtiter wells by overnight drying. The antibody binding was determined by ELISA using polyclonal (BP1) and monoclonal antibody (5D2). Average of replicate absorbance values at 60 min of substrate incubation was plotted against DNA concentration by linear regression (Sigmaplot).
modified (±)-anti-BPDE-DNA (Pulkrabek et al. 1977). This modified sample had substantially high absorbance at 260 nm as well as 350 nm allowing an easy and reliable estimation of absolute adducts level. The value of this standard was also confirmed by immunoassay with another radioactively labeled anti-BPDE modified DNA as an additional reference (Rodriguez and Loechler, 1993b). All the adduct values quantitated by various assays in the present study were expressed in reference to this 0.45% modified DNA standard. As shown in Table 2, the adduct levels obtained from differential spectra and quantitation by polyclonal antibodies were identical up to 25 μM (±)-anti-BPDE dose. Nevertheless, data obtained by ELISA with PAb BP1 continued to exhibit a dose dependent linear response at lower (±)-anti-BPDE concentrations. In contrast, the monoclonal antibodies showed a non-linear response and the calculated modification levels were several-fold lower than the determinations by polyclonal antibodies.

Quantitation of (±)-anti-BPDE DNA adducts in ISB

Immuno-slot blot assays are highly suited for the non-competitive detection of very low levels of carcinogen-DNA adducts due to the elevated binding of antigen (~0.5-1 mg DNA/mm²) to NC membrane filters compared to micro-titer plates (~20-30 ng/well) (van Schooten et al. 1987).
Table 2. Estimation of adducts in (±)-anti-BPDE modified DNA.

<table>
<thead>
<tr>
<th>Dose (µM)</th>
<th>Modification Level (Adducts/10^6 nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diff. Spectra</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>4500</td>
</tr>
<tr>
<td>50</td>
<td>2600</td>
</tr>
<tr>
<td>25</td>
<td>1590</td>
</tr>
<tr>
<td>12.5</td>
<td>1180*</td>
</tr>
<tr>
<td>6.25</td>
<td>609*</td>
</tr>
<tr>
<td>3.125</td>
<td>439*</td>
</tr>
</tbody>
</table>

A series of modified DNA was prepared by in vitro treatment of calf thymus DNA with (±)-anti-BPDE. After complete removal of unreacted carcinogen the adduct levels were determined by spectrophotometry and quantitative non-competitive ELISA. *A350 values for these samples were beyond the reliable detection limit.
Polyclonal and monoclonal antibodies were utilized to determine their sensitivity of binding to (±)-anti-BPDE DNA adducts in ISB assays. Varying concentrations of DNA (0.4 ng to 500 ng), reacted with a range of (±)-anti-BPDE doses (0.001 mM to 1 mM), were immobilized to NC filters along with suitable reference standard and processed for determination of antibody binding (Figure 14). The adduct levels were quantitated by comparing the slot intensities of a set of modified DNA samples with laser densitometrically determined intensity values obtained with concurrently run reference standard. The slot color intensity of modified DNA increased proportionally with the amount of immobilized antigen (Figure 10, top to bottom) as well as the extent of modification (Figure 14, left to right). The binding of polyclonal antibodies was stringently specific to modified DNA and could be easily observed with samples treated with 1 nM (±)-anti-BPDE. In ISB assay, a slight non-specific binding of polyclonal antibodies to high amounts of unmodified DNA (> 0.5 µg DNA/slot) was completely abolished by the addition of 0.5 M NaCl during antibody incubation (Figure 14, lane 1). Nevertheless, no loss of specific antibody binding to adducted DNA was seen at this ionic strength. The antibody binding responses of DNA modified with varying doses (lanes 2-5) were well within the slot intensities observed with the standard reference run on the same filter (lane 6). Therefore, the intensity comparisons of various slots provide an easy estimate of adduct levels in corresponding samples. Evaluation of monoclonal
Fig. 14. Detection of (±)-anti-BPDE-DNA adducts by immuno-slot blot assay. A representative filter demonstrating specificity and sensitivity of antibody binding in ISB assay is shown. Serial dilutions of DNA (1:2 reduction) starting at 500 ng (lanes 1-3) or 50 ng (lanes 4-6) were immobilized to nitrocellulose filters and polyclonal antibody BP1 binding determined as described in chapter II. Lanes contain unmodified control DNA (lane 1), DNA modified in vitro with 1 nM (lane 2), 10 nM (lane 3), 100 nM (lane 4), 1 μM (lane 5) of (±)-anti-BPDE and standard reference containing 1.87x10² adducts/10⁶ nucleotides (lane 6).
antibodies, in parallel ISB assays, exhibited a very low binding affinity with (±)-anti-
BPDE modified DNA (data not shown). Thus, monoclonal antibodies could not be
used effectively for adduct quantitation at doses below 1 μM (±)-anti-BPDE.

Effect of modification level on the binding efficiency of antibodies

The efficiency of antibody binding to (±)-anti-BPDE-DNA modified to
varying adduct frequencies was compared in non-competitive ISB assay. As shown
in Figure 15, polyclonal antibodies exhibited an adduct concentration dependent
linear binding response for a series of DNA modified at an adduct/nucleotide ratio
of 4.5x10⁻³ (high), 1.87x10⁻⁴ (intermediate) and 6x10⁻⁷ (low). Interestingly, polyclonal
antibodies did not discriminate between the modified samples on the basis of
adduct frequency but showed specific binding proportional to absolute adduct
concentration within DNA immobilized to the nitrocellulose filter (Figure 15). The
sample of lowest modification showed slightly decreased antibody binding
resulting in ~23% underestimation (4.74x10⁻⁷ adduct/nucleotide) compared to
radioactivity measurement. Besides lower overall antibody binding, monoclonal
antibodies gave a variable response in adduct quantitation of DNA with different
modification frequencies. Thus, a gross underestimation of adducts was evident at
all the adduct levels tested. Negligible and unquantifiable binding was seen in
particular for DNA bearing the lowest level of (±)-anti-BPDE-DNA adducts.
Fig. 15. Effect of (±)-anti-BPDE modification level on antibody binding. Increasing concentrations of DNA containing 4500x10^3 adducts/10^6 nucleotides (■, □), 187x10^2 adducts/10^6 nucleotides (●, ○), and 0.6 adducts/10^6 nucleotides (▼, ▼) were immobilized to give indicated amount of adducts per slot of nitrocellulose filters. A range of concentrations of unmodified DNA was analyzed in parallel. Binding of polyclonal and monoclonal antibodies was determined by quantitative ISB assay. For low modified DNA ([3H]-BPDE treated calf thymus DNA), small peak intensity values obtained with corresponding concentrations of control unmodified DNA were subtracted from the modified DNA values.
It should be noted that quantitation of adducts in low modified DNA was achieved by immobilizing relatively higher amounts of DNA (~0.85 mg for 1.6 fmol) on the filter slots in ISB assay. Hence, the assay can be enhanced further by increasing DNA adsorption and/or signal amplification to provide specific detection of still lower levels of (±)-anti-BPDE modification by using polyclonal antibodies.

Dose response relationship of (±)-anti-BPDE-DNA adduct formation

Quantitation of (±)-anti-BPDE-DNA adducts induced by exposure of DNA to different doses of carcinogen gave identical values in ELISA and ISB with polyclonal antibodies (Figure 16). Moreover, owing to the high capacity of DNA binding to the filters, ISB was capable of detecting DNA modifications at carcinogen doses below 1 nM. The lower detection limit, under the present assay conditions, was in the range of 2 adducts/10⁷ nucleotides induced by in vitro treatment of DNA with 0.5 nM (±)-anti-BPDE. A similar dose dependent linear induction of adducts was also observed for in vivo modified DNA from human cells. However, the absolute adduct levels for DNA treated in vivo with the same carcinogen dose were lower than those obtained in vitro. The binding responses shown in Figure 16 with (±)-anti-BPDE dose vs. DNA reactivity were reproducible in several independent experiments.
Fig. 16. Dose response relationship of (±)-anti-BPDE induced adducts in non-competitive immunoassays. Adducts in DNA modified with a wide range of (±)-anti-BPDE doses in vitro (PBS) and in vivo (DMEM) were quantitated by non-competitive ELISA and ISB using PAb BP1. The adduct level of each modified DNA was quantitated from duplicate analysis of two to three different concentrations falling within the linear response range of the modified standard (1.87x10² adducts/10⁶ nucleotides). The data shown are from three to four independent experiments. The lines were calculated by the linear regression best fit analysis using data from corresponding in vitro and in vivo treatments.
Dose dependent differential repair of anti-BPDE-DNA adducts in human fibroblast cells

The ability of non-competitive ISB assays to quantitate very low levels of (±)-anti-BPDE induced DNA damage in very small amounts of DNA (<1 μg) allowed the comparison of adduct repair in human cells exposed to a range of genotoxin doses. The extent of repair was measured as the loss of antibody binding sites immuno-analyzed for a constant amount of DNA from the exposed cells. Figure 17 shows the repair of (±)-anti-BPDE-DNA adducts in repair-proficient normal human fibroblast cells as a function of post-treatment time at various levels of initial genotoxic damage. At very low doses (0.02 and 0.1 μM) of carcinogen, normal human fibroblasts were able to eliminate ~50% of the initial damage (0.88 and 3.44 adducts/10^6 nucleotides) in 4 and 8 h, respectively. There was no significant decrease in the comparable number of initial adducts (1.92 and 3.74 adducts/10^6 nucleotides) in repair-deficient xeroderma pigmentosum XP12BE fibroblast cells (Table 3). Notably, the repair rate for comparatively higher levels of initial genotoxic lesions in cellular DNA was found to be lower. At intermediate dosage of incipient DNA damage (~20 adducts/10^6 nucleotides), repair-proficient normal cells removed approx. 40 and 60% of the initial lesions in 8 and 24 h, respectively. At higher levels of DNA damage (105 and 176 adducts/10^6 nucleotides), only 33% and 20% decrease in the respective initial adducts was observed at 24 h post-treatment. Repair in XP12BE cells was consistently absent at higher initial levels of (±)-anti-BPDE induced
DNA damage (Table 3). The index of cellular DNA replication, as measured from changes in the specific radioactivity of DNA prelabeled with \[^{3}H\]dThd did not show genome duplication during the periods of repair analysis (Table 4). This indicates that the decrease in the number of antibody binding sites was not due to the dilution of the antigenic adducts via DNA replication but due to active cellular repair processes.
Fig. 17. Repair of bulky (±)-anti-BPDE adducts in human cells. Confluent [³H]dThd prelabeled cultures of normal fibroblast cells (HF1) were treated with 0.02, 0.1, 0.4, 2.0, 4.0 mM (±)-anti-BPDE as described in chapter II. After carcinogen removal, cells were incubated for various post-treatment times before harvesting and DNA isolation. ISB analysis of DNA modification was performed within the linear response range of the standard DNA (1.87x10² adducts/10⁶ nucleotides). Repair was expressed as the loss of adduct binding sites and normalized to initial damage of 0.88, 3.4, 21, 105 and 176 adducts/10⁶ nucleotides induced at various doses. Except for 0.02 mM dose, data shown for each plot are the mean ± SD of two to three separate experiments.
Table 3. Post-treatment loss of anti-BPDE adducts in repair-proficient and -deficient cells.

<table>
<thead>
<tr>
<th>Dose (mM)</th>
<th>Time (hr)</th>
<th>HF1 Cells</th>
<th>XP12BE Cells</th>
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<td>Modification (Add./10⁶ nucl.⁹)</td>
<td>Repair (%)</td>
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<tr>
<td></td>
<td>4</td>
<td>92.06±9.44</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>82.48±4.09</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>71.23±0.98</td>
<td>33</td>
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<td>4.0</td>
<td>0</td>
<td>176.7±0.9</td>
<td>0</td>
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<tr>
<td></td>
<td>4</td>
<td>157.4±5.6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>168.5±16</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>143.12±3.8</td>
<td>19</td>
</tr>
</tbody>
</table>

ᵃAdduct levels were quantitated using the linear portion of a reference standard containing 187 adducts/10⁶ bases as described in Fig. 15. Values represent ± S.D. from two to three separate experiments for each dose.
ᵇValues derived from a single experiment.
N.D., not determined.
Table 4. Absence of DNA replication in confluent cultures of carcinogen treated human cells at various times of repair analysis.

<table>
<thead>
<tr>
<th>Dose (mM)</th>
<th>Time labeling (hr)</th>
<th>HF1 Cells</th>
<th>XP12BE Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific Activity (dpm/ng DNA)</td>
<td>labeling (%)</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>13.88±0.68</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12.95±0.07</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>12.6±0.42</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>13.5±0.28</td>
<td>97</td>
</tr>
<tr>
<td>0.4</td>
<td>0</td>
<td>9.45±0.53</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.19±0.21</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>9.20±0.55</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>9.06±0.08</td>
<td>96</td>
</tr>
</tbody>
</table>

The fibroblast cells were uniformly labeled by growing in medium supplemented with 15-20 nCi/ml of [3H]dThd. After achieving confluence, the medium was replaced with Hank's buffer and cells were treated with the carcinogen as described. Treated cells were incubated in DMEM medium without serum for indicated times. DNA was quantitated by two independent methods and radioactivity of several microgram aliquots determined by scintillation counting. The data are average of replicate treatments.
BIOCHEMICAL MEDIATORS OF DNA DAMAGE INDUCED p53 STABILIZATION

Dose dependence and time course of p53 accumulation after (±)-anti-BPDE treatment

DNA damage serves as an important trigger to activate the cellular responses such as cell cycle arrest, DNA repair and apoptosis (Nelson and Kastan, 1994; Kastan et al. 1991). To establish a dose-response relationship between (±)-anti-BPDE induced DNA damage and p53 induction, excision repair-proficient human fibroblast (OSU-2) and lymphoblastoid cells (GM2184C) were treated with increasing concentrations of the carcinogen (0.05 to 1.0 μM). The DNA damage levels as detected by non-competitive immuno-slot blot assays, for varying doses used in these experiments, ranged from 3.76 to 107 adducts/10⁶ nucleotides in normal lymphoblastoid cells (Table 5). To correlate DNA damage with p53 protein accumulation, whole cell extracts from treated and untreated control cells were analyzed using antibodies specific for different epitopes of the tumor suppressor protein. Results with low passage normal human diploid fibroblasts (OSU-2), showed a rapid and sustained accumulation of the p53 protein in response to (±)-anti-BPDE treatment. A brief treatment of the cells with the genotoxin resulted in a p53 induction response that was apparent with doses as low as 0.05 μM.
Table 5 Carcinogen dose dependent induction of (±)-anti-BPDE DNA adducts in normal human lymphoblastoid cells.

<table>
<thead>
<tr>
<th>Dose (μM)</th>
<th>Adducts/10⁶ nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>3.76 ± 0.31</td>
</tr>
<tr>
<td>0.1</td>
<td>8.76 ± 0.91</td>
</tr>
<tr>
<td>0.5</td>
<td>49.5 ± 0.25</td>
</tr>
<tr>
<td>1.0</td>
<td>107 ± 7.72</td>
</tr>
</tbody>
</table>

Normal lymphoblastoid cells were treated with the indicated doses of the carcinogen for 30 minutes, lysed and processed for DNA isolation. The adduct levels (± SEM) were quantitated by the non-competitive immuno-slot blot assay as described in chapter II.
Failure of p53 accumulation upon treatment of cells with the inactivated carcinogen (hydrolyzed in aqueous media) indicated that the response was due to the interaction of the reactive epoxide metabolite with the genome leading to DNA damage. The extent of p53 accumulation increased proportionally with the dose of the carcinogen. However the p53 induction response was saturable at all the time points from treatment with doses higher than 0.5 μM (Figure 18, top and center). Time course experiments revealed that the tumor suppressor protein begins to accumulate as early as 2.5 hours and remains at elevated levels for at least 24 hours after exposure (Figure 18, center). Control cells treated with vehicle alone did not indicate any change in the intracellular levels of the p53 protein. Cell lysates from transformed HeLa cells, containing high constitutive levels of p53, served as a positive control for specific antibody based detection by western blotting. The p53 protein specific binding of antibodies was further established by using extracts of p53 null HL-60 cells which demonstrated absence of any immuno-reactive protein bands at 53 Kd (data not shown).

For subsequent studies to relate the effects of DNA damage on cell cycle arrest and apoptosis, lymphoblastoid cells grown in suspension cultures provided an useful cellular model. In order to establish that the p53 induction response was similar to that of fibroblasts, lymphoblastoid cells were evaluated under similar conditions. Genotoxin treated normal and repair deficient (XPA) lymphoblastoid cells displayed an identical response in which the p53 protein
Fig. 18. Carcinogen dose and time-dependent accumulation of p53 protein in fibroblasts (top and center) and lymphoblastoid cells (bottom) after (±)-anti-BPDE treatment. Exponentially growing cells were treated with the indicated doses of the carcinogen for 30 minutes and lysed at various time points. Cellular extracts (equivalents of 1.2 X 10^5 cells / lane) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and visualized by enhanced chemiluminescence as described in chapter II.
levels were increased in a dose and time dependent manner in repair-proficient and repair-deficient lymphoblastoid cells (Figure 18, bottom panel and data not shown).

Modulation of p53 accumulation by inhibition of poly(ADP-ribose) polymerase

It is now well documented that exposure to various physical and chemical carcinogenic DNA damaging agents cause an increase in poly (ADP-ribose)polymerase (PARP) activity within minutes of genotoxic insult (Whitacre et al. 1995). The synthesis of poly(ADP-ribose) polymers has been described as a necessary accompaniment for efficient DNA repair processes (Satoh and Lindahl, 1992). The periodic proximity and similarity of PARP activity induction and elevated p53 protein levels suggest that PARP may be a potential DNA damage inducible candidate involved in the post-translational modification of the p53 protein conferring increased half-life (Whitacre et al. 1995). To evaluate the importance of PARP for the p53 induction response, cells with normal PARP activity but different repair capacities were treated with the PARP specific inhibitor, 3-aminobenzamide (3-AB) before and after exposure of cells to (±)-anti-BPDE. As shown in Figure 19 (top), pre-incubation of normal lymphoblastoid cells with 3-AB led to a decrease in the overall induction of the p53 protein compared to cells treated with (±)-anti-BPDE alone. Densitometric
quantitation of the films, exposed to non-saturating densities, revealed a 50-60% decrease in the intensity of the p53 protein bands at 4 and 8 h after DNA damage. The treatment of cells with 3-AB before carcinogen exposure also led to a concomitant decrease in the (±)-anti-BPDE-DNA adduct levels by ~60% (Figure 19), indicating that the p53 accumulation was clearly a consequence of the induced DNA damage. However, the treatment of the cells with the PARP inhibitor, after genotoxic insult also resulted in a decreased p53 induction response (Figure 19). Thus poly ADP-ribosylation itself may be directly involved in the pathway for the stabilization of the p53 protein. Since PARP activity in XPA cells has been shown to be decreased due to the lowered amount of excision repair mediated strand breaks after alkylation damage, repair-deficient (XPA) lymphoblastoid cells were also treated under identical conditions. An analogous decrease in p53 levels was noticed in these cells when 3-AB was present prior to and after (±)-anti-BPDE treatment (Figure 19, center). In addition, pre-incubation of XP lymphoblastoid cells with 3-AB also resulted in a concurrent decrease in the (±)-anti-BPDE DNA adduct levels (Figure 20). The decrease in p53 levels was not due to a reduced amount of protein loaded in the gel as indicated by the fast green dye (FG) staining of the lower portion of a representative protein blot at ~35 Kd range (Figure 19, bottom).
Fig. 19. Effect of 3-aminobenzamide (3-AB) on p53 accumulation. Exponentially growing normal lymphoblastoid (top) and XPA lymphoblastoid cells (center) were treated with 0.5 μM (±)-anti-BPDE in the presence (pre) or absence (post) of 3-aminobenzamide (5 mM), resuspended in fresh media containing 3-AB and p53 accumulation was monitored after 0, 4 and 8 h. Control cells (lanes 1-3) were treated with 1% ethanol and then incubated with 5 mM 3-AB (post). Fast green (FG) staining of the bottom portion of the protein blot from normal lymphoblastoid cells to indicate equal protein loading is shown (bottom panel).
Fig. 20. Effect of 3-aminobenzamide pre-treatment on the levels of DNA damage in normal and XP lymphoblastoid cells. The (±)-anti-BPDE adduct levels were quantitated by immuno-slot blot assay as described in chapter II.
Induction of p53 levels by inhibitors of DNA replication

While various types of DNA lesions are shown to elevate the intracellular levels of p53, the initiating pathways responsible for this effect remain unclear. The role of DNA strand breaks in triggering p53 levels has been suggested but not proven unequivocally (Nelson and Kastan, 1994; Hess et al. 1994). To investigate the role of replication mediated DNA strand breaks at the polymerase pause sites due to (±)-anti-BPDE-DNA adducts, repair-deficient lymphoblastoid cells were incubated with inhibitors of DNA replication immediately after exposure to the carcinogen at doses of 0.125 μM and 0.5 μM. Since these cells are deficient in the excision repair pathway, DNA damage and the inhibition of replication would not lead to an increase in the formation of DNA strand breaks either through repair or replication (Nelson and Kastan, 1994). The results shown in Figure 21 describe the effect on p53 accumulation when the ribonucleotide reductase inhibitor, hydroxyurea and the DNA replication inhibitor, AraC were included in the post incubation period after carcinogen exposure. The treatment of cells with (±)-anti-BPDE resulted in an enhancement (~2 fold) of the p53 protein accumulation despite the block of cellular replication (Figure 21). Interestingly, in control untreated cells, block of replication with HU and AraC, stimulated a p53 accumulation response that was detectable in 4 and 8 h as opposed to vehicle treated cells not blocked for replication (Figure 21). This indicates that the perturbation of DNA synthesis
might itself be a trigger for p53 induction. In separate experiments, treatment of cells with AraC alone caused an accumulation of p53 protein levels analogous to the response seen in combination with HU (data not shown).

<table>
<thead>
<tr>
<th>AraC + HU</th>
<th>Time [hr]</th>
<th>BPDE [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 21. Effect of inhibition of DNA replication on DNA damage induced p53 accumulation. Repair deficient lymphoblastoid cells (XPA) were treated with the indicated doses of (±)-anti-BPDE and incubated in the presence (+) or absence (-) of hydroxyurea (2 mM) and AraC (10 μM) for 4 and 8 h and processed for p53 protein levels as described in Figure 18. Control cells were treated with 1% ethanol.
Effect of Calphostin-C on DNA damage induced p53 levels

It has been recently suggested that the phosphorylation of the tumor suppressor protein might be necessary for its stabilization after DNA damage (Hupp and Lane, 1994). To investigate the role of phosphorylation on p53 stabilization/induction, fibroblasts (OSU-2) and lymphoblastoid cells (GM2184C) were pre-treated for 1.5 h with 300 nM of the PKC inhibitor, calphostin-C and the induction patterns of the p53 protein were monitored subsequent to (±)-anti-BPDE treatment. Pre-treatment of both the fibroblast and lymphoblastoid cells with calphostin-C led to a complete abrogation of the elevated p53 response observable as a result of cellular DNA damage (Figure 22, top and center). This failure of p53 accumulation response by damage was observed at 4 and 8 h post-incubation after carcinogen treatment. Once again the decrease in the amount of the p53 protein was not due to changes in the total amount of protein loaded as indicated by the fast green staining of the protein blots (Figure 22, bottom). However, at the 8 h post treatment time, there was a slight increase in the levels of the p53 protein in the control cells treated with calphostin-C alone (Figure 22).

Cell lineage dependent cellular response to DNA damage

Earlier reports have shown that various effects and the subsequent fate of a cell in response to DNA damage, i.e., to undergo cell cycle arrest and/or
apoptosis that occur is dependent on factors like cellular histotype, extent of
genotoxic damage and functional status of the relevant gene products i.e., p53
and bcl2 (Canman et al. 1994; Di Leonardo et al. 1994; Lowe et al. 1993; Fan et al.
1994; Liebermann et al. 1995). To delineate the differences in the post-damage
responses of distinct cellular histotypes, exponentially growing fibroblasts (OSU-
2) and lymphoblastoid cells (GM2184C) were treated with (±)-anti-BPDE and
analyzed for inter-nucleosomal DNA fragmentation. As shown in Figure 23,
agarose gel electrophoresis of cellular DNA from carcinogen treated
lymphoblastoid cells showed DNA fragmentation characteristic of apoptosis
(Figure 23, lanes marked L-N). Time course experiments revealed that the
lymphoblastoid cells undergo cell cycle arrest before the induction of apoptosis
at approximately 24 h (data not shown). In contrast, agarose gel analysis of
DNA from fibroblasts did not indicate any detectable inter-nucleosomal DNA
fragmentation (Figure 23, lanes marked OSU-2). Morphological analysis of the
lymphoblastoid cells also revealed cellular changes characteristic of apoptosis
while the fibroblasts exhibited an increase in cellular size resembling large
senescent cells (data not shown). To substantiate the differences in the above
mentioned responses, cells were treated with the carcinogen and their transition
through the cell cycle was analyzed by flow cytometry at various post-treatment
times. The mitotic inhibitor nocodazole (0.4 μg/ml) was added immediately to
cultures after the genotoxin treatment to avoid confounding results due to cells
escaping the G2/M phase (Bae et al. 1995). It may be noted that, non-adherent fibroblasts were collected along with the trypsinized cells for the flow cytometric analysis. As shown in Figure 24 (top), normal fibroblasts and lymphoblastoid cells treated with ethanol vehicle alone showed a typical cell cycle profile of exponentially growing cultures with a higher fraction of the cells in the G1 phase analyzed at 24 h. Nocodazole treatment of these cells caused the expected shift and accumulation of control cells at the G2/M phase (Figure 24, center). However, treatment of fibroblasts to an initial dose of 1 μM of the carcinogen prevented the progression of cells from the G1 phase and caused cell cycle arrest at the G1/S boundary. About 82% of the original G1 population was arrested at the G1 phase in fibroblast cells treated with 1 μM of the carcinogen while the G1 fraction had decreased to 25% in the control cells incubated with nocodazole (Figure 24, center and bottom left). In contrast to fibroblasts, lymphoblastoid cells were able to activate programmed cell death observed at 24 h following carcinogen exposure (1.0 and 0.5 μM) and apoptotic cells appeared as a distinct population with a lower DNA content (Figure 24, bottom right and data not shown). Furthermore, analysis of fibroblasts treated with higher doses (up to 4 μM) of the carcinogen and isolated after longer post-treatment intervals (48 h) did not indicate any evidence of apoptosis (data not shown).
Fig. 22. Abrogation of the p53 induction response by treatment of human cells with the PKC inhibitor, calphostin-C. Exponentially growing normal fibroblast (top) and lymphoblastoid cells (center) were pre-incubated with 300 nM of calphostin-C for 1.5 h and then treated with the indicated dose of the carcinogen. Cell lysates were prepared at the indicated time points and p53 protein was detected using a mixture of antibodies as described in materials and methods. The bottom panel shows the protein blot of the lymphoblastoid cellular extracts stained with fast green (FG) to indicate equal protein loading.
Fig. 23. Effect of (±)-anti-BPDE treatment on DNA fragmentation in fibroblasts (OSU-2) and normal lymphoblastoid (L-N) cells. Cells were treated with the indicated doses of the carcinogen for 30 minutes and incubated in fresh media for 24 h. Fragmented DNA was separated and analyzed by agarose gel electrophoresis as described in chapter II. Lanes marked M are 1-kbp ladder.
Fig. 24. Cell cycle analysis of carcinogen treated human cells in culture. Exponentially growing normal fibroblast (OSU-2) and lymphoblastoid cells (L-N) were treated with 1.0 μM (±)-anti-BPDE for 30 minutes and incubated in fresh media containing 0.4 μg/ml nocodazole for 24 h. The cells were stained with propidium iodide for DNA content analysis. The G1, S-phase, G2/M and apoptotic cells are indicated for the various treatment schedules. Flow-cytometric analysis was performed as described in chapter II.
Fig. 24.

DNA CONTENT
EFFECT OF p53 MUTATIONS ON CELLULAR RESPONSES TO DNA DAMAGE

(±)-anti-BPDE induced elevation of p53 levels in Li-Fraumeni (LFS) fibroblasts

Recent studies indicate that the C-terminal domain of the tumor suppressor protein is involved in the stabilization of the protein in response to DNA damage (Hupp and Lane, 1994). In addition, studies have also indicated that sequence specific DNA binding of p53 results in an increased stability of the protein (Liu and Pelling, 1995). To analyze the effects of mutations within the DNA binding domains on p53 protein stabilization, immortalized human fibroblasts, derived from Li-Fraumeni syndrome (LFS) patients, with homozygous mutations at codons 184 and 248 of the p53 gene were treated with increasing doses of (±)-anti-BPDE. Normal fibroblasts were also treated simultaneously to compare the p53 elevation patterns. The initial adduct levels induced by a brief treatment of the carcinogen ranged from ~30 to 140 adducts (Table 6). The cell lysates were analyzed for increase in p53 protein levels after 6 hours post treatment with the carcinogen. As shown in Figure 25, Li-Fraumeni fibroblasts were proficient in inducing their (mutant) p53 protein levels after carcinogen treatment. However, the extent of induction varied between the normal and the mutant fibroblast cells. Cells with the homozygous mutation at
codon 184 (041) showed the lowest p53 induction response while the response of the codon 248 mutant cell line (087) was similar to that of the normal cells. In the immortalized cells, the immunodetection of the p53 protein exhibited a doublet at 53 Kd which was similar to that obtained with He La cell lysates (Figure 18, Chapter V). Equal protein loading was confirmed by fast green dye staining of the lower portion of protein blot (data not shown).

Effect of DNA damage on cell cycle arrest in Li-Fraumeni fibroblasts

To determine if mutations in the DNA binding domain of p53 were able to abolish the cell cycle arrest response after DNA damage, exponentially growing normal and LFS fibroblasts were treated with various doses of (±)-anti-BPDE (1.2 and 0.6 µM) for 30 minutes and analyzed for cell cycle arrest using flow cytometry. The cells were resuspended in fresh medium containing the mitotic inhibitor nocodazole (0.4 µg/ml) for reasons described in Chapter V. As shown in Figure 26, cells treated with nocodazole alone showed a gradual decrease in the G1 fraction at 8 h and 22 h due to the accumulation of cells at the G2 phase. However, cells treated with the carcinogen showed a higher proportion of the G1 population indicating the activation of cell cycle arrest. This response was qualitatively similar to all the three cell lines. However, the 041 cell line was sensitive to nocodazole treatment as a substantial fraction (~20%) of the control cells appeared as a nonviable cells. To further substantiate the results, the LFS cells were treated with a lower dose of the
carcinogen (0.6 μM) and their transition through the cell cycle was studied. The LFS cells were able to undergo cell cycle arrest at the G1-S phase even at doses (0.6 μM) that gave an initial adduct level of ~50/10^6 bases (Table 7).
Table 6. Carcinogen dose dependent induction of (±)-anti-BPDE DNA adducts in human fibroblast cells.

<table>
<thead>
<tr>
<th>Dose (µM)</th>
<th>Adducts/10⁶ nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HF1</td>
</tr>
<tr>
<td>0.3</td>
<td>42.4 ± 1.2</td>
</tr>
<tr>
<td>0.6</td>
<td>73.0 ± 1.3</td>
</tr>
<tr>
<td>1.2</td>
<td>142.1 ± 4.5</td>
</tr>
</tbody>
</table>

Exponentially growing cells were treated with the indicated doses of the carcinogen for 30 minutes, lysed and processed for DNA isolation. The adduct levels (± SD) were quantitated by the non-competitive immuno-slot blot assay as described in Chapter II.
Fig. 25. (±)-anti-BPDE dose dependent accumulation of p53 protein levels in normal and Li-Fraumeni fibroblasts. Exponentially growing cells were treated with the indicated doses of the carcinogen for 30 minutes, rinsed with phosphate buffered saline, incubated in fresh medium and lysed after 6 h. Cellular extracts (equivalent to 1.2 x 10^5 cells/lane) were separated by SDS-polyacrylamide gel electrophoresis and processed for p53 immunoblot analysis as described in chapter II. Representative blot of two separate experiments that gave similar results is shown.
Table 7. Cell cycle arrest response in LFS cells treated with intermediate doses of (+)-anti-BPDE (0.6 μM).

<table>
<thead>
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<th>Time (h)</th>
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<th>0.0</th>
<th>0.6</th>
<th>0.6</th>
</tr>
</thead>
<tbody>
<tr>
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<td>37.8 (100)</td>
<td>37.8 (100)</td>
<td>50.0 (100)</td>
<td>50.0 (100)</td>
</tr>
<tr>
<td>8</td>
<td>22.1 (58)</td>
<td>31.6 (83)</td>
<td>22.6 (45)</td>
<td>39.6 (79)</td>
</tr>
<tr>
<td>18</td>
<td>16.2 (42)</td>
<td>25.4 (67)</td>
<td>11.1 (23)</td>
<td>39.5 (79)</td>
</tr>
</tbody>
</table>

Exponentially growing cells were treated with the indicated doses of the carcinogen for 30 minutes and resuspended in fresh medium containing 0.4 μg/ml of nocodazole. At the indicated time points the cells were trypsinized and fixed in 75% ice-cold ethanol and analyzed by flow cytometry as described in chapter II. The fraction of G1 population in the total population is indicated and the %G1 population that is arrested with respect to the 0 h untreated sample is given in parentheses.
Fig. 26. Induction of cell cycle arrest in LFS fibroblasts following treatment with (±)-anti-BPDE. Exponentially growing fibroblasts were treated with 1.2 μM (hatched bars, 4-6) of the carcinogen for 30 minutes and resuspended in fresh medium containing 0.4 μg/ml nocodazole. Control cells (empty bars, 1-3) were treated with 1% ethanol. Cells were recovered at the indicated time points and analyzed by flow cytometry as described in chapter II. The % G1 population was calculated by normalizing the G1 fraction of the 0 h samples to 100%.
ENANTIOMER ADDUCT CONFORMATIONS ARE DETERMINANTS OF ANTIBODY SPECIFICITY

When DNA interacts with (±)-anti-BPDE in vitro or in vivo, the resulting spectrum of modifications represent both the variable reactivity at several DNA base nucleophilic sites and the steric configuration of the reacting carcinogen. The immunological reactivity of antibodies, so far developed against anti-BPDE modified DNA, is primarily directed towards the major reaction product at N² of guanine base (Poirier, 1984). Additionally, the procedures of induction and analysis have mostly favored its detection, e.g., using protein conjugated modified deoxyguanosine or heavily modified DNA as antigen for screening of the antibodies. Among the remaining mix, the BPDE adducts are either unstable e.g., BPDE-N7-dG or constitute very small proportion e.g., N⁶-dA adduct. The lower reactivity of polyclonal antibodies against (-)-anti-BPDE-N²-dG, seen in our
experiments, might also be due to low levels of this adduct in the initial (±)-anti-BPDE modified DNA antigen. However, antibodies against minor adducts can be elicited by a systematic use of suitably designed DNA antigens (preferably synthetic oligodeoxynucleotides containing specific adducts). In contrast to polyclonal antibodies, small numbers of clones producing monoclonal antibodies, against (-)-anti-BPDE-N^2-dG or any other minor DNA adduct, can be selected by use of deliberately and carefully chosen modified antigenic DNA. It appears that the MAb 5D2 obtained upon screening with (±)-anti-BPDE-DNA antigen, fortuitously selected such a clone with specificity towards the (-)-enantiomer adduct. For achieving defined base adduct specificity, antibodies have also been developed against (±)-anti-BPDE modified guanine base made antigenic upon linking to a protein (Santella et al. 1984). In these experiments, however, attention was not given to the stereospecific nature of the BPDE-dG adduct and consequently the reason for the observed specificity of the resulting antibodies can only be conjectured. One such monoclonal antibody, 8E11, showed a higher affinity for the cognate anti-BPDE-dG mono-adduct than the anti-BPDE-DNA and also showed a lesser degree of affinity for anti-BPDE-DNA than antibodies generated against modified DNA (Santella et al. 1985). Among those antibodies developed against modified DNA, the best response, in terms of both sensitivity and specificity, was provided by the MAb 5D2 requiring 17 fmol of (±)-anti-BPDE DNA and more than a 5000-fold greater amount of modified guanine base to achieve 50 % inhibition in competitive
ELISA (Santella et al. 1985). This high affinity for modified DNA, rather than
differential enantiomer adduct specificity (not known initially), was the basis of
selecting the MAb 5D2 in our present comparative study.

Polyclonal antibodies, developed initially for the quantitative assessment of
BP-adducts, cross react with a number of structurally related PAHs modified DNA
(Santella et al. 1990b). The antisera developed against anti-BPDE recognize
chrysene-1,2-diol-3,4-epoxide modified DNA more efficiently than anti-BPDE
modified DNA (Newman et al. 1990). Nevertheless, these cross reacting antibodies,
apparently recognizing related BPDE adducts, provide a general index of PAH
exposure in epidemiological studies (Newman et al. 1988; Poirier, 1984; Santella et al.
1990a; Perera et al. 1987). An obvious alternative, i.e., development of a series of
monoclonal antibodies with stringent specificities, has not overcome the problem of
promiscuous antibody specificity for PAH modified DNA (Newman et al. 1990;
Santella et al. 1990b). Molecular modeling studies have been invoked to suggest that
the structural similarity of the adduct immunological epitopes, e.g., between
benzo(a)pyrene and benzo(a)chrysene moiety, was the primary antigenic
determinant for cross recognition of antibodies developed against either adduct
(Weston et al. 1990; Newman et al. 1990). While the correlations of antibody binding
and adduct structure seem to explain an apparently broad antibody specificity, these
data as well as mechanistic inferences need to be more critically evaluated in view of
characteristic adduct conformations. It is conceivable, for example, that the
monoclonal antibodies screened against the mixed (+)-adduct containing antigens were actually specific for one or the other enantiomer adduct of various PAHs (Newman et al. 1990; Weston et al. 1990; Santella et al. 1984; Santella et al. 1985; van Schooten et al. 1987). Thus the observed differences of antigen-antibody binding might very well be due to those specificity variations. Therefore, accurate deductions about adduct epitope recognition and mechanisms can be made only upon comparisons of antibody binding with the corresponding enantiomer adducts of different PAHs.

The covalent adducts of various BPDE epoxides have been categorized as site I or site II (Geacintov et al. 1991). Considerable evidence, based on potential energy minimization studies and spectroscopy, indicates that the type of antigenic modification in the (+)- and (-)-anti-BPDE-oligomers and (+)-anti-BPDE-DNA, used in our study, belong to site II class adducts (Geacintov et al. 1991; Santos et al. 1992). The pyrenyl ring of the (+)- and (-)-trans-anti-BPDE is tilted towards the 5'-end of the (+)-anti-BPDE modified strand and towards 3' end of the (-)-anti-BPDE modified strand (Singh et al. 1991; Santos et al. 1992; Cosman et al. 1993). These ring orientations should expectedly interact with various proteins, including IgG molecules in a considerably different manner. The demonstration of differential cleavage of (+)- and (-)-anti-BPDE adduct containing duplex oligomers by spleen and snake venom phosphodiesterases provides experimental support for this distinguishing protein-DNA interaction (Cheh et al. 1990). The data showing a clear
discrimination by various IgG proteins of rabbit and mouse origin, are also compatible with differential adduct-protein interaction. However, it is notable that the orientations for different enantiomeric adducts have been depicted for the duplexed B-DNA (Singh et al. 1991; Santos et al. 1992). Previous studies have not indicated, with any degree of certainty, whether the characteristic adduct conformation in duplexes are manifested in the denatured, single-stranded conformation of the modified DNA and adduct containing oligomers. The data thus show that the characteristic adduct preferences of polyclonal and monoclonal antibodies are maintained in duplexed antigens, albeit with relatively lower binding efficiency. Enhancement of binding to modified DNA upon denaturation has been reported in earlier competitive and non-competitive immunoassays (Newman et al. 1990; Santella et al. 1985; Wani et al. 1984). The duplex antigen binding data is consistent with the model placing the pyrenyl moiety external and accessible to interact with the IgG molecule. Decrease in binding could very well be due to the burial of certain portions of the antigenic epitopes within the minor groove of the DNA duplex. Nevertheless, the distinct differences of antibody binding to (+)- and (-)-adducts in single-stranded DNA antigens immobilized to two different types of matrices, show that the characteristic (+)- and (-)- adduct conformations are manifested irrespective of single- or double-stranded nature of DNA.

The studies do not particularly address the antibody specificity for adducts of BPDE derived from syn-stereoisomer or the adducts of cis-configuration. It is very
likely that antibodies can be developed (by selecting antigens containing distinct adducts to elicit immunogenic response or antibody screening) against these DNA base adducts. Such truly mono-specific antibodies of known chemical and stereochemical adduct affinity can be utilized in various immunoanalytical studies for the investigation of precise origin and fate of an adduct or mechanism of protein-DNA adduct interaction. Understanding of these phenomena is critical to many experimental studies e.g., selective repair of BPDE adducts in mammalian cells (Chen et al. 1992), nature of adducts being recognized by cellular processing proteins in vivo or those being used as analytical tools in vitro (Thomas et al. 1988) and, of course, the nature and origin of culprit adducts being identified with an ever-increasing vigor in epidemiological studies (Harris, 1991; Santella et al. 1990b; Poirier, 1993). Lastly, the sequence specific binding studies with racemic as well as (+)- and (-) enantiomeric species of BPDE show the dependence of reactivity on guanine base flanking sequence (Rill and Marsch, 1990). Developing antibodies, against appropriately designed oligodeoxynucleotide sequences containing strategically situated specific adducts, to qualitatively and quantitatively distinguish the non-random base modifications, would help us understand the basis of the differences in the biological effects of the enantiomers of anti-BPDE.
QUANTITATIVE ASSESSMENT OF (±)-*anti*-BPDE DNA ADDUCTS SHOW
DOSE DEPENDENT DIFFERENCES IN REPAIR RATES

Various quantitative immunoanalytical approaches utilizing carcinogen-DNA adduct specific antibodies have proven to be valuable in risk assessment and adduct processing studies (Perera et al. 1988; Everson et al. 1988). Polyclonal and monoclonal antibodies specific for (±)-*anti*-BPDE-DNA adducts were used in non-competitive immunoassays to determine low levels of DNA damage induced *in vitro* and *in vivo*. Non-competitive ELISA showed limited utility for quantitating adducts resulting from treatments at doses below 0.1 mM *anti*-BPDE. However, in ISB assays, polyclonal antibodies exhibited greater than 100-fold binding affinity and detected as low as 2 adducts/10^7 nucleotides. As shown in chapter 3, these polyclonal and monoclonal antibodies differentially recognize the (+)- and (-)-*anti*-BPDE-N^2-dG adducts in DNA (Venkatachalam and Wani, 1994). Thus, the higher binding affinity and sensitivity of detection by polyclonal antibodies could very well be attributed to their preference for the (+)-*anti*-BPDE-N^2-dG adduct that constitutes the majority of the adducts induced by racemic *anti*-BPDE (Meehan and Straub, 1979; van Schooten et al. 1987). Previous reports have indicated that the binding efficiency of various polyclonal and monoclonal antibodies varies with the level of DNA modification. These binding variations of 1.4 to 11-fold (Santella et al. 1988) or
2.5 to 5-fold (van Schooten et al. 1987) have been implicated in the faulty estimation of low frequency adduct containing DNA in competitive immunoassays. The differences in the antibody affinity for highly and sparsely modified DNA were suggested to be due to the presence or absence of adduct clusters of variable antigenicity within the DNA sequences (van Schooten et al. 1987). It may be noted that these studies did not specifically assess the (+)- or (-)-anti-BPDE N2-dG and (+)- or (-)-anti-BPDE-N6-dA adduct preferences of the antibodies. Our data show that polyclonal antibody-based quantitation by non-competitive immunoassays is independent of adduct frequency in DNA. On the contrary, drastic variations of monoclonal antibody binding did result in gross adduct underestimation by the non-competitive ISB assay. Thus the differences in the antibody binding efficiency against DNA samples of different adduct frequency may very well depend upon the nature of the antibody used in the assay. An underestimation of 23% for the [3H]-BPDE modified sample by our polyclonal antibodies is consistent with its mono-adduct preference for (+)-anti-BPDE-N2-dG as this lesion comprises about 80% of the total adduct population (van Schooten et al. 1987; Meehan and Straub, 1979). Thus, the quantitative linear dose response observed with the polyclonal antibodies essentially reflects their binding specificity for the major (+)-anti-BPDE-dG adduct present in the samples. Since the minor adduct decreases proportionately with lower DNA modification, the (-)-anti-BPDE-N2-dG adduct preference of monoclonal antibody 5D2 would be expected to give lower binding response as well as adduct
underestimation. The specificity towards the minor adduct also limits the sensitivity of the monoclonal antibody for detecting low levels of DNA adducts generated by racemic (±)-anti-BPDE. However, it should be possible to design a study by selecting a monoclonal antibody of stringent specificity for (+)-anti-BPDE-dG adduct and determine its sensitivity in non-competitive assays. This could further clarify the extent of contribution by the type of the antibody or the immunoassay in the quantitative assessment of (±)-anti-BPDE modified DNA.

The bulky genotoxic lesions like pyrimidine dimers and (±)-anti-BPDE-DNA adducts are known to interfere with normal DNA transactions and their removal is indispensable for normal cellular functions. Early studies on (±)-anti-BPDE-DNA adduct repair in human cells have relied on determining the loss of DNA bound radiolabeled carcinogen or disappearance of Uvr ABC excision nuclease cleavage sites (Koostra, 1982; Yang et al. 1980; Chen et al. 1992; Van Houten et al. 1986). Problems with quantitative detection of adducts at low levels have limited the scope of the previous reports. Interpretation of these data is further complicated due to differences in the reactivity of (±)-anti-BPDE with cellular DNA under different treatment conditions and rapid inactivation of the carcinogen in aqueous media. A review of the literature reveals inconsistencies of carcinogen dose to DNA damage relationship shown for anti-BPDE under in vivo conditions (van Schooten et al. 1987; Kurian et al. 1992; Van Houten et al. 1986). The contrasting results could be due to various extents of medium-mediated carcinogen quenching which may modify the
effective dose delivered to the cellular nucleus. Therefore, the comparison of the rates of adduct repair based upon the initial DNA damage rather than the carcinogen dosage would be more meaningful. The sensitive non-competitive immunoassay utilizing polyclonal antibodies of mono-adduct specificity enabled the repair analysis of the major (+)-anti-BPDE-N²-dG adduct at very low levels of initial damage. The repair rate observed with high carcinogen dose (2 mM, corresponding to 105 adducts/10⁶ nucleotides) is consistent with earlier reports of the persistence of >50% adducts at 24 h for similar initial adduct level (Chen et al. 1992; koostra, 1982).

The absence of repair in XP12BE cells agrees with previous observations indicating the inability of these cells to excise bulky anti-BPDE lesions (Van Houten et al. 1986; koostra, 1982). Furthermore, the results show that the eventual persistence of adducts is directly linked to the initial genotoxic insult. Repair proficient human cells efficiently removed lower levels of damage from their genome and the repair rate gradually decreased with the increase in the extent of initial damage. A decreased repair activity at higher doses may be due to an overall saturation of the repair enzyme activity and/or a direct inactivation of cellular repair proteins (Wei et al. 1991). The differential repair rates observed for the damage range of 0.88 to 176 adducts/10⁶ nucleotides could explain the dose dependent differences of mutational types induced by (+)-anti-BPDE (Wei et al. 1991). It has been speculated that altered ratio of adenine vs. guanine adducts, as a function of carcinogen dose, forming increased adenine adducts at lower doses might account for these mutational data.
Decreased repair rates, leaving higher amounts of unrepaired guanine adducts after heavy initial damage, would be responsible for a high frequency of GC→TA transversions seen at higher doses (Wei et al. 1991). At the same time, at low doses, adenine adducts and their corresponding mutations will predominate due to the efficient removal of guanine adducts. To date, the repair of anti-BPDE-dA adduct in DNA has not been studied in any detail. Mutational data suggest a decreased or absence of repair of this lesion in mammalian cells (Wei et al. 1991). However, this will have to be evaluated by direct adduct analysis and with relevant human cell types.

Epidemiological studies have shown an increased incidence of lung cancer as a function of tobacco smoke exposure (Islam and Schottenfeld, 1994; Falk et al. 1992). Slow adduct removal at high exposure levels has potential implication in the predisposition of heavy tobacco smokers to increased risks of cancer. It should be noted, however, that the processing of DNA damage is a complex cellular phenomenon involving various key factors responsible for adduct recognition, incision and excision (Sancar, 1994). Emerging evidence suggest that DNA repair may be intimately related to the interplay of several cellular processes including transcription (Hanawalt, 1994), cell cycle arrest (El-Deiry et al. 1994), tumor suppressor gene induction and apoptosis (Nelson and Kastan, 1994). In-depth studies integrating various cellular, molecular biological and toxicological approaches are necessary to delineate the complexity of responses to DNA damage.
MODULATION OF p53 ACCUMULATION RESPONSE BY INHIBITORS OF PKC AND PARP

Accumulation of cellular p53 protein in response to a variety of agents that damage DNA has been demonstrated in several earlier reports (Kastan et al. 1991; Hess et al. 1994; Bae et al. 1995; Bjelogrlic et al. 1994; Hall et al. 1993). While the kinetics of the p53 induction response has been shown to be similar in repair-proficient and repair-deficient cells, the biochemical and molecular events that lead to the increase in p53 levels have not been established clearly (Lu and Lane, 1993; Abrahams et al. 1995). Consistent with earlier studies, the results show an identical mode of p53 induction after DNA damage with repair-proficient and repair-deficient cells (Lu and Lane, 1993; Abrahams et al. 1995; Kaspian and Baird, 1996). However, in contrast to the reported findings showing the requirement of DNA strand breaks for the p53 induction response in XP cells (Nelson and Kastan, 1994), our data indicate that the increase in p53 levels may not be mediated solely via strand breaks. The induction and super-induction of p53 in XP lymphoblastoid cells treated with replication inhibitors in the absence and presence of (±)-anti-BPDE-DNA damage suggest that p53 elevation may result from any perturbation to the DNA replication machinery and/or structural distortions to the DNA helical structure. However, these results do not rule out the possibility of an increase in the amount of strand breaks at the sites of the
stalled replication enzyme leading to an enhanced effect on p53 accumulation. Nonetheless, previous studies have shown that PARP is necessary for the ligation of DNA strand breaks (Chatterjee and Berger, 1994). If DNA strand breaks are the only necessary trigger for the p53 elevation response, our experiments with the PARP inhibitor would have shown an increase in the above mentioned response in repair-proficient cells due to the lack of strand break ligation. These results, showing a decrease in the p53 accumulation response in the presence of the PARP inhibitor, argue against the role of DNA strand break formation as the only initiating factor in the p53 response pathway. Therefore, p53 might serve as a common cellular regulatory mediator for intracellular events specifically related to DNA replication that may potentially result in heritable genetic changes. This assumption is consistent with earlier findings which have indicated that DNA replication inhibitors can induce cellular p53 levels (Khanna and Lavin, 1993; Hess et al. 1994). Interestingly, the ability of p53 to bind structural distortions in DNA has recently been demonstrated (Lee et al. 1995). Though these results were obtained in vitro, it is likely that p53 might be capable of binding gross structural distortions in the DNA as encountered with UV and bulky chemical lesions within the cell.

In spite of a plethora of published reports concerning the role of p53 in the DNA damage response pathway (Nelson and Kastan, 1994; Kastan et al. 1991; Kastan et al. 1992), the early steps in the signal transduction mechanism
remain mostly hypothetical. The data, with PARP and PKC inhibitors demonstrate that more than one pathway may be in place to ensure the elevation of p53 levels in response to DNA damage. The 50-60% reduction in p53 levels in cells incubated in the presence of the PARP inhibitor 3-AB, indicate that poly(ADP-ribosylation) of p53 might be an important factor in stabilizing p53 levels. However, an earlier study has shown an enhancement of \((t)\text{-anti-BPDE}\) induced cellular p53 levels by 3-aminobenzamide (Stierum et al. 1995). The analytical methods and the dosage of the carcinogen (2.5 \(\mu\)M) used in these studies may be a reason for these contrasting effects. As shown in the results, the decrease in p53 protein was detectable only at lower exposure times of the immuno-blots that did not lead to saturating densities of the signal.

Furthermore, poly ADP-ribosylation of the chromatin has been implicated in various nuclear processes like transcriptional regulation and DNA repair (Satoh and Lindahl, 1992; Lautier et al. 1993). The reduced levels of carcinogen-DNA adduction in cells pre-incubated with 3-AB show that the dynamic nature of the chromatin can also modulate the extent of DNA damage caused by bulky carcinogens and is consistent with an earlier report (Kurian et al. 1992).

Nevertheless, the lowering of p53 levels upon post-damage incubation with 3-AB suggest that p53 is a potential candidate for modification by poly ADP-ribosylation. The increased half-life of poly ADP-ribosylated p53 protein would provide an unequivocal proof for this assumption. Another explanation for the
relative reduction in post-damage p53 levels may be the obstruction of the DNA binding capacity of the tumor suppressor protein due to the lack of poly ADP-ribosylation of histones. A recent report has documented the reduced p53 induction response in cells deficient in PARP synthesis thus confirming the potential role of poly ADP-ribosylation in this process (Whitacre et al. 1995).

Further studies are needed to address the role of PARP in stabilizing p53 levels.

The results with the PKC inhibitor, calphostin-C appear to indicate that phosphorylation of p53 might be the primary mechanism by which the protein is stabilized in the nucleus following DNA damage. However, the final conclusion about the role of protein kinase C in DNA damage induced signal transduction pathway(s) that lead(s) to p53 stabilization cannot be elucidated from the present data. Recent data also indicate that phosphorylation might be involved in the regulation of the post-damage p53 response and the ability of the tumor suppressor protein to bind its consensus sequences in the DNA (Zhang et al. 1994; Price and Calderwood, 1993). Consistent with the role of p53 phosphorylation, the AT (ataxia-telangiectasia) gene product, a PI-3 kinase, has been suggested to be involved in the signal transduction pathway leading to the stabilization of p53 levels (Savitsky et al. 1995; Khanna et al. 1995; Khanna and Lavin, 1993). This has been further substantiated by the abrogation of the sequence specific DNA binding capacity of p53 by a specific inhibitor of PI-3 kinase (Price and Youmell, 1996). The results are consistent with the report
showing that calphostin-C can down-regulate p53 induction after gamma and UV irradiation in normal and ataxia-telangiectasia cells (Khanna and Lavin, 1993). A direct role of PKC in regulating p53 function has been demonstrated by in vitro studies in which phosphorylation of the carboxy terminus of p53 leads to the activation of the DNA binding function of the protein (Hupp and Lane, 1994). However, in spite of the evidence that p53 phosphorylation is involved in the DNA damage response, the sequence of events that link DNA damage and the down-stream stabilization of p53 remains unclear. Since the activity of kinases can in turn be regulated by phosphorylation, the role of PKC and PI-3 kinase in the p53 induction response may either be a direct one involving p53 phosphorylation, or an indirect one in which one of the proteins is regulated by the other. In addition, it is important to note that the observed decrease in p53 levels after calphostin-C treatment is not due to the decreased binding of antibodies to the unphosphorylated forms of p53 as the mixture of antibodies (clones 1801 and DO-7) used in this study do not discriminate between the phosphorylated and the unphosphorylated forms of p53 (Kumar and Spandau, 1995). While the stabilization of p53 protein and its binding to consensus sequences (after DNA damage) might be mutually exclusive, earlier reports have indicated that the binding of p53 to its consensus sequences in the DNA leads to a change in the protein conformation that is similar to the mutant form (Halazonetis et al. 1993) and also confers resistance to ubiquitin mediated
proteolysis (Molinari and Milner, 1995). Thus, the inhibition of PKC activity might in turn perturb the ability of p53 to bind damaged DNA thereby increasing the degradation of the tumor suppressor protein which leads to a decrease in cellular p53 levels.

The cellular response to DNA damage is a complex phenomenon that is highly dependent upon various factors such as extent of damage, differentiation status of the cell and cellular type. Indeed, the results show that cell cycle arrest and apoptosis occur in a cell type dependent fashion. The prolonged cell cycle arrest exhibited by fibroblasts even at higher doses indicate that these cells might be resistant to apoptotic cell death induced by DNA damage. This is in agreement with earlier reports that have shown that fibroblasts do not undergo apoptosis after DNA damage (Canman et al. 1994; Di Leonardo et al. 1994). While the lymphoblastoid cells and fibroblast cells appear to sustain a similar post-damage p53 induction response, the outcome of these events namely, cell cycle arrest and/or apoptosis seem to depend on cell type specific factors. It is of interest that the lymphoblastoid cells do not show a typical oligo-nucleosomal DNA fragmentation as encountered with treatment of HL-60 cells (Venkatachalam et al. 1993). The DNA fragmentation patterns suggest the presence of high molecular weight DNA fragments ranging from several kilobases to several hundred kilobases and are characteristic of early stages of programmed cell death (Walker et al. 1994).
DNA DAMAGE RESPONSES IN p53 MUTANT CELLS ARE SIMILAR TO NORMAL CELLS

The p53 tumor suppressor protein has been identified as a multifunctional transcription factor that integrates various cellular responses to DNA damage (Kastan et al. 1995). In addition, recent data also suggest that this protein may play a role in the process of nucleotide excision repair (Wang et al. 1995; Ford and Hanawalt, 1995). The structural analysis of p53 reveals that the protein’s central core domain, essential for its transactivation function, is the target for hundreds of mutations. Interestingly, the two other domains of the protein, the N-terminal activation domain that binds to the basal transcription factors (TFIID and TFIIH) and the C-terminal domain involved in protein tetramerization and stabilization have the least number of mutations. The studies described here with the two LFS cell lines (with homozygous mutations in the DNA binding domain) were aimed at elucidating the effects of specific alterations in the p53 protein on the processes of, (a) p53 protein accumulation and (b) initiation of cell cycle arrest. Our data indicate that the stabilization of the p53 protein after DNA damage and its specific binding to DNA sequences may not be interrelated. These results further substantiate the role of other domains in the stabilization of the protein. Notably, earlier studies have shown that mutant p53 proteins have a higher half-life than their normal counterparts. However, these studies involve mutations that destabilize the p53
protein structure. It is of interest to note that the induction of the contact mutant (codon 248) was comparable to that of the normal while the codon 184 mutant protein induction was significantly lower. The basis for these observations are not clear. The codon 248 mutant has been shown to lack any DNA binding capacity to the p53 consensus sequences. This is due to the role of Arg\textsuperscript{248} in the wild type protein in making specific DNA contacts. The effect of mutation at codon 184 on protein structure and DNA binding ability is not known and awaits further analysis.

The cellular response to DNA damage is a fundamental necessity that safeguards the genome. The importance of the faithful propagation of genetic material would require fail-safe mechanisms involving several surrogate effectors. While the versatile role of p53 in a multitude of processes might serve to protect the genome, studies have shown that eukaryotic cells can circumvent p53 deficiency via other mechanisms to counteract the effects of DNA interacting agents (Johnson \textit{et al.} 1994; Macleod \textit{et al.} 1995). In addition, accumulation of point mutations in a transgene target has been shown to be similar in cells that are deficient in p53 in comparison to cells that harbor wild type p53 (Sands \textit{et al.} 1995). The results with LFS cells demonstrate that DNA damage induced cell cycle arrest can occur even in the absence of transcriptionally active p53. These results are consistent with the recent findings that the transcriptional target of p53 i.e., the CDK inhibitor p21 can be regulated in a p53 dependent and independent manner (Macleod \textit{et al.} 1995; Johnson \textit{et al.} 1994). Whether p21 plays a role in the induction of cell cycle arrest in
LFS cells subjected to DNA damage by (±)-anti-BPDE remains to be seen. Nevertheless, these results indirectly question the role of p53 in the cellular response to DNA damage. If mutations in p53 do not perturb the cell cycle arrest responses, what is the exact role of p53 in maintaining genomic integrity? Recent data regarding the role of p53 in the excision repair process seem to imply that the p53 protein may play a direct role via interacting with the multi-functional transcription factor TFIID involved in replication and repair (Wang et al. 1995). However, studies conducted with cell free systems do not suggest an apparent role of p53 in NER (Sancar, 1995). In addition, two studies have shown opposite roles of p53 in gene specific repair (Ford and Hanawalt, 1995; Wang et al. 1995). The contrasting roles of p53 in NER may in part be due to the different effects of the p53 mutations on the tertiary structure of the protein necessary for its cellular functions. While these studies have utilized cell lines with different mutations in the DNA binding domain of p53, it is necessary to emphasize that the interactions of p53 with TFIID (hypothesized to play a role in NER) do not involve the core domain. However, one cannot rule out the possibility that mutations in the core domain may be necessary to stabilize the p53-TFIID interactions or mutations in the DNA binding domains may destabilize the protein structure thereby abolishing the above mentioned interactions. Therefore, a systematic analysis of the role of mutations on specific cellular responses (involving p53) and the correlation of the results with the structural studies would be necessary to explain the genuine role of p53.
SUMMARY

The ability of a carcinogen to cause mutations depends upon various factors that include the spatial distribution of adducts, the susceptibility of specific sequences to carcinogen adduction, the local conformation of the adduct and the heterogeneity of repair within a gene. The aim of the above mentioned studies was to integrate the various aspects of the cellular responses to DNA damage using a multidisciplinary approach. The ultimate carcinogen, (±)-anti-BPDE was used as the model DNA damaging agent to study the effect of DNA damage at the cellular and the genetic level. The development of polyclonal antibodies that bound preferentially to the major carcinogenic adduct formed by (+)-anti-BPDE enabled us to quantitatively correlate the effects of DNA damage. In addition, comparison of DNA damage responses in in vitro models of distinct histotypical characteristics (lymphoblastoid vs. fibroblasts) and genetic backgrounds (normal p53 vs. mutant p53) were made.

The differential binding of antibodies to the major and minor enantiomer adducts of anti-BPDE with guanine indicates that the characterization of antibody specificity towards these species would be a necessary step for their rational use as biomonitoring tools. The exceptional specificity of these biomolecules in differentiating various adducts (guanine vs. adenine adducts) points out to the possibility of the precise analysis of adduct processing in biological systems. This
would in turn lead to the identification of the biologically relevant DNA lesions. In addition, the differential binding of antibodies to enantiomer adducts should pave way for creative approaches for their use in the structural analysis of carcinogen DNA adducts. The results also demonstrate the utility of non-competitive immunoassays with polyclonal antibodies of high adduct specificity and affinity for the detection and monitoring of very low levels of DNA damage induced in vitro and in vivo. Suitable sensitivity refinements of these assays can be performed for their routine application in adduct processing and molecular epidemiological studies. The quantitative assessment of in vivo DNA damage processing demonstrates the influence of DNA lesion burden on the overall extent of repair of bulky DNA damage. Assessment of the role of variable repair rates of bulky adducts in eliciting contrasting mutational spectra in mammalian cells could provide critical mechanistic insights regarding hazards associated with low dose human exposures.

The biochemical mechanisms within the cell that lead to the stabilization of the p53 tumor suppressor gene are yet to be discerned with certainty. Our studies show that DNA damage induced by the carcinogenic metabolite, (±)-anti-BPDE leads to a rapid and sustained increase in cellular p53 levels. The primary mode of p53 protein stabilization might occur via the phosphorylation of the tumor suppressor protein and poly(ADP-ribosylation) might serve as secondary means of regulation. In addition, the results demonstrate that DNA strand
breaks are not the only necessary alterations responsible for the p53 elevation response. The immediate response of p53 protein stabilization accompanies the downstream processes of cell cycle arrest and/or apoptosis in a cell lineage dependent fashion.

One of the recurring themes in evolution is the development of adaptive mechanisms by organisms for their enhanced survival in adverse environments. Thus it is likely that higher organisms might have developed several auxiliary mechanisms (at the molecular level) to carry out functions that are fundamental to the cell’s (and in turn the organism’s) survival. Our results with p53 mutant cell lines indicate the existence of additional mechanisms (independent of p53) to mediate the cellular responses to DNA damage for essential maintenance of the integrity of the genome.

CONCLUSIONS

The subtle conformational differences exhibited by enantiomer-DNA adducts at the molecular level are distinguished by antibodies. Specificity towards the major adduct, (+)-anti-BPDE-N2-dG adduct results in an enhanced increase in the sensitivity of the antibody. Polyclonal antibodies though consisting of various proportions of IgG molecules of varied specificities can discern the structural differences of anti-BPDE enantiomer adducts. Non-competitive immunoassays
using high capacity antigen binding matrices can detect very low levels of DNA
damage (~2 addicts/10^6 bases). However, the sensitivity and the reliability of the
non-competitive immunoassays were solely dependent upon the major adduct
specificity of the antibody used. Normal fibroblasts show a DNA damage
dependent difference in the extent of the removal of (±)-anti-BPDE-DNA adducts.
Fibroblasts also exhibit a prolonged cell cycle arrest response after (±)-anti-BPDE
induced DNA damage. In contrast lymphoblastoid cells activate programmed cell
death after DNA damage.

The p53 tumor suppressor protein stabilization mediated by (±)-anti-BPDE is
subject to modulation primarily by phosphorylation pathways. Poly(ADP)
ribosylation plays a secondary role in the stabilization of p53 levels. Cells with
mutant p53 are also proficient in several cellular DNA damage response pathways.
Development of antibodies with mono-adduct preferences allows the validation of
the structural differences reported by physico-chemical methods and the
quantitative assessment of cellular responses to DNA damage.
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