CO-CULTIVATION OF CELLS AS A PROMISING TOOL IN CANCER RESEARCH *IN VITRO*

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WIM M. F. JONGEN

CO-CULTIVATION OF CELLS AS A PROMISING TOOL IN CANCER RESEARCH *IN VITRO*

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 12 februari 1988
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

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Promotoren : dr. J.H. Koeman, hoogleraar in de toxicologie.
dr. L. den Engelse, bijzonder hoogleraar in de biochemische oncologie in het bijzonder de chemische carcinogenese, Rijksuniversiteit Leiden

NN08201, 1199

STELLINGEN

1. Gap junctions spelen een belangrijke rol bij de weefseldistributie van reactieve intermediairen.
Dit proefschrift.

2. Bij onderzoek naar het vermogen van stoffen om intercellulaire communicatie te remmen is het gebruik van celtypen met een adequaat vermogen tot biotransformatie essentieel.
Dit proefschrift.

3. De antimutagene effecten van β-NF kunnen niet worden toegeschreven aan het enzym-inducerend vermogen van deze verbinding.
Dit proefschrift.

4. Beïnvloeding van biotransformatie processen door indolen kan, afhankelijk van de keuze van het precarcinogeen, leiden tot zowel een groter als een kleiner effect van blootstelling en de term "inhibitors", algemeen gebruikt om dit type effect te categoriseren, dient te worden vervangen door "modulators".
Ramel et al., Mutation Res., 168, 47.
Pence et al., (1986), J. Natl. Cancer Inst., 77, 269
Dit proefschrift.

5. Bij onderzoek naar mutagene en carcinogene eigenschappen van stoffen voorkomend in voedingsmiddelen dient meer aandacht te worden besteed aan matrixeffecten.
Jongen and Dorgelo, (1986), Neth. J. Agric. Sci., 34, 395.
Jongen et al., (1987), Fd. Chem. Toxic., 25, 141.

6. De rol van endogene vorming van N-nitroso verbindingen bij het ontstaan van kanker bij de mens kan pas reëel worden geschat wanneer de bijdrage van niet-vluchtige en cyclische N-nitroso verbindingen voldoende is uitgezocht.
V. Broekhoven et al., (1987), Proc. IXth Int. Symp. on N-nitroso Compounds, IARC Scient. Publ., 84, 360.
Wakabayashi et al., (1985), Mutation Res., 143, 17.

7. Voedingsadviezen m.b.t. de preventie van kanker zijn pas gerechtvaardigd als ze gebaseerd zijn op inzicht in de oorzakelijke verbanden tussen de betreffende voedingsfactoren en de waargenomen effecten.

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8. De conclusie van Peraino et al. dat het beschermend effect van gelijktijdige toediening van phenobarbital en levercarcinogenen berust op een verandering van de biotransformatie stoelt niet op experimenteel bewijs.
   Peraino et al., (1983), Multistage Hepatocarcinogenesis, In: T.J. Slaga, (Ed.), Mechanisms of Tumor Promotion, Vol. I, CRC Press, 1.

9. De hardnekkige pogingen om met steeds andere combinaties van mutageniteitstoetsen de voorspellende waarde t.a.v. carcinogene potentie van stoffen te vergroten getuigen van een gebrek aan inzicht in de complexiteit van het proces.

10. De veranderingen in de financieringsstructuur van het wetenschappelijk onderzoek leiden tot het prematuur uitdragen van onderzoeksresultaten en dragen op die manier bij aan een negatieve beeldvorming over de waarde van het wetenschappelijk onderzoek.

11. De wijze waarop in Nederland het tweede fase onderwijs wordt ontwikkeld getuigt meer van burgerlijke koopmansgeest dan van liefde voor de wetenschap.

12. Het autorijden wordt zuur betaald.

Stellingen behorend bij het proefschrift: Co-cultivation of cells as a promising tool in cancer research in vitro.

Wim M.F. Jongen, 12 februari 1988.

CONTENTS

1. **CHAPTER I** 9
   GENERAL INTRODUCTION
2. **CHAPTER II** 36
   PRINCIPLES OF THE TEST METHODS USED IN THE INVESTIGATIONS
3. **CHAPTER III** 43
   CO-CULTIVATION OF CELLS AS A PRACTICAL TOOL FOR MUTAGENICITY TESTING
4. **CHAPTER IV** 59
   TRANSPORT OF REACTIVE INTERMEDIATES IN A CO-CULTIVATION SYSTEM:
   THE ROLE OF METABOLIC COOPERATION
5. **CHAPTER V** 76
   CO-CULTIVATION OF PRIMARY CHICK-EMBRYO HEPATOCYTES AND V79 CHINESE
   HAMSTER CELLS AS A MODEL FOR METABOLIC COOPERATION STUDIES
6. **CHAPTER VI** 96
   THE USE OF THE CO-CULTIVATION SYSTEM TO STUDY MECHANISMS OF CANCER
   MODULATING COMPOUNDS
7. **CHAPTER VII** 132
   SUMMARY AND CONCLUDING REMARKS
8. **CHAPTER VIII** 139
   SAMENVATTING EN DISCUSSIE
9. **CURRICULUM VITAE** 146

Aan Ine, Freek en Joost,
aan mijn ouders

VOORWOORD

Het tot stand komen van een proefschrift kan alleen maar lukken dankzij de inzet van vele personen en het resultaat dient dan ook beschouwd te worden als een gemeenschappelijk product.

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CHAPTER I

GENERAL INTRODUCTION

THE CANCER PROCESS

Cancer is the name for a number of different types of diseases which have in common that a group of cells has escaped growth control and grow more or less autonomously. Such a group of cells almost always is clonal i.e. originates from one deviating cell. There is considerable epidemiological and experimental evidence showing that the process of tumor formation in humans and experimental animals proceeds stepwise. Such events may be caused or at least largely be influenced by exposure to certain chemicals, various forms of radiation and viruses.

Studies in experimental animals showed that in the stepwise development of tumors both genetic and epigenetic factors play an important role. Also for the human situation there is sufficient evidence to assume that similar processes contribute to the incidence in tumor formation. The involvement of genetic factors in the etiology of human cancer has been observed in various studies. The existence of familial derived predisposition for certain types of cancer like retinoblastoma or polyposis coli has been established and at the present a significant number of different hereditary traits have been described which predispose to cancer. The genetic alterations underlying these traits include both point mutations and chromosome aberrations (McKusick, 1983 ; Mulvihill, 1984).

Epidemiological data provided further evidence that genetic changes in somatic cells are an important causative step in the onset of carcinogenesis (Friesen et al., 1985; Bartsch and Montesano, 1984).

Among members of a Chinese population the high incidence of oesophageal cancer is associated with the presence of increased amounts of carcinogenic N-nitroso compounds present in the food. Analysis of the DNA from oesophageal cells revealed that persons bearing tumors had substantially higher levels of modified DNA bases than non-affected individuals (Umbenhauer et al., 1985).

On the other hand, the apparent multistep nature of chemical carcinogenesis and the often long latency time between the first exposure to a carcinogen and the manifestation of tumors indicates that epigenetic mechanisms play also an important role in this process.

On the Carribean island Curacao the black and creole population suffers from an exceedingly high rate of oesophageal cancer compared with the incidence on surrounding islands. Through epidemiological investigations it was established that on the island, as part of local life-style, the fresh green aromatic leaves of the bush Croton flavens L. are used to make a 'bush tea' drunk as an everyday beverage.

The leaves of this plant contain a large number of non-mutagenic, tumor promoting agents including 12-O-tetradecanoyl-13-phorbolacetate (TPA) the strongest known tumor promoter (Hecker et al., 1983; 1984).

Since the discovery of Rehn in 1895, that aniline workers developed a high rate of bladder cancer much attention has been paid to chemical carcinogenesis. On the basis of these studies a theory has been developed which implies that chemical carcinogenesis proceeds according to a sequence of three more or less distinct phases: the initiation phase, the promotion phase and the progression phase (Hecker et al., 1984; Emmelot and Scherer, 1980; Goldsworthy and Hanigan, 1987).

In this context initiation can be defined as a relevant irreversible change in the genome of a cell leading to a change in the genetic properties of that cell. Promotion is that part of the process whereby initiated cells in a tissue or organ develop focal proliferations such as nodules, papillomas and polyps. In the progression phase, probably due to additional genetic damage, one or more of these focal proliferations undergo a cellular evolution to malignant neoplasm.

Some chemical compounds have initiating capacities but exposure to these compounds alone does not lead to the induction of tumors. Other compounds cannot initiate the process of carcinogenesis but are able to make initiated cells develop into visible tumors. In addition there are the so-called complete carcinogens which might be described as having both initiating and promoting capabilities. Exposure to the latter compounds alone can lead to the induction of tumors.

The functional differences between initiators and promoters arises from their mode of action. Initiators react with the DNA of the cells thus causing gene mutations or chromosomal damage. In fact they change the genotype of the cell. The view is held that pure promoters cannot bind to DNA or give rise to mutations. Their mode of action is a stimulation of cell proliferation of initiated cells which can be brought about by various mechanisms like cell killing or specific mitogenic stimuli.

INITIATION

Initiation is the genetic event which is considered to be the first step in the onset of chemical carcinogenesis. Although a chemical that can bind covalently with DNA will do so largely at random, only specific lesions will result in genetic alterations that are relevant for the cancer process. In this respect the discovery of the existence of human cellular oncogenes in the beginning of the 1980s clearly was a major step forward. Oncogenes are a family of genes which are involved in cellular processes regulating growth and differentiation (Yarden et al., 1986; Dmitrovsky et al., 1986). Under normal conditions they are expressed only during restricted periods of differentiation and organ development. Many human tumors contain activated oncogenes which show enhanced expression or synthesize altered gene products. This activation results from chromosome rearrangements, insertion of foreign DNA sequences or point mutations (Capon et al., 1983; Rechavi et al., 1982; Santos et al., 1983).

The initiation phase comprizes two distinct steps, the induction of the molecular lesions and the fixation of these lesions by DNA replication.

The vast majority of chemical mutagens, either with or without metabolic activation, react with cell constituents including DNA.

These alterations in the genome can be classified in two categories:

a. Point mutations

Point mutations are changes at the gene level and two types can be recognized.

Base pair substitutions: Alkylating agents produce chemical changes in nucleotides. At the next replication the altered base may select a "wrong" partner e.g. an alkylated guanine selects thymine instead of cytosine and after two rounds of replication the GC base pair has been substituted by an AT base pair. Another possibility is that the alkylated base is removed either spontaneously or enzymatically and that "error prone" repair puts in the wrong base.

Frame shift mutations: Compounds may produce frame shifts by insertion or deletion of one or several contiguous bases within a gene. Frame shifts can beproduced either by covalent binding or by intercalation of flat aromatic structures.

b. **Chromosome aberrations**

Primary damage to individual chromosomes consists of breakage of chromatids which results from a discontinuity of both strands of the DNA in a chromatid. Chromosome breaks may undergo three different fates with quite different consequences for the cell.

1. Enzymatic repair processes restitute the original situation.
2. The fragments remain unjoined in the cells. This almost always results in cell death at the next or one of the folowing mitoses.
3. The fragments are rejoined in an order different from the old one resulting in chromosome rearrangements like translocations, inversions and deletions.

Fixation of lesions in the DNA is held to be an essential step in initiation. One way to bring about fixation of these lesions is cell proliferation. In experimental animals cell proliferation plays an important role in the fixation of altered DNA sequences and, depending on the type of organ or tissue, fixation occurs automatically or must be brought about with exogenous stimuli. For example, in organs or tissues like colon, skin and bone marrow cell proliferation is an ongoing property and fixation of lesions in the DNA occurs automatically. In adult animals for instance in the liver, due to the quiescent nature of this organ with respect to cell proliferation, fixation of DNA lesions will be largely increased by exogenous stimuli like partial hepatectomy, induction of liver cell necrosis or treatment with chemical mitogens. In neonate or very young animals this need for cell proliferation is fulfilled automatically because almost all organs contain dividing cells (Farber and Sarma, 1987). Various types of DNA damage caused by chemical carcinogens elicit DNA repair. This role of the various DNA repair enzyme systems is essential in the preservation of the genetic properties (Lehman and Karran, 1981; Maher, 1979). Basic "error proneness" of the processes involved in the processes underlying this preservation may also lead to fixation of DNA lesions (Singer and Kroger, 1979; Scherer, 1984).

PROMOTION

Promotion is the process whereby an initiated tissue or organ develops focal proliferations of phenotypically altered cells. Promotion of initiated cells can be brought about by a variety of factors like wounding, bacterial and

viral infection, hormones, growth factors and by xenobiotic compounds (Slaga, 1984).

Animal studies showed that exposure to promoters must be repeated and not to much spaced in time. The process of promotion, in its first phase, is reversible to a certain extent (Verma and Boutwell, 1980; Hecker et al., 1984). In addition promoters appear to act only above a certain threshold level (Verma and Boutwell, 1980). The proliferative stimulation necessary for the promotion of initiated cells can be brought about by either cell killing or specific mitogenic stimuli. Evidence exists, demonstrating that unless a DNA damaging agent produces significant cell killing, as well as mutations, exogenous promoters must be present in order to obtain tumors (Frei, 1976; Trosko and Chang, 1984). This implies that the only difference between an initiator and a "complete" carcinogen might be the ratio between the initiating capacity and the toxic potential. At cytotoxic levels of a xenobiotic compound the surviving cells, including the initiated cells, are forced to repopulate the necrotic tissue. The same mode of action also holds for treatments like partial hepatectomy.

In addition to this aspecific type of effect promotion can be brought about by specific mitogenic stimuli. For instance growth factors or hormones have been shown to behave like promoters (Desser-Wiest, 1979; Sonnenschein and Soto, 1980; Paranjpe et al., 1980; Takizawa and Hirose, 1978; Yoshida et al, 1980) and other types of promoters can interfere with growth factor pathways e.g. by influencing receptor affinity and binding sites (Farrar et al., 1985; Fearon et al, 1985; Lee and Weinstein, 1980; Wolf et al., 1984).

In vitro studies have shown that many of these proliferative stimulations are triggered by a promoter-membrane interaction. Structural and functional changes in cellular membranes have been reported, activation or inhibition of various plasma membrane bound enzymes, modulation of ion transport and transport of small molecules through membranes (for reviews see Yamasaki, 1984; Mufson, 1984; Trosko and Chang, 1984). Much of the knowledge obtained on the mode of action of tumor promoters has come from studies with phorbolesters and their derivatives (Hecker et al., 1984; Yamasaki, 1985). The mode of action of other types of tumor promoters has been studied much less intensively. The reported diversity of effects of tumor promoters on cultured cells is enormous and those considered to be relevant for understanding the mechanisms of the process of tumor promotion shall be reviewed in a bird's eye view.

Specific binding to cellular receptors

Biochemical studies have shown that phorbolesters have special interactions with cellular membranes. Phorbolesters which are active tumor promoters stimulate the phospholipid metabolism in vitro and in vivo (Suss et al., 1971; Kreiblich et al., 1971; Rohrschneider et al., 1972) whereas non active phorbol derivatives don't affect this metabolism (Suss et al., 1972).
Direct research towards the mechanisms of this interaction became possible when Driedger and Blumberg (1980) reported the existence of specific receptors localized in the plasma membrane. Since then a large number of eukaryotic cells were shown to possess this specific receptor (Delclos et al., 1980; Verma et al., 1985; Shoyab et al., 1981; Jaken et al., 1981).
Binding was not observed in prokaryotic cells (Driedger and Blumberg, 1980) and erythrocytes (Shoyab et al., 1981). Binding characteristics of phorbolester-sensitive cells proved to be very similar for a wide range of cell types and tissues suggesting a fundamental role of the receptor in multicellular organisms. (Shoyab and Todaro, 1980). Further characterization of the binding site has revealed that the phorbolester receptors co-purify with protein kinase C, a phospholipid dependent enzyme. In vitro and in vivo studies have shown that the active phorbolester derivatives activate this enzyme (Niedel et al., 1983; Nishizuka, 1984; Castagna et al., 1982).
In conclusion, it seems reasonable to suppose that protein kinase C is part of the phorbolester receptor.
Protein kinase C is a Ca^{2+} and phospholipid dependent enzyme (Nestler and Greengard, 1983). The enzyme is present in all eukaryotic cells investigated (Kuo et al., 1980; Kikkawa et al., 1982; 1983) and is constituted of two functional differing peptides which can be separated by Ca^{2+} dependent thiol proteases (Kikkawa et al., 1982).
The hydrophobic part binds to cellular membranes and the hydrophilic part carries the catalytic centre. The enzyme has been found both in membranes and in cytosolic fractions.
Activation occurs through translocation of the cytosolic fraction towards the plasma membrane where binding occurs (Kraft and Anderson, 1983). Further research revealed that in most cells protein kinase C is present in the cytosol in an inactive form which, after stimulation, binds to cellular membranes (Nishizuka, 1984). When hormones or growth factors interact with receptors on cellular membranes the inosotol phospholipid turnover is transiently activated and produces diacylglycerol which activates protein kinase C. Tumor promoting

phorbolesters like TPA are intercalated into the membrane, substitute for diacylglycerol and permanently activate protein kinase C, in spite of the presence of a feed back control mechanism (Farrar et al., 1985; Fearon et el., 1985; Miyake et al., 1984; Wolf et al., 1985).
Activation of protein kinase C triggers a cascade of biochemical responses like ornithine decarboxylase actvity, receptor binding of epidermal growth factor, synthesis of prostaglandins and cyclic GMP, production of arachidonic acid, activation of transglutaminase and inhibition of intercellular communication. Furthermore the phorbol ester mediated differentiation of HL60 cells probably proceeds via protein kinase C.
In addition to the active phorbolesters also teleocidine and aphlysiatoxine, both strong tumor promoters in the mouse skin, induce similar effects (Sugimura and Fujiki, 1983).
On the other hand known tumor promoters like phenol, oleic acid, saccharin and cyclamate don't influence phorbol ester binding to cellular receptors and known inhibitors of tumor promotion like retinoic acid, dexamethasone and disulfiram don't influence this binding (Shoyab and Todaro, 1980).

Modulation of cell differentiation

Already in 1954, Berenblum hypothesized that the mechanisms of tumor promotion in mouse skin could involve a delay in cell maturation (Berenblum, 1954). Indeed phorbolesters tumor promoters are able to modulate a variety of cell differentiation programmes resulting in both inhibitory and stimulating effects. (Diamond et al., 1980; Yamasaki, 1984). It seems reasonable to suppose that only inhibition of cell differentiation is responsible for tumor promotion, since accumulation of continuous dividing stem cells is attained (Yamasaki, 1980). Beside this, initiation-promotion studies in experimental animals show that the promotion of initiated cells is always associated with de-differentiation (Scherer, 1984).
The reversibility of phorbolester mediated inhibition of cell differentiation has been studied the most extensively in Friend erythroleukemia cells (FELC) (Rovera et al., 1977; Yamasaki; 1984) and chick embryo cells (Holtzer et al., 1982; Payette et al., 1980). It was shown that exposure to the phorbolesters did not influence cell division rate and normal FELC proliferation (Yamasaki et al., 1982). This suggests that only gene expression which is directly related to differentiation is influenced and not that necessary for maintenance of cell proliferation.

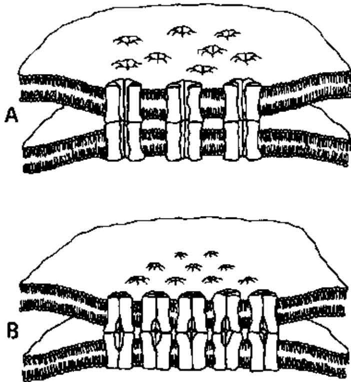
Phorbolester mediated inhibition of cell differentiation is observed in a tremendous diversity of differentiation programmes affected (Yamasaki, 1984). For instance a single compound like TPA can inhibit differentiation of cells that are committed for erythroid, chondrogenic, melanogenic, myeblastoid, neuroblastoid and epidermal lineages. What are the mechanisms by which such a variety of programmes are selectively influenced ? For one it is well established that intercellular communication plays an important role in the control of cellular differentiation. Secondly it has been reported that many tumor promoters inhibit intercellular communication (Yotti et al., 1979; Murray and Fitzgerald, 1979; Trosko et al., 1982). Therefore it seems likely that phorbolester mediated inhibition of intercellular communication itself is the cause of aberrant gene expression.

Inhibition of intercellular communication

Communication between cells in a living organism is supposed to play a fundamental role in the regulation of cell proliferation and differentiation (Loewenstein, 1979). There are two major pathways for intercellular communication. It can take place via the intercellular space and is then regulated by production and transmission of signal molecules like hormones and growth factors which act through receptor binding to cellular membranes. Between neighbouring cells, permeable intercellular junctions can be formed which allow for a direct passage of signal molecules (Trosko and Chang, 1984).
In vivo these gap junctions occur in most mammalian cell types. Exceptions to this rule are the striped muscle cells and some nerve cells (Loewenstein, 1979, Larsen, 1983). The relative amount of cellular membrane involved in gap junction formation may vary in time and between tissues, ranging from 0.05% for the BHK cell line to 7-8% for granulocytes in the follicles of rabbits (Yee and Revel, 1978; Yancey et al., 1982; Larsen, 1983). In addition to gap junctions formed between homologous cells also heterologous gap junctions can be formed. Cells and cell lines from different organs and even of different vertebrates can be manipulated in vitro to form gap junctions (Michalke and Loewenstein, 1971; Overton, 1974; Epstein and Gilula, 1977; Lawrence et al., 1977; Gaunt and Shubak-Sharpe, 1979; Hooper, 1982; Jongen et al., 1987). The formation of gap junctions between cells of different origin indicates the fundamental role of this type of junction in intercellular communication. The transport capability allows for the passage of neutral and negatively charged molecules to a molecular weight up to 1000 and also small positively charged

molecules. The upper limit for positively charged molecules is unknown probably due to binding to cytoplasmic constituents (Simpson et al., 1977; Bennett and Goodenough, 1978). Transport of various types of compounds occurs like electrolytes, low molecular nutrients, waste products and signal molecules for growth and differentiation. Beside this gap junctions are essential for synchronisation of contractile movements in heart- and smooth muscle tissue (Larsen, 1983; Peracchia, 1980).

Figure I



Tentative model of vertebrate gap junction architecture in coupled (a) and uncoupled (b) conditions. Intramembrane particles composed of six subunits span the membrane thickness and bind to similar particles of the adjoining membrane. Channels (1.5-2.0 nm wide) span the junctional membranes at the center of the particles providing cytoplasmic bridges for cell-to-cell diffusion of small molecules. The intramembrane particles form loose and disordered arrays in coupled junctions (a) and more ordered packings in uncoupled junctions (b), up to crystalline (hexagonal) arrays with a periodicity of 8.5 nm. (From Peracchia and Bernardini, 1984).

Figure II

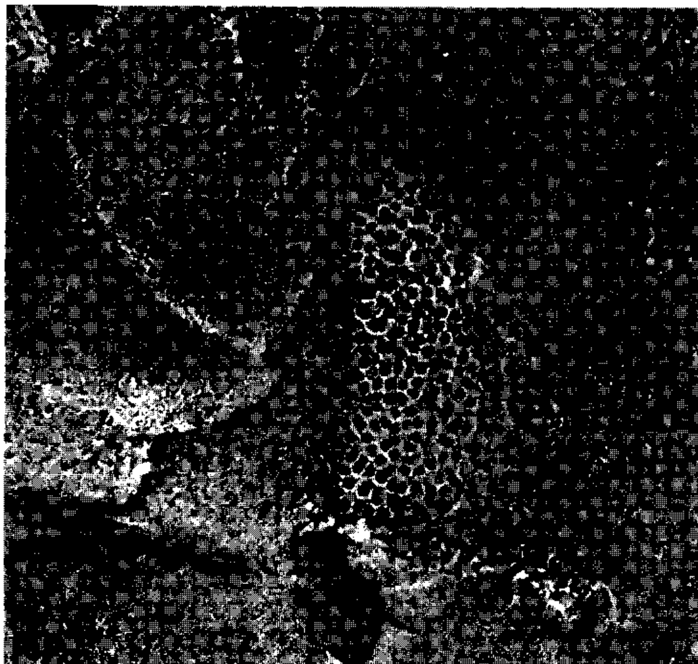


Foto taken from sectioned, freeze-etched material obtained from cultured primary chick-embryo hepatocytes. Picture shows gap junction (Enlargement 186.340 x). Foto prepared by P.T.J. van der Zandt.

Evidence that tumor promoters can inhibit intercellular communication was first obtained, almost simultaneously, by Yotti et al. (1979) and Murray and Fitzgerald (1979). Studies of Yancey et al. (1982), Kalimi and Sirsat (1984) and Jongen et al. (1987) have shown that addition of the phorbolester TPA decreased the number of gap junctions in cultured cells as well as in experimental animals (mouse skin).

Inhibition of intercellular communication has been studied in a variety of cultured cells by means of transfer of labeled nucleotides, transfer of poisonous base analogues, electrical coupling and dye transfer methodes (Yotti et al. , 1979; Murray and Fitzgerald, 1979; Loewenstein, 1979; Williams et al., 1981; Telang et al., 1982; Trosko et al., 1980, 1983; Enomoto et al., 1984, a, b).

Evidence has been obtained which shows that many types of promoting agents inhibit intercellular communication and that the <u>in vivo</u> tumor promoting capability correlates well with the capability to inhibit intercellular communica-

tion (Trosko et al., 1982). Moreover, there is a good correlation between phorbolester mediated inhibition of intercellular communication and enhancement of cell transformation in BALB/c 3T3 cells (Yamasaki, 1984). Chemically transformed cells have deviating communication characteristics from those of nontransformed cells. In some instances they don't communicate at all (Fentiman et al., 1979; Wiener and Loewenstein, 1983) while in other cases they don't communicate with surrounding non-transformed cells. Also, antitumor promoting agents like retinoic acid and dexamethasone antagonize the phorbolester mediated inhibition of intercellular communication (Shuin et al., 1983; Yamasaki, 1984; Nishino et al., 1984). Recently, several oncogene products such as from the src and ras now have shown to be associated with a decrease in gap junctional communication (Atkinson and Sheridan, 1984; Azarnia and Loewenstein, 1984; Chang et al., 1985). Beside this it is well known that partial hepatectomy and repeated wounding can act as tumor promoters in the two stage model. Therefore the observation that under these circumstances a decrease in both the number and the size of gap junctions is found supports the hypothesis that inhibition of intercellular communication plays an essential role in tumor promotion.

PROGRESSION

Progression is that part of the process whereby one or more focal proliferations undergo a cellular evolution to malignant neoplasm. During this evolution these cells acquire several characteristics which can be divided in the following categories (Ponten, 1987; Scherer, 1984):

- infinite growth. There is an indefinite expansion of the net cell number in a tumor resulting in a progressive increase in tumor burden.
- genetic instability. Within a tumor new abnormal genotypes are created and fixed implying a considerable risk of loosing important proliferation and differentiation controls.
- irregular migration. Cells acquire invasive properties which means that they have the potential to migrate, settle and grow at abnormal locations in the organisms. In experimental animals progression is accomplished by addition of initiating agents like urethane or dimethylbenzanthracene which induce additional genetic changes (Farber and Sarma, 1987; Hecker et al., 1984).

The development of malignant tumors from pre-existing preneoplastic lesions or from benign tumors is one of the least studied processes in carcinogenicity studies in experimental animals (Farber and Sarma, 1987).

THE USE OF INTACT CELLS AS METABOLIZING SYSTEM; OBJECTIVES OF THE INVESTIGATIONS

Biotransformation of xenobiotics

Living organisms are continually exposed to a variety of naturally occurring and synthetic chemicals. If these compounds are lipophilic they are generally made more hydrophilic by metabolic transformations, mainly oxidations or hydroxylations, and subsequent conjugations, to facilitate excretion. This capacity to deal with lipophilic xenobiotics used to be considered a defense mechanism of the organism. The last decades, however, an overwhelming amount of information has been gained, which indicates that in the process of conversion of the non-reactive parent compounds to hydrophilic metabolites reactive intermediates can be formed which may have toxic effects. In this connection it was the concept of the 'ultimate carcinogen' as an electrophilic species, which binds covalently to the DNA, that has led to the realisation that the majority of known initiating carcinogens is not reactive per se but must be transformed enzymatically into reactive products (Brookes and Lawly, 1964; Miller, 1970). This process of metabolic transformations can be divided in two phases. Phase I reactions introduce new functional groups into the lipophilic compounds converting them into highly reactive products which can bind covalently to nucleophilic molecules (Wright, 1980). These transformations are oxidative processes like hydroxylations, epoxydations, oxidative dealkylations and deaminations, reductions and hydrolyses. Phase II metabolism comprizes synthetic reactions with small endogenous molecules that are coupled to functional groups originally present or introduced by phase I metabolism. The main phase II reactions are glucuronic acid conjugation, mercapturic acid formation, sulfate conjugation, methylation, acetylation and amino acid conjugation. It should be emphasized that even if phase II enzymes in the vast majority of cases provide an efficient protection against toxic intermediates in several cases cause activation of the parent compound into highly reactive products (Irving, 1979; Rannug et al., 1978; van Bladeren et al, 1980a, b; Jongen et al., 1982).

Modulation of biotransformation

The ability of mutagens to exert their effects depends largely upon the balance between activating and deactivating enzymes. Any change in this

balance will result in a change in genotoxic effects. Many environmental factors are known to be able to induce or inhibit the metabolism of xenobiotics. Some well known enzyme inducers are phenobarbital, 3-methylcholanthrene, mixtures of polychlorinated biphenyls and cigarette-smoke condensate. The influence of dietary related factors may be apparent from the observation that when rats were fed with a guinea-pig diet the B(a)P hydroxylase activity was 10 fold enhanced when compared with the activity on a normal diet (Hietanen and Vainio, 1973). Many compounds, naturally occurring in the human diet, modulate the biotransformation of several precarcinogens, resulting in a reduced tumor incidence (Ramel et al., 1986). This implies compounds which inhibit the formation of electrophilic intermediates by acting as inducers of alternative pathways of metabolism as well as compounds which change the balance between activating and deactivating enzyme systems (Mirvish, 1981; Newmark and Mergens, 1981; Wattenberg, 1979, 1980, 1983; Wood et al., 1982).
Studies performed sofar have shown that there is a good correlation between the enzyme inducing capability of several dietary related compounds and their potential to inhibit carcinogenesis (Sparnins et al, 1982). As Ramel (1986) pointed out, most work in this area is still on a basic and exploratory level and a basic knowledge of the mechanism behind such interactions is evidently of great importance before this information can be used for practical actions.

Isolated hepatocytes in 'in vitro' studies

The liver is the organ predominantly involved in the biotransformation of many xenobiotics. For practical purposes, in the majority of _in vitro_ studies liver homogenates or subcellular liver fractions have been used to mimic _in vivo_ metabolism.
However the use of this kind of preparations has several disadvantages. Due to homogenization and fractionation of the cells the balance between activating and deactivating enzyme systems is disturbed. Especially conjugation reactions, which are located in different cellular compartments are much less operative. Also, these preparations are equipped artificially with cofactors needed for optimal enzyme functioning (Brouns, 1981).
The disadvantages mentioned may be overcome by the use of intact cells as metabolizing system. Isolated hepatocytes resemble in their structural and biochemical properties the _in vivo_ situation much more closely than subcellular fractions (Glatt et al., 1981; Green et al., 1977; Jones et al., 1978;

Kuroki and Drevon, 1978; Nordenskjöld et al., 1981; Strom et al., 1981; Neis, 1986). The value of the use of intact cells for the assessment of the genotoxic effects of xenobiotics has been shown extensively by several authors (McMahon, 1980; Brouns, 1981; Gould, 1983; Neis, 1986; Roberfroid, 1980).
When freshly isolated hepatocytes are cultured in vitro they have to adapt to the new environment. This adaptation takes about 24 hours (Princen et al., 1984). Unfortunately, when primary hepatocytes are cultured they show a rapid decline in their biotransformational capacity e.g. cytochrome P450 catalyzed activities. To overcome this disadvantage several methods have been applied like addition of hormones, addition of enzyme inducers and co-cultivation with epithelial cells (Holme et al., 1983; Paine, 1980; Malansky and Williams, 1982; Blaauboer et al., 1985; Quillouzo et al., 1985). In contrast to parenchymal cells isolated from mammmalian liver, chick-embryo hepatocytes maintain their initial levels of cytochrome P450 containing monooxygenases in vitro for at least 72 hours. Moreover, induction in vitro with known enzyme inducers like phenobarbital, 3 methyl-cholanthrene or Aroclor 1254 leads to a 2-2.5 fold increase in cytochrome P450 content which is comparable with the in vivo situation (Althaus et al., 1979; Sinclair et al., 1979; Topp and van Bladeren, 1986). This makes these cells a potentially useful tool to study the effects of modulating compounds.
Sofar several sudies, using primary chick-embryo hepatocytes as metabolizing system, have shown that these cells are capable of bioactivation of a wide range of premutagens belonging to different chemical classes (Bruggeman and van der Hoeven, 1985; van der Hoeven et al., 1984; Jongen et al., 1985, 1986, 1987).

OBJECTIVES OF THE INVESTIGATIONS PRESENTED IN THIS THESIS

Research on the relation between nutrition and cancer is still largely at an exploratory level. There is a lack of basic kowledge on the mechanisms behind such a relation. Obviously, mechanistic studies towards that relation require the use of adequate experimental systems. The use of primary chick-embryo hepatocytes, co-cultivated with V79 Chinese hamster cells, offers a potentially useful model. The investigations presented in this thesis are directed to develop a model system enabling the study of effects of chemicals on various important aspects of the cancer process, in particular mutagenicity, metabolic cooperation and modulation of drug metabolism. For this purpose attention has been focussed on four aspects:

- The relation between the sensitivity of the co-cultivation system, the genetic endpoint and the type of xenobiotic.
- Development of a model system to study the effects of agents known to modulate experimental carcinogenesis.
- A detailed study on the transport of reactive intermediates from the metabolizing cells into the target cells.
- Development of a model system to study the process of metabolic cooperation between the primary chick-embryo hepatocytes and the V79 Chinese hamster cells.

The results of these studies are presented in the chapters II-V and are based on already published papers and papers that are submitted or 'in press'.

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CHAPTER II

PRINCIPLES OF THE TEST METHODS USED IN THE INVESTIGATIONS

MUTAGENICITY TESTING

In the last two decades a large number of short-term test systems have been developed with the aim to detect chemical mutagens and carcinogens. These can be divided in two main categories: systems using prokaryotic cells (bacteria) which are able to detect point mutations and small deletions and systems with eukaryotic cells (fungi, yeast, mammalian cells) which are able to detect both point mutations, small deletions and chromosome aberrations.
In the present investigations two short-term test systems with mammalian cells have been applied: the sister chromatid exchange (SCE) test and the test on forward mutation at the hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) locus.

Sister chromatid exchanges

Sister chromatid exchanges are the result of reciprocal exchanges between sister chromatids at a homologous locus. In 1958, Taylor described the existence of this phenomenon in plant chromosomes using autoradiographic methods. Protocols applied nowadays usually include the use of 5-bromodeoxy-uridine, a thymidine analog. Incorporation of this compound for two rounds of replication results in chromosomes consisting of one unifiliarly substituted chromatid and one bifiliarly substituted chromatid. These chromatids stain differentially with Giemsa or fluorescent dyes. The best results are obtained with a combination of these (Zakharow and Egolina, 1972; Latt, 1973; Korenberg and Freedlender, 1974; Perry and Wolff, 1974). Although the molecular mechanisms of exchanges are largely unknown as yet, there are good indications that SCEs and chromosomal aberrations are caused by the same type of chromosome damage (Wolff, 1977). The test system is a sensitive indicator of the effects of chemical mutagens (Perry and Evans, 1975; Evans, 1982; Carrano et al., 1978; Latt et al., 1981).

Figure I

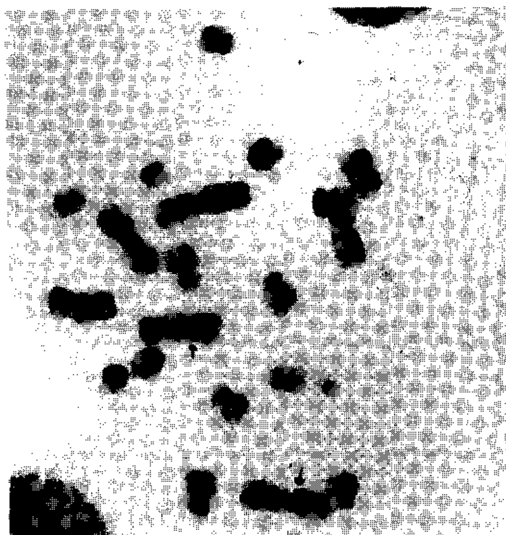

Foto showing metaphase of V79 Chinese hamster cell containing differential stained chromatids. Arrows indicate reciprocal exchanges between homologous sister chromatids.

Forward mutation on the HGPRT locus

Cultured mammalian cells follow two routes to provide in their need for purines and pyrimidines. In addition to the 'de novo' synthesis from simple precursors, cells have evolved salvage pathways for the recycling of bases from degraded DNA or RNA. Hypoxanthine and guanine are salvaged by the dual purpose hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT), an enzyme coded for by a gene on the X chromosome (Polani et al., 1981). When cells are cultured in the presence of the poisonous base analogue 6-thioguanine this compound will be incorporated into nucleic acids ultimately leading to cell death. Only mutated cells which lack the enzyme activity will survive in this selective medium. Because this is only a salvage pathway mutant cells can grow normally by the use of the 'de novo' synthesis.
The general procedure is to expose cells to the mutagen and then allow them to grow in a non-selective medium for a longer period (expression time) which depends on the combination of cell type used and compound tested. After this

the cells are replated in a selective medium in which only the specific mutant will grow (van Zeeland and Simons, 1975 , 1976a, 1976b).

IN VITRO TEST SYSTEMS FOR TUMOR PROMOTION

So far two groups of test systems have been applied primarily for in vitro testing of tumor promoters. One group of test models (cell transformation) uses irreversible functional and morphological changes as endpoint whereas the other group measures inhibition of intercellular communication as endpoint.

Inhibition of intercellular communication

Several systems have been developed to measure inhibition of intercellular communication using different techniques. Trosko et al. (1982) co-cultivated the wild type of the V79 Chinese hamster cell line with a HGPRT deficient V79 mutant. In this case the existence of metabolic cooperation between neighbouring cells can be shown by the use of mutant cells which lack the salvage pathway to phosphorylate purines and pyrimidines. When these cells are co-cultured with wild type cells in the presence of poisonous purine or pyrimidine analogues, the effect on the survival of the mutant cells is an index for metabolic cooperation.These mutants, when cultured alone, will survive in a selective medium in the presence of 6-thioguanine. When they are co-cultured with the wild type cells, the phosphorylated metabolite of 6-thioguanine is transferred through gap junctions from the wild type to the mutant cells. Ultimately this results in cell death for both cell types. When inhibition of metabolic cooperation occurs, the mutant cells will survive and form colonies. By varying exposure levels dose-effect relationships can be obtained which are indicative for the level of intercellular communication (Yotti et al., 1979).
Another method to show metabolic cooperation has been presented by Subak-Sharpe et al. (1969). In this system too wild type cells are co-cultured with HGPRT deficient mutants. During co-cultivation the cells are treated with a labeled purine or pyrimidine. After washing off the label, the mutants will only then incorporate label when the phosphorylated purine or pyrimidine is transferred into the mutant cells through gap junctions. This transfer can be quantitated by the use of autoradiographic methods.
Pitts and Simms (1977) introduced a variant on this method which does not require the use of mutant cells. Donor cells are treated with a labeled precursor for instance uridine, and then washed with unlabeled medium. The uri-

dine, remaining in the cells, is metabolized to the nucleotide. During co-cultivation with non-labeled cells transfer through gap junctions can be measured. Also here, quantitation of transfer is done with autoradiographic methods (Fitzgerald and Murray, 1979; Hunter and Pitts, 1981).

Two techniques which are based on direct measurement of intercellular communication are electrical coupling and microinjection of a fluorescent dye.

When electrical coupling is used, microelectrodes are placed on two adjacent cells. When cells are connected through junctional channels, the electrical current put onto the electrode in one cell will result in a current into the second cell which can be measured with a second electrode. If cells don't communicate, the current towards this second electrode will stay below the detection level.

Microinjection uses the transfer of a fluorescent dye. The compound is injected into a single cell within a few seconds. The plasma membrane is impermeable for this type of compounds and transfer to surrounding cells is measured after 10 min by counting the fluorescent cells. Using this method it has been shown that inhibition of intercellular communication is reversible and that the amount of inhibition of compounds at equimolar doses correlates well with their in vivo promoter activity (Yamasaki, 1984; Enomoto et al., 1984a, b).

Figure II

Foto showing fluorescing V79 Chinese hamster cells. Cells are micro-injected with Lucifer yellow. Left panel shows communicating cells. Right panel shows non-communicating cells.

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CHAPTER III

This chapter is based mainly on the following publication:

Jongen, W.M.F., Hakkert, B.C. and van der Hoeven, J.C.M. (1985), Genotoxicity testing of cigarette-smoke condensate in the SCE and HGPRT assays with V79 Chinese hamster cells, Fd Chem. Toxic., 23, 603-607.

CO-CULTIVATION OF CELLS AS A PRACTICAL TOOL FOR MUTAGENICITY TESTING

ABSTRACT

The cytotoxic and mutagenic effects of B(a)P, DMN, CSC and TPA, compounds from diffent chemical classes, were investigated in the co-cultivation system. Plating efficiency, sister chromatid exchanges (SCEs) and forward mutations at the HGPRT locus were scored.

Benzo(a)pyrene [B(a)P] and dimethylnitrosamine (NDMA) were positive in both assays but only after metabolic activation. Cigarette-smoke condensate (CSC) was negative in the SCE assay both with and without metabolic activation. In the HGPRT assay no direct mutagenicity of CSC was observed but after metabolic activation there was a considerable increase in the number of mutants. The phorbolester 12-0-tetradecanoylphorbol-13-acetate (TPA) was not mutagenic at the concentrations tested. Comparison of a number of metabolizing systems, showed that B(a)P with non-induced, intact chick hepatocytes induced a comparable number of mutants as B(a)P with liver from Aroclor 1254 pretreated rats. The chick liver homogenate was considerably less active on B(a)P. DMN was weakly positive with all three systems, the intact hepatocytes giving the highest number of mutants. When CSC was tested, all three metabolizing systems generated similar numbers of mutants.

In this study the use of primary chick-embryo hepatocytes showed the best performance as an activation system. The co-cultivation system seems to be a useful, practical tool in mutagenicity testing.

INTRODUCTION

When short term in vitro genotoxicity test systems are used the choice of the metabolizing system can critically affect the results. In the majority of studies liver homogenates or subcellular liver fractions are used to mimic in vivo drug metabolism. Homogenate fractions, however, may give a false representation, both qualitatively and quantitatively of the overall biotransformation process occurring in the cells from which they are prepared. The subcellular fractions cannot account for rate limiting factors such as uptake of chemicals, their intracellular distribution and the release of metabolites by the intact cell. In addition, homogenization and fractionation of tissue may modify the micro environment. Furthermore, to express their oxydative capacity, subcellular preparations are usually equipped with very high, non physiological levels of NADPH or NADPH regenerating factors (Brouns, 1981).

One of the major shortcomings of subcellular preparations is that they lack the capacity to carry out sequential reactions like microsomal oxydation followed by conjugation. Conjugation reactions catalyzed by enzymes located in different cellular compartments and depending on the generation of different cofactors are much less operative in these subcellular fractions (Selkirk, 1977; Bock et al., 1976; Wright, 1980). Despite such restrictions the results of in vitro tests using subcellular activation systems are valuable in that they provide preliminary indications that a mutagenic agent may pose a genotoxic risk in vivo (Wright, 1980).

In studies directed towards metabolic processes involved in the biotransformation of premutagens many of the above mentioned disadvantages may be overcome by the use of intact cells as metabolizing system. Isolated hepatocytes resemble in their structural and biochemical properties the in vivo situation much more than subcellular fractions (Brouns et al., 1979; Glatt et al., 1981; Green et al, , 1977; Jones et al., 1978; Kuroki and Drevon, 1978; Nordenskjöld et al, , 1981; Strom et al., 1981). Unfortunately, when mammalian hepatocytes are cultured in vitro, the levels of the cytochrome P450 containing mono-oxygenases decline fairly rapidly except after addition of hormones or in cocultivation with epithelial cells (Holme et al., 1983; Paine, 1980; Malansky and Williams, 1982; Quillouzo et al., 1985). In contrast to parenchymal cells isolated from mammalian liver, chick-embryo hepatocytes maintain their initial levels of cytochrome P450 containing mono-oxygenases in vitro for at least

72 h. Moreover induction, both in vivo and in vitro, with known enzyme inducers like phenobarbital, 3 methylcholanthrene or Aroclor 1254 leads to induction rates comparable with the in vivo situation (Althaus et al., 1979; Sinclair et al., 1979; Topp and van Bladeren, 1986). Sofar several studies, using primary chick embryo hepatocytes as metabolizing system, have shown that these cells are capable of bioactivation of a wide range of premutagens belonging to different chemical classes (Hoeven van der et al., 1984; Bruggeman and van der Hoeven, 1985; Jongen et al., 1985; 1986).
The purpose of the study presented in this chapter was twofold. The mutagenic effects of the model compounds, selected for the present investigations, were studied in the co-cultivation system with two different endpoints, the SCE assay and the HGPRT assay. Additionally, the sensitivity of this test system was compared with that of two different subcellular homogenates.

MATERIALS and METHODS

Materials. Cigarette-smoke condensate (CSC) was prepared according to Groenen (1981) and was a gift from the CIVO Institutes TNO, Zeist, The Netherlands. Benzo(a)pyrene (B(a)P), which was used as positive control, was obtained from Sigma Chemical Co., St Louis, MO, USA. CSC and B(a)P were dissolved in concentrations of 8.0 and 2.0 mg/ml, respectively, in dimethylsulfoxide (DMSO) obtained from E. Merck AG, Darmstadt, FRG. 6-thioguanine (6-TG) was obtained from Fluka AG Buchs Sg, Switzerland. Eggs containing chick embryos (Hubbard strain) were purchased from De Zeeuw, Bennekom, The Netherlands.

Isolation of primary chick embryo hepatocytes. Hepatocytes were isolated under sterile conditions from 15-day-old chick embryos. The procedure followed was essentially the same as that described by Debets et al. (1980). In summary, the livers were perfused in situ with a Ca- and Mg-free solution of Hanks' BSS containing 0.04% EDTA and HEPES (20mM, pH7.4). The livers were removed, transferred to a Petri dish containing the perfusion solution and sliced in small pieces, which were transferred into an Erlenmeyer flask and enzymatically dissociated with dispase (2 mg/ml) for 10 min at 37° C. The dissociated cells were collected and the remaining liver material was treated with dispase again. The cells were pooled, centrifuged (300g for 4min) and resuspended in a buffered solution of ammonium chloride to remove erythrocytes. After 4 min the

cells were centrifuged (300g for 4 min), resuspended in Williams E medium (supplemented with 10% newborn-calf serum and 2 mM glutamine) and plated.

Co-cultivation. Five ml of a hepatocyte suspension ($6x10^5$ hepatocytes/ml) were plated in a 6-cm diameter Petri dish and incubated at 37° C in a humidified atmosphere containing 5% CO_2. After 4 hr the medium was replaced to remove unattached cells. After 24 hr the medium was replaced by Ham's F10, supplemented with 10% newborn- calf serum and $1x10^6$ V79 Chinese hamster epithelial cells were added. 2 hr later the test compound was added. The test compound was removed after 24 hr, the cells were washed twice with physiological salt solution, dissociated with trypsin (0.2%) and suspended in Ham's F10 medium, supplemented with 10% newborn-calf serum, penicillin (50 U/ml) and streptomycin (50 µg/ml). For the SCE test $1x10^5$ V79 cells were plated onto microscopical slides, $2.5x10^5$ cells were transferred to tissue culture flasks for the HGPRT test and 200 cells were plated in 6-cm diameter Petri dishes to estimate survival.

Preparation of liver homogenates. Rat liver homogenate was prepared from 3-month-old male Wistar rats injected ip with a suspension (200 mg/ml) of Aroclor 1254 in olive oil at a dose of 500 mg/kg body weight. Five days after injection the animals were anaesthetized with diethylether and the livers were perfused with a phosphate buffer (0.1 M, pH 7.4; 0.1mM EDTA) and removed. Livers were weighed, cut into small pieces, rinsed with buffer and transferred into a Potter apparatus. After homogenizing in buffer (3ml/g liver) at 1200 rpm the homogenate was centrifuged for 15 min at 9000 g. The supernatant (S-9) was collected and supplemented with buffer to a total volume of 4ml/g liver and stored in liquid nitrogen at -196° C. Liver homogenate was prepared from 15-day-old chick embryos which were removed from their shells and decapitated. The livers were perfused in situ with a Ca- and Mg-free physiological salt solution containing EDTA (0.04%) and HEPES (20mM; pH7.4). Preparation of the homogenate was as described for rat liver homogenate.

Pre-incubation assay. A mixture containing 1 ml of cell suspension (10^6 V79 cells/ml), 0.2 ml S-9 mix and 10 µl of the solution containing the test compound was pre-incubated for 2 hr at 37° C in a shaking waterbath. After centrifugation for 4 min at 300 g the supernatant was discarded and the pellet was resuspended in a physiological salt solution containing HEPES (20mM, pH7.4). After repeating centrifugation the cells were resuspended, counted

and the appropriate number of cells was plated to assay plating efficiency, SCE induction and HGPRT deficient mutants. The S-9 mix contained 30% S-9 and the composition of the co-factors was as described by Ames (1975).

SCE test. The SCE assay was performed according to Perry & Wolff (1974) with slight modifications. Microscope slides were washed in ethanol, sterilized and placed in 10-cm diameter Petri dishes. One ml of a cell suspension containing $1x10^5$ V79 cells was applied to the slides and incubated for 2 hr for the cells to attach. Then 9 ml of medium containing bromodeoxyuridine (11.1 μm) was added, the cells were incubated in the dark for 24 hr and subsequently treated with colchicine (0.1 μg/ml) for 4 hr. To obtain visible chromosomes the cells were washed with Hanks' BSS and allowed to swell in a 75 mM KCl solution for 15 min at room temperature. Fixation was done in methanol/acetic acid (3:1, v/v) followed by air drying overnight. Staining was performed as described by Jongen et al. (1981). For each experiment all exposures were carried out in duplicate and at each dose level two slides were prepared. On each slide 25 metaphases were scored. Each experiment was repeated at least once. Because the V79 cell line is pseudodiploid the number of SCEs was expressed as the number of SCEs/chromosome.

Gene mutation test at the HGPRT locus. The experiments on forward mutation were slight modifications of those described by van Zeeland & Simons (1975 & 1976a, b). After exposure to various doses of the model compounds, survival was measured by seeding 200 cells in a 9-cm diameter Petri dish. After incubation for 7 days the colonies were fixed in methanol, stained in Giemsa and counted. Simultaneously with cell seeding for survival $2.5x10^5$ cells were plated and subcultured every second day to maintain a logarithmically growing cell population. After an expression time of 7 days, cells were seeded into 9-cm Petri dishes, (10^5 cells/dish) in medium supplemented with 6-TG (10 μg/ml). For estimation of the plating efficiency 200 cells were seeded. Mutant selection dishes were incubated for 9 days and plating efficiency dishes for 7 days before fixation (in methanol), staining (10 min in 10% Giemsa) and counting of the colonies. Each point was carried out in duplicate. At each dose, cells were plated into five dishes. Each experiment was repeated at least once. An effect was considered to be positive when the induced mutation frequency was at least three times higher than the spontaneous mutation frequency for that specific experiment (Bradley et al., 1981).

RESULTS

The data on survival of the V79 cells after exposure to the selected compounds, with and without the presence of different metabolizing systems, is presented in Table I. Because DMSO was used as solvent different concentrations of this compound were also tested. Concentrations of 3% and higher appeared to be cytotoxic and therefore in subsequent experiments the concentration of DMSO did not exceed 1%. Without metabolic activation only the highest concentration of CSC tested (64 µg/ml) showed cytotoxic effects whereas after metabolic activation, irrespective of the system, all concentrations applied showed some cytotoxic effects. However no dose response-relationship was observed in any of the three metabolizing systems. For B(a)P differences in survival were observed between the different metabolizing systems. The largest decrease in plating efficiency was found in the presence of primary chick-embryo hepatocytes whereas the smallest decrease was observed in the presence of liver homogenate, prepared from the same chick embryos. For NDMA the primary chick-embryo hepatocytes gave a comparable reduction in survival as the rat-liver homogenate. Again the liver homogenate prepared from chick embryos gave the smallest reduction in survival. When the phorbolester TPA was tested no cytotoxic effects were observed with or without the presence of metabolizing systems. CSC at concentrations up to 64 µg/ml does not induce mutants on the HGPRT locus in V79 cells without metabolic activation (Table II). However after metabolic activation there was a dose related increase in the number of mutants, and the increase was similar in each of the metabolizing systems. In contrast, for B(a)P there were considerable differences between the metabolizing systems with regard to the number of mutants induced: the chick-liver homogenate induced a substantially smaller number of mutants than did the two other systems Therefore the observed cytotoxicity of B(a)P seemed to be reflected in the number of mutants. Also for NDMA there appeared to be differences. In the presence of rat-liver homogenate no mutagenic effects were observed. Chick-liver homogenate induced a borderline increase in the number of mutants. The only clear-cut positive results were obtained when primary chick-embryo hepatocytes were used. No positive effects were observed when TPA was tested either with or without different metabolizing systems. In the SCE test a small increase was observed in the number of SCEs in V79 cells after exposure to CSC in the co-cultivation system with primary chick-embryo

Table I. **Survival of V79 Chinese hamster cells after exposure to various compounds without metabolic activation and after activation with different metabolizing systems.**

PLATING EFFICIENCY (%)

Exposure		no activation	activation with: Hepatocytes	Chick S-9	Rat S-9
Control		94	93	100	95
DMSO	10	98	93	99	94
(µl/ml)	30	84	nt	nt	nt
	50	46	nt	nt	nt
CSC	16	nt	87	nt	78
(µg/ml)	24	nt	73	nt	nt
	32	96	72	77	73
	64	79	82	73	66
B(a)P	1.0	nt	55	95	92
(µg/ml)	10.0	nt	17	73	39
DMN	1000	nt	81	87	70
(µg/ml)	1500	nt	39	80	45
	2000	nt	32	77	37
TPA	10	96	99	95	95
(ng/ml)	50	95	95	92	100
	100	97	97	96	96
	1000	95	95	97	94

nt= not tested. DMSO=dimethylsulfoxide CSC= cigarette-smoke condensate B(a)P= benzo(a)pyrene DMN= dimethylnitrosamine TPA= 12 O-tetradecanoylphorbol-13-acetate. Survival is based on 200 cells/plate. Values are means of duplicate tests. Exposure without activation and with co-cultivation with primary chick-embryo hepatocytes was for 24 hr. Exposure with preincubation with rat or chick-embryo S-9 was for 2 hr.

Table II. Mutagenicity of various compounds in the HGPRT assay with V79 Chinese hamster cells without metabolic activation and with activation with different metabolizing systems.

MUTAGENICITY (mutants/10^5 survivors)

Exposure		no activation	activation with: Hepatocytes	Chick S-9	Rat S-9
Control		1.6±0.6	1.6±0.5	1.2±0.7	1.5±0.4
DMSO (µl/ml)	10	1.7±0.8	1.7±0.7	1.5±0.3	1.6±0.6
CSC	16	nt	6.8±1.3	nt	nt
(µg/ml)	24	nt	7.1±1.1	nt	nt
	32	2.7±1.0	11.9±1.8	6.5±1.5	8.9±1.4
	64	2.6±0.7	16.3±1.1	14.3±0.9	16.2±1.9
B(a)P	1.0	nt	12.2±1.8	3.7±1.1	9.4±2.1
(µg/ml)	2.5	nt	19.7±2.1	nt	nt
	5.0	nt	30.1±3.0	nt	nt
	10.0	nt	48.6±4.7	13.5±1.9	41.9±3.2
DMN	1000	nt	3.4±0.8	1.0±0.2	2.5±0.7
(µg/ml)	1500	nt	6.9±0.6	1.5±0.5	2.7±1.1
	2000	nt	7.3±1.6	3.4±1.4	3.1±0.6
TPA	10	1.4±0.3	2.3±1.1	1.5±0.6	0.8±0.1
(ng/ml)	50	2.1±0.5	1.9±0.9	1.4±1.0	2.0±0.7
	100	2.3±1.1	3.2±1.3	3.0±0.9	1.7±0.3
	1000	1.9±0.9	2.4±1.2	2.5±1.0	2.1±0.9

nt= not tested. DMSO= dimethylsulfoxide; CSC= cigarette-smoke condensate; B(a)P= benzo(a)pyrene; DMN= dimethylnitrosamine; TPA= tetradecanoylphorbol-acetate. Values are means (±SEM) of duplicate tests. Exposure without activation and with co-cultivation with primary chick-embryo hepatocytes was for 24 hr. Exposure with rat and chick-embryo S-9 was for 2 hr.

Table III. **Induction of sister chromatid exchanges in V79 Chinese hamster cells by various compounds without metabolic activation and after co-cultivation with primary chick-embryo hepatocytes.**

Exposure		SCEs/CHROMOSOME No activation	Co-cultivation
Control		0.27±0.01	0.26±0.01
DMSO	10	0.27±0.03	0.25±0.03
(μl/ml)			
CSC	16	0.35±0.02	0.39±0.04
(μg/ml)	24	nt	0.36±0.05
	32	0.38±0.02	0.38±0.05
	64	0.41±0.04	0.41±0.04
B(a)P	1.0	nt	0.34±0.03
(μg/ml)	2.5	0.30±0.03	0.80±0.03
	5.0	nt	0.96±0.03
	10.0	0.28±0.02	1.21±0.06
DMN	1000	0.23±0.02	0.88±0.07
(μg/ml)	1500	0.24±0.03	1.74±0.07
	2000	0.28±0.03	2.73±0.11
TPA	10	0.28±0.01	0.25±0.02
(ng/ml)	50	0.28±0.03	0.25±0.03
	100	0.24±0.03	0.27±0.02
	1000	0.24±0.03	0.23±0.04

SCE= sister chromatid exchange; DMSO= dimethylsulfoxide; B(a)P= benzo(a)pyrene DMN= dimethylnitrosamine; TPA= 12-O-tetradecanoylphorbol-13-acetate. Values are means (±SEM) of duplicate tests. The cells were exposed to the test compounds for 24 hr.

hepatocytes (Table III). Without metabolic activation the same increase was observed. Furthermore there is no dose- response relationship. Exposure to B(a)P and DMN induced a considerable increase in the number of SCEs. Also in the SCE test TPA did not generate any positive effect with or without metabolic activation.

DISCUSSION

The genotoxicity of CSC has been studied using V79 Chinese hamster cells in two test systems with different endpoints. In the HGPRT test a clear positive effect was found after metabolic activation with any one of the three metabolizing test systems: co-cultivation with primary chick-embryo hepatocytes, preincubation with S-9 prepared from rat or chick-embryo livers. No clear differences in mutagenic or cytotoxic effects were observed between the different metabolizing systems. A small increase in the number of SCEs was observed after exposure to CSC irrespective of metabolic activation (co-cultivation with primary chick-embryo hepatocytes). The increase was consistent in all experiments but never exceeded 40%. This raises the question whether it is justified to ascribe this positive effect to a reaction of CSC with the DNA of the cells.

Firstly, no influence of biotransformation on the number of SCEs has been observed in the present or other (De Raat, 1979) studies. This is in contrast with the data obtained with the Salmonella/mammalian microsome assay (DeMarini, 1983) and the HGPRT test (Clive et al., 1979) in which positive effects were only obtained with metabolic activation. Secondly, no dose-response relationship was found. Finally, the concentrations of CSC used in this study also show cytotoxic effects and it is possible that toxic effects influence the cell cycle and thus cause an increase in the number of SCEs (Jongen et al., 1981). Comparison of our data with those reported in the literature shows that although De Raat et al.(1979) found an increase in the number of SCEs the higher concentrations of DMSO used in that study are cytotoxic and may have a considerable influence on the number of SCEs. Hopkin & Evans (1979) did find positive effects of CSC on the induction of SCEs in human lymphocytes _in vitro_. These results were confirmed by other studies (Madle etal., 1981; Sorsa et al., 1982; Vijayalaxmi & Evans, 1982). Possibly the metabolizing capacity of the lymphocytes plays an essential role. When the results of the different metabolizing systems are compared for B(a)P, Aroclor 1254 pretreated rat-liver homogenate induces a comparable number of mutants as non-induced primary chick-embryo hepatocytes. S-9 prepared from chick-embryo liver is much less active in inducing mutants than the intact cells. One possible explanation for this difference is, that due to the stability of the metabolizing system _in vitro_, longer exposure times can be applied with the

intact cells. Rat-liver homogenate appeared to be able to metabolise DMN to cytotoxic intermediates as can be seen in the survival data. However, in contrast to the results obtained with primary chick-embryo hepatocytes, these effects were not reflected in the mutagenicity data. No significant increase in the number of mutants was observed. When the data, obtained with systems derived from chick-embryo were compared, the use of intact liver cells appeared to generate a higher number of mutants than the liver homogenate. For both endpoints used no effect of TPA was observed. In the literature conflicting data on the induction of SCEs by TPA have been reported. Some laboratories found an increase in the number of SCEs (Kinsella and Radman,1978; Gentil et al., 1980; Nagasawa and Little, 1979) but others did not observe such effects (Loveday and Latt, 1977; Thompson et al., 1980). The apparent contradiction in observed effects can be ascribed to the presence of the enzyme superoxide dismutase (SOD) in the serum of the culture medium. If the enzyme is present in sufficient amounts it prevents the interaction of superoxide radicals, formed by interaction of TPA with the cellular membrane, with the DNA of cells (Nagasawa and Little, 1981; Yamasaki, 1985).

In conclusion, we have demonstrated that CSC, B(a)P and DMN are positive in the HGPRT assay with V79 cells after metabolic activation but that CSC, in contrast to B(a)P and DMN, is negative in the SCE assay after metabolic activation in a co-cultivation system with primary chick-embryo hepatocytes. These results suggest that the SCE assay with V79 cells does not offer an adequate endpoint for measuring the genotoxic properties of this complex mixture. The use of intact hepatocytes, compared with subcellular fractions, offers a more sensitive test system for the compounds selected in this study.

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CHAPTER IV

This chapter is based on the following publications:

1. Jongen, W.M.F., Hakkert, B.C. and van der Hoeven, J.C.M., (1985), Genotoxicity testing of cigarette-smoke condensate in the SCE and HGPRT assays with V79 Chinese hamster cells, Fd Chem. Toxic., 23, 603-607.

2. Jongen, W.M.F., Hakkert, B.C. and van de Poll, M.C.M., (1986), Inhibitory effects of the phorbolester TPA and cigarette-smoke condensate on the mutagenicity of benzo(a)pyrene in a co-cultivation system, Mutation Res., 159, 133-138.

3. Jongen, W.M.F., van der Leede, B.J.N., Chang, C.C. and Trosko, J.E., (1987), The transport of reactive intermediates in a co-cultivation system: the role of intercellular communication, Carcinogenesis, 9, 1239-1243.

TRANSPORT OF REACTIVE INTERMEDIATES IN A CO-CULTIVATION SYSTEM: THE ROLE OF METABOLIC COOPERATION

ABSTRACT

The transport of reactive intermediates from primary chick-embryo hepatocytes to V79 Chinese hamster cells was investigated using two test systems with different genetic endpoints (SCE and HGPRT assay). When the V79 cells were co-cultivated with the hepatocytes at a distance of 1 mm, the mutagenic effects of B(a)P were reduced with ca. 75% compared with the normal co-cultivation conditions, in which there is direct cell-cell contact between primary chick-embryo hepatocytes and V79 cells. Addition of TPA and CSC inhibited the mutagenic effects of B(a)P in both assays with 50% when the two cell types were in direct contact. No influence of TPA on the number of B(a)P induced SCEs was observed in a pre-incubation assay using Aroclor 1254 pretreated rat liver homogenate. CSC appeared to be able to inhibit metabolic cooperation between V79 cells. When the transport studies were done with the metabolic cooperation deficient mutant V79 cell line MC^{-}-27, in addition to B(a)P, also DMN was used. The inhibitory effects of TPA on wild type V79 cells were completely comparable to those obtained with the mutant cell line. No difference in intrinsic sensitivity for mutagenic effects was found between the V79 wild type cells and the V79 MC^{-}-27 cells. The results strongly suggest the occurrence of transport of reactive intermediates in this co-cultivation system and the involvement of metabolic cooperation pathways in this transport.

INTRODUCTION

In the field of mutagenicity testing liver homogenates or subcellular liver fractions are commonly used to mimic *in vivo* metabolism. For several reasons, indicated in the previous chapter, results obtained with these homogenates and fractions may not be representative either qualitatively or quantitatively for the overall biotransformation process in intact cells (Wright, 1980). To avoid several of these disadvantages, systems have been developed which make use of intact cells as metabolizing system. Also when cell-mediated metabolic activation is used interpretation of mutagenicity data obtained with these systems may be complicated. In principle the transport of reactive intermediates may take place via two routes, namely via cell-cell contact where gap junctions act as gates or via the medium.

In case of transport through the medium the cellular membrane of the metabolizing cell will represent an additional barrier for the reactive intermediates to reach DNA of target cells (Brouns et al., 1979; Glatt et al., 1981). Also the composition of the culture medium, such as for instance the type and amount of serum, may interfere with the transfer of these metabolites (Kuroki and Drevon, 1978). In all studies published to date it is generally assumed that in cell-mediated metabolism the transport of reactive intermediates occurs through the medium.

The existence of gap junctions between homologous and heterologous cells has been shown by several authors (Epstein and Gilula, 1977; Hooper, 1982; Hunter and Pitts, 1981). The presence of these junctions permits the free exchange between cells of small molecules and ions but not of macromolecules (Simpson et al., 1977).This suggests that also (metabolites of) xenobiotics will be transported in this way. Compounds effecting cellular membranes are known to influence the transfer of molecules from one cell to neighbouring cells. Several tumor-promoting compounds, especially the phorbolesters, inhibit metabolic cooperation between cells (Trosko and Chang, 1983; 1984).

The aim of the study presented in this chapter was to investigate the transport of reactive intermediates. This includes experiments on the route of transport of active metabolites from B(a)P and DMN. The role of transport of metabolites through gap junctions was studied by the use of TPA and the metabolic cooperation deficient mutant cell line MC^{-}-27.

MATERIAL and METHODS

Material

The phorbolester 12-O-tetradecanoylphorbol-13-acetate (TPA) was obtained from Dr Peter Borchert, Chemical Carcinogenesis Institute, Eden Prairie, HN 55344, U.S.A. All other compounds were obtained as described under M.& M. in chapter III.

Methods

Test of inhibition of metabolic cooperation

The test protocol for this assay was a modified version of that described by Yotti et al.(1979): 2×10^5 6-TG sensitive V79 cells together with 200 6-TG resistant V79 cells were plated in 6-cm diameter Petri dishes in replicates of five. After 18 hr the test compound was added and 30 min later 6-TG (10 μg/ml) was added. Seventy-two hours after application of the test compound the medium was replaced and 7 days after plating the colonies were fixed(in methanol) stained (10 min in 10% Giemsa) and counted. Inhibition of metabolic cooperation was expressed as the difference in percentages between the number of colonies grown with and without application of the test compound and corrected for the spontaneous mutation frequency of the 6-TG-sensitive cells. Each test was repeated at least once.

Isolation of metabolic cooperation deficient mutants of Chinese hamster V79 cells

A non-revertable 6-thioguanine-resistant ($6TG^r$) mutant cell line of Chinese hamster V79 cells (380-23), obtained from Dr. E.H.Y. Chu of the University of Michigan, was mutagenized by a combined treatment of 5-bromodeoxyuridine (10^{-4}M for 1 day) and black light (90 min) (Chu et al., 1972). The surviving colonies developed in a week were replated and irradiated by UV light (10 J/M^2). These mutagenized 6 TG^r cells were selected four times by 6-thioguanine (20 μg/ml) in the presence of high density of wild type 6-TG^s cells. The cell numbers per plate (9cm) were 5×10^4 6-TG^r and 2×10^6 6-TG^s cells in the first two selections, 1×10^4 6-TG^r and 5×10^6 6-TG^s cells in the third selection and 400 6-TG^r and 5×10^6 6-TG^s cells in the fourth selection. After the last selec-

tion, 28 surviving 6-TG^r colonies were randomly selected and tested for their ability to metabolically cooperate with 6-TG^s cells. The results indicate that all these clones except one are deficient in metabolic cooperation. Seven most deficient clones were further tested and confirmed as deficient in metabolic cooperation. One of these clones, MC$^-$-27, was used for experiments in this report. The isolation of the mutant cell line was performed by Dr. C.C. Chang, East Lansing, Michigan, USA.

Determination of intercellular communication by scrape-loading and dye transfer

The method recently developed by El-Fouly et al. (1987) was used. The cell monolayer was plated overnight and rinsed with phosphate buffered saline (PBS) before the addition of Lucifer yellow (0.05% in PBS). The dye solution was scrape-loaded at room temperature using a metal or wooden probe and left on the cells for 3 minutes before it was decanted. The plates were then rinsed with PBS to remove the background fluorescence and the cells examined for Lucifer yellow transfer under a Nikon epifluorescence phase microscope illuminated with an Osram HBO 200 W lamp.

Exposure at a distance

Primary chick-embryo hepatocytes were plated in 9-cm diameter Petri dishes ($6x10^6$ cells/dish). Simultaneously, $5x10^4$ V79 Chinese hamster cells were plated onto microscopical slides and incubated overnight. The next day, 1 mm-thick pieces of glass were placed in the dishes containing the hepatocytes and the slides carrying the V79 cells were placed on top of these glass pieces with the V79 cells directed towards the hepatocytes. After exposure the V79 cells were dissociated with trypsin, collected and treated as described for the SCE assay. Exposure conditions were identical as described for co-cultivation.

All other methods are described in chapter III

RESULTS

First of all, the question whether direct or proximate contact between the hepatocytes and the V79 cells is necessary for an adequate transfer of reactive intermediates was studied. The induction of SCEs by B(a)P using co-cultivation was compared with a system in which the two cell types were kept separate. The data in Table I show that when the two cell types are kept at a distance of 1 mm the number of SCEs is considerably lower. This indicates that direct or proximate contact between the two cell types is required for adequate genotoxicity measurement.

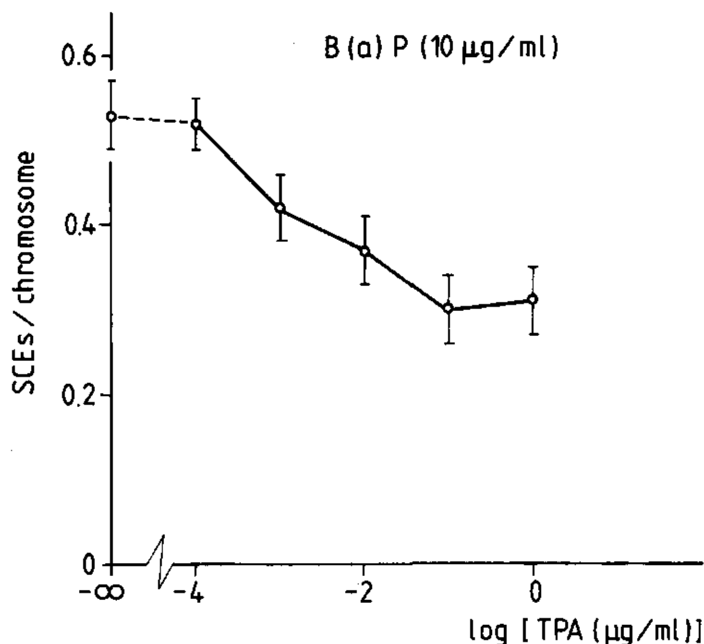
Table I **The effect of cell distance on the mutagenicity of B(a)P in a co cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells.**

Exposure	SCEs/chromosome	
B(a)P (ug/ml)	co-cultivation	At distance of 1 mm
0	0.25±0.02	0.30±0.03
2.5	0.67±0.04	0.41±0.05
5.0	0.82±0.04	0.43±0.04
7.5	1.21±0.06	0.55±0.02

Data represent mean ± SEM. Experiments carried out in triplicate.

TPA is a well known tumor promoter and it is known to inhibit metabolic cooperation between cells. When different concentrations of this phorbolester were added to the co-cultivation system, 30 min before the cells were exposed to B(a)P an increasing dose of TPA caused a dose-related decrease in the number of SCEs (Fig I). TPA itself had no effect on the number of SCEs in V79 cells at concentrations up to 5 μg/ml (data not shown).
Comparison of the HGPRT assay and the SCE assay showed that for both genetic endpoints approximately the same percentage of inhibition was found (Table II).

Figure I: **Inhibitory effects of TPA on B(a)P induced SCEs in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells.**



Control value has been subtracted (0.27±0.03). Values are given with their S.E.M.

CSC did inhibit B(a)P-induced mutagenicity in both assays when tested in the co-cultivation system. If the inhibitory effect in the HGPRT assay is corrected for the mutagenicity of the mixture itself, the inhibition is quan titatively comparable with that caused by TPA. Of course this does not imply a qualitative equivalence between TPA and CSC since the doses differ by 300-fold.

Because these data indicated that CSC was capable of inhibition of metabolic cooperation the effects of this complex mixture on metabolic cooperation between V79 cells was studied. The data are presented in Figure II and show that at non-cytotoxic doses a dose related increase in the number of surviving colonies was found, indicating that CSC indeed inhibits metabolic cooperation between cells.

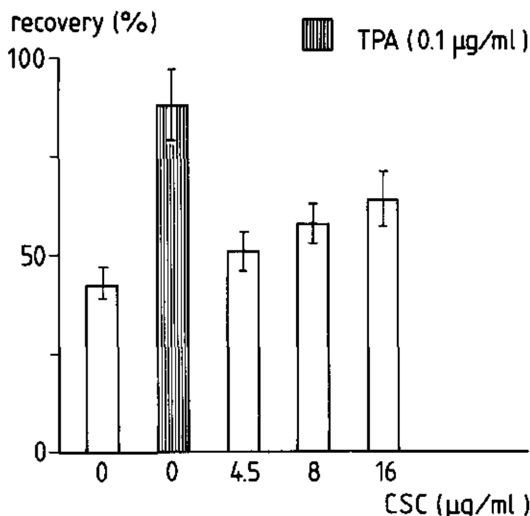
Table II **Inhibitory effects of TPA and CSC on B(a)P induced mutagenicity in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells.**

B(a)P	CSC	TPA	HGPRT assay		SCE assay
			Survival (%)	Mutants/10^5 survivors	SCEs/chromosome
-	-	-	97	2.1±0.6	0.27±0.04
-	+	-	87	12.3±1.9	0.40±0.04
+	+	-	67	43.9±2.0	0.65±0.06
-	-	+	101	2.2±0.7	0.27±0.02
+	-	+	71	29.3±1.6	0.69±0.07
+	-	-	20	67.0±2.6	1.01±0.07

Exposure to the various compounds was: B(a)P: 10 μg/ml; CSC:32 μg/ml; TPA:0.1 μg/ml. Data are means of duplicate tests and are given with their SEM.

To find out whether non-specific membrane effects play a significant role in the inhibitory effect of TPA, a suspension of V79 cells was exposed to B(a)P in a preincubation assay. In this situation metabolic cooperation is not possible. After addition of TPA no significant difference in the number of SCEs was observed (Table III)

Figure II: **Influence of cigarette-smoke condensate on the metabolic cooperation between 6-TG resistant and 6-TG sensitive V79 Chinese hamster cells.**



For each dish 200 6-TG resistant and $4x10^5$ 6-TG sensitive cells were plated. The recovery was calculated on the basis of 100% plating efficiency for the 6-TG resistant cells. The plating efficiency of the 6-TG sensitive cells was 95%. Values are means of five replicates for one representative experiment and are given with their SD.

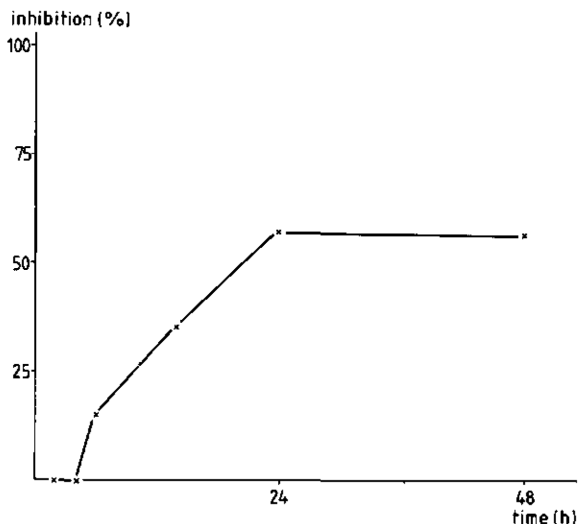
Table III **Mutagenicity of B(a)P in a preincubation assay with V79 wild type cells with and without addition of TPA and MC⁻-27 cells.**

Exposure	V79 Wild Type		V79 MC⁻-27
B(a)P (µg/ml)	TPA (0.1 µg/ml)		
	-	+	
0	0.26±0.03	0.25± .02	0.20±0.04
2.5	0.58±0.04	0.60±0.03	0.62±0.03
5.0	0.75±0.04	0.76±0.04	0.72±0.05

Data are means of duplicate tests and are given with their SEM

The inhibitory effects were strongly related to the culture time of the cells (Fig. III). After 6 hr of co-cultivation a significant increase in the percentage of inhibition was observed. The percentage gradually increased with longer culture times until a maximum of ca. 50% inhibition was reached after 24 hr. Upon longer culture times no further increase was observed.
The MC^{-}-27 Chinese hamster V79 cell mutant was found to be deficient in metabolic cooperation (Table IV).

Figure III: Effect of culture time on the occurrence of transport of reactive intermediates through gap junctions formed between primary chick embryo hepatocytes and V79 Chinese hamster cells.



Data are calculated from the average values of different experiments carried out in duplo. Control values ranged between 0.21-0.25 SCEs/Chromosome. The percentage inhibition was calculated after subtraction of the appropriate control values.

Table IV **Metabolic cooperation between 6-TG^r and 6-TG^s Chinese hamster cells.**

6-TG^r cells	6-TG^s cells	Relative recovery of 6-TG^r cells (%)
MC^+:		
500	0	100
500	5x10^5	30
500	1x10^6	10
500	2x10^6	2.4
MC^--27:		
500	0	100
500	5x10^5	98
500	1x10^6	88
500	2x10^6	89

6-Thioguanine was added at a concentration of 20 µg/ml. Cells were co-cultured in 9-cm diameter Petri dishes for seven days.

The defect in intercellular communication of this cell line was further confirmed by an experiment using the scrape-loading and fluorescent dye transfer technique. As shown in Fig. IV, the fluorescent Lucifer yellow stayed in initially loaded MC^--27 cells in contrast to wild type MC^+ cells which transfer Lucifer yellow from initially loaded cells to contiguous neighbouring cells.

The transport of reactive intermediates was also studied with the metabolic cooperation deficient mutant V79 cell line MC^--27. To extend the observations done sofar to a compound from a different chemical class also the mutagenicity of dimethylnitrosamine (DMN) was studied. At different time points after start of the co-cultivation, the mutagenicity of B(a)P and DMN in the V79 wild type cells with and without addition of TPA was compared with the effects in the MC^--27 cells (Table V).

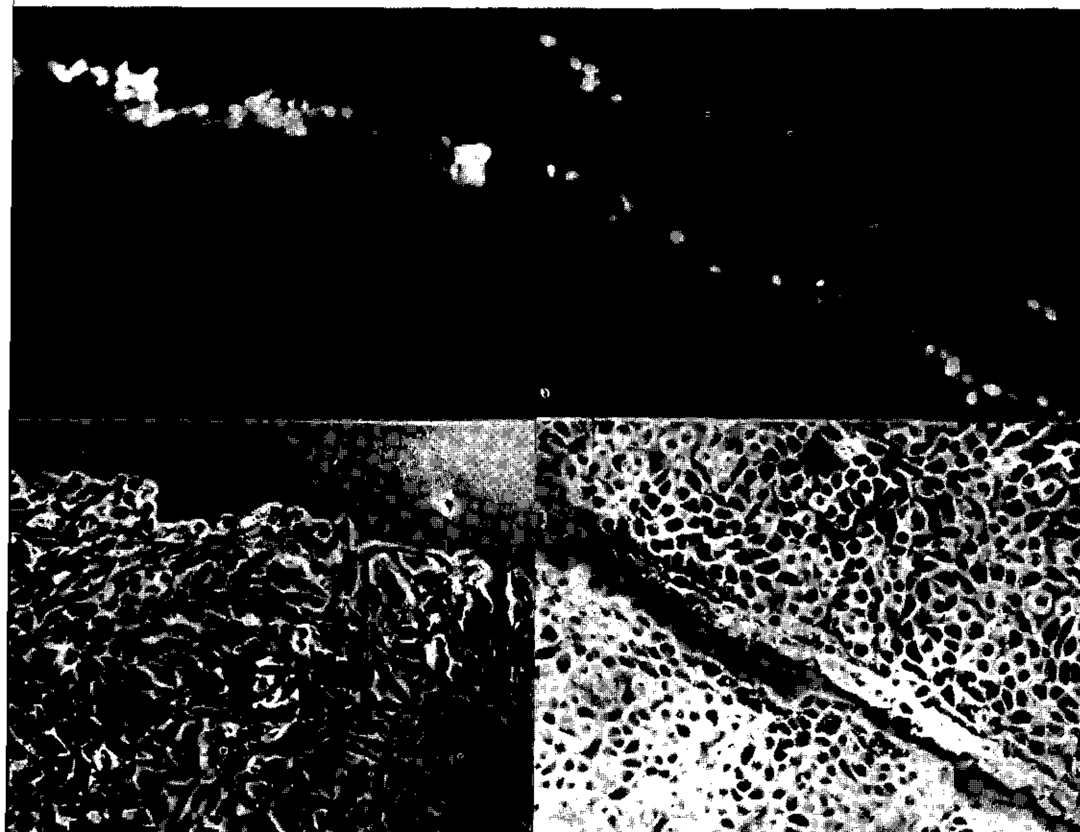
Table V **Induction of SCEs by B(a)P and DMN in V79 wild type cells and V79 MC^{-}-27 cells with and without addition of TPA after cocultivation with primary chick-embryo hepatocytes.**

		SCEs/CHROMOSOME			
		TPA(100 ng/ml)			
		-		+	
Exposure	time	WT(*)	MC-$^{-}$27(**)	WT(*)	MC^{-}-27(**)
Control	24 hr	0.19±0.02	0.21±0.03	0.22±0.02	nt
B(a)P (7.5 μg/ml)		0.83±0.04	0.52±0.05	0.43±0.04	0.41±0.03
DMN (1.0 mg/ml)		0.88±0.11	0.39±0.07	0.42±0.09	nt
Control	48 hr	0.20±0.02	0.24±0.02	0.23±0.02	nt
B(a)P (7.5 μg/ml)		1.19±0.10	0.70±0.06	0.67±0.04	0.70±0.05
DMN		1.24±0.11	0.78±0.11	0.76±0.12	nt

*) = Wild Type. (**) = Metabolic Cooperation deficient mutant cell line. Data are mean ± SEM. Experiments carried out in triplicate. Exposure to B(a)P and DMN was for 4 hr.

The inhibitory effects of TPA were completely comparable to those obtained with the mutant cell line. Addition of TPA to the MC^{-}-27 cells had no influence on the number of SCEs. In addition preincubation experiments were performed to compare the intrinsic sensitivity of the two cell lines for the mutagenicity of B(a)P. No difference in mutagenic effect, measured as SCEs/chromosome was observed (Table III). When different culture times were compared the overall mutagenic effects were higher after 48 hr. However no difference was observed in the percentage of inhibition between the two culture times for both compounds. Apparently the amount of reactive intermediates transported through gap junctions is proportional to the total amount formed.

Figure IV: Photographs of Chinese hamster V79 cells showing positive dye transfer in wild type MC^+ cells (A) and absence of dye transfer in MC^--27 cells (B). Cells in the same area were photographed under regular (bottom) or fluorescent light (top).



DISCUSSION

When the number of induced SCEs in different experiments are compared at equal dose levels of B(a)P considerable differences are observed. However within each experiment pooled cell suspensions were used and the data between replicates were consistently identical. This discrepancy may be due to quantitative differences in the metabolic capacity of the freshly isolated chick-embryo hepatocytes. The data presented in this study strongly suggest a significant

role of metabolic cooperation in the transfer of reactive intermediates in this co-cultivation system. Several other studies have also implicated gap junctional communication in the toxicity of polycyclic aromatic hydrocarbons (Reiners and Herlick, 1985; Jongen et al., 1986, 1987; Moore et al., 1986; Madle et al., 1987). The mutagenic potential of compounds may be underestimated if the compounds or their metabolites are capable of inhibiting metabolic cooperation. The finding that CSC inhibits metabolic cooperation between cells may be an important factor in the quantification of the mutagenicity data obtained with this mixture. Probably, the number of mutants observed in the HGPRT assay does not reflect quantitatively the mutagenic potential of CSC after activation by intact primary chick-embryo hepatocytes. This may explain why for CSC in the HGPRT test no difference in mutagenicity between the three metabolizing systems was observed whereas for B(a)P considerable differences were observed, depending on the type of system used (Chapter I).
The mutagenic effects of B(a)P and DMN are reduced by approximately 50% when metabolic cooperation between the two cell types is inhibited. Still metabolites appear to be able to penetrate the cellular membranes causing mutagenic effects in V79 cells. It is known that depending of the type of metabolite formed, intermediates may be selectively retained by the hepatocytes (Jones et al., 1978). For both compounds examined, transport occurred through the heterologous gap junctions. The proportion of the mutagenic metabolites going through the gap junctions was the same for both compounds and independent of the total amount of metabolites formed.
Therefore a preliminary conclusion may be that this type of transport is not restricted to specific types of ultimate mutagens but is a phenomenon of a more general nature. This observation may have consequences as to which cells in a given tissue are at risk to become 'neoplastic' upon exposure to an initiating agent. For example, when the V79 epithelial cells are co-cultivated with primary chick embryo hepatocytes and then exposed to 10 μg/ml B(a)P, the toxicity measured as plating efficiency, results in 80% cell death (Jongen et al., 1985; 1986). On the other hand when the metabolism of B(a)P is studied, the same hepatocytes are capable of metabolizing concentrations up to 50 μg/ml without biochemical or morphological signs of intoxication (Topp and van Bladeren, 1986). Thus drug metabolizing cells like the primary chick-embryo hepatocytes are protected more efficiently against toxic intermediates than non-metabolizing cells like the V79 cells. The finding that reactive intermediates of both B(a)P and DMN are transported through gap junctions suggests that under these circumstances they do not inhibit metabolic cooperation. Both

compounds are considered to be initiating carcinogens in animal studies. Inhibition of metabolic cooperation has been postulated as an essential step in tumor promotion by several authors (Trosko and Chang, 1984; Yamasaki, 1984a; Yamasaki et al., 1984b). One possible explanation for this apparent discrepancy is that the concentrations at which these compounds exert carcinogenic effects in animals are cytotoxic at a cellular level (Frey, 1976; Trosko and Chang, 1981). Probably these cytotoxic effects induce mitogenic stimuli _in vivo_.

Tachikawa et al. (1986) have reported that dimethylbenzanthracene (DMBA) did cause a decrease in both gap junctional communication and in gap junctions in cultured primary lingual epithelium as measured by electrophysiological and freeze fracture studies, respectively. However, since cell viability studies were not conducted, one can not conclude that DMBA modulated gap junctions under non-cytotoxic conditions.

In contrast to these findings other groups have not seen any inhibition of metabolic cooperation by complete carcinogens (Noda et al., 1981; Telang et al., 1982; Enomoto et al., 1981). Possibly the cell types applied in these studies were not capable to metabolize these carcinogens efficiently.

In conclusion it can be stated that the combined use of the wild type V79 cells and the MC^{-}-27 cells in co-cultivation with primary chick embryo hepatocytes provides a potential promising _in vitro_ model to study the role of gap junctional intercellular communication in the transport of reactive intermediates.

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CHAPTER V

This chapter is based mainly on the following publication:

Jongen, W.M.F., Sijtsma, S.R., Zwijsen, R.M.L. and Temmink, J.H.M., (1987), A co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells as a model for metabolic cooperation studies, Carcinogenesis, 9, 767-772.

CO-CULTIVATION OF PRIMARY CHICK-EMBRYO HEPATOCYTES AND V79 CHINESE HAMSTER CELLS AS A MODEL FOR METABOLIC COOPERATION STUDIES

ABSTRACT

In this chapter the role of metabolic cooperation was investigated in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells. A morphological study showed that, in addition to the gap junctions formed between homologous cells, also gap junctions were formed between the primary chick-embryo hepatocytes and the V79 Chinese hamster cells. The number of gap junctions present in this system decreased in the following order: hep.-hep.> V79-V79> hep.-V79. Under control conditions this number was constant during a co-cultivation period of 48 hr. The heterologous gap junctions allowed the passage of [^{3}H] labeled hypoxanthine. Addition of the phorbolester TPA inhibited this transfer in a dose related way. Electron microscopy studies with sectioned material showed that inhibition of transfer was parallelled by the disappearance of all gap junctions. There was a remarkable difference between the response time of the different types of gap junctions. Those formed between V79 cells had disappeared after 20 min, whereas those formed between the hepatocytes had disappeared after 12 hr. The heterologous gap junctions behaved more or less like those between hepatocytes. After exposure times longer than 7 hr the transfer of [^{3}H] hypoxanthine was restored partly and also morphologically the gap junctions reappeared. Studies with Mitomycine C indicated that this adaptation may be ascribed to the mitotic index of the cells. Cigarette-smoke condensate, which is also an inhibitor of intercellular communication did not reduce the number of gap junctions. Dimethylbenzanthracene, at non-cytotoxic concentrations, inhibited the transfer of labeled nucleotides and may be the first example of an indirect acting inhibitor of intercellular communication.

INTRODUCTION

The mechanisms underlying the process of tumor promotion are not well understood as yet. Phorbolesters, especially 12-O tetradecanoylphorbol-13-acetate (TPA) have been widely used to study the mechanisms of tumor promotion. These esters exert an extraordinarily wide range of biological effects both in vivo and in vitro. In recent years increasing evidence has been obtained which suggests that modulation of cell proliferation and differentiation are important factors. Cell proliferation and differentiation are supposed to be processes in which intercellular communication via gap junctions plays an essential role.

Evidence that tumor promoters can inhibit intercellular communication was first obtained by Yotti et al., (1979) and Murray and Fitzgerald (1979). Studies of Yancy et al. (1982) and Kalimi and Sirsat (1984) have shown that addition of TPA decreased the number of gap junctions in cultured cells as well as in mouse skin. Inhibition of intercellular communication has been studied in a variety of cultured cells by means of inhibition of metabolic cooperation, electrical coupling and dye transfer methods (Yotti etal., 1979; Murray and Fitzgerald, 1979; Loewenstein, 1979; Williams et al., 1981; Telang et al., 1982; Trosko et al., 1980;1983; Enomoto et al., 1984a, b). An increasing data base has been obtained which indicates that many types of promoting agents inhibit intercellular communication. Moreover, there is a good correlation between phorbolester mediated inhibition of intercellular communication and enhancement of cell transformation in BALB/c3T3 cells (Yamasaki, 1984). Chemically transformed cells do not communicate with surrounding non-transformed cells and reported anti tumor promoting agents antagonize the phorbol ester mediated inhibition of intercellular communication (Shuin et al., 1983; Yamasaki et al., 1984; Nishino et al., 1984; Boreiko et al., 1987).

So far only very little attention has been paid to the role of metabolism in the formation of compounds with promoting activities in vitro, since most cell cultures used in this type of research do not possess significant levels of biotransformation enzymes. In this respect the use of primary chick-embryo hepatocytes co-cultivated with V79 Chinese hamster cells offers a promising model. Several studies have shown that these hepatocytes maintain their biotransformation capacity in culture for several days and that they are able to metabolize a wide range of indirect acting mutagens and carcinogens (van der Hoeven et al., 1984; Bruggeman and van der Hoeven, 1985; Jongen et al., 1985,

1986; Topp and van Bladeren, 1986). In the previous chapter it was demonstrated that addition of TPA or cigarette-smoke condensate to this co-cultivation system inhibited the transfer of mutagenic intermediates from the metabolizing cells to the target cells (Jongen et al., 1986). This observation suggested that metabolic cooperation occurred between the two cell types and that transfer of intermediates of benzo(a)pyrene and dimethylnitrosamine metabolites also took place through gap junctions.
The purpose of this chapter was twofold. The first aim was to investigate whether functional gap junctions were really formed between the two cell types. The second aim was to study the possibility to develop a test system for inhibition of metabolic cooperation using this co-cultivation system.

MATERIAL and METHODS

Material.

[^{3}H] labeled hypoxanthine was obtained from Amersham, England. The photographic emulsion NTB2 was from Kodak, Rochester, U.S.A.. Mitomycin C was obtained from Sigma, St Louis, MO, U.S.A.. Petriperm dishes were obtained from Heraeus, Hanau, FRG.

Methods.

Cell culturing

In the experiments for the electron microscopy studies the cells were co-cultured in Hams F10 (Flow) supplemented with 10% newborn calfserum (Gibco). For the transfer studies of labeled nucleotides, the cells were co-cultured in Dulbecco's MEM (Flow) without hypoxanthine supplemented with 10% newborn calfserum (Gibco) and 5% of a non-essential amino acids solution (Flow). To all media penicillin (50 units/ml) and streptomycin (50 μg/ml; Gist-Brocades) were added.

Electron microscopy studies

Transmission electron microscopy of sectioned material.

V79 cells were brought on microscopical cover slips placed in sterile tissue culture multi-well dishes. After 4 hr the medium was removed and 1 ml of a suspension of primary chick-embryo hepatocytes was added ($5x10^6$ hepatocytes /ml). Four hours later the medium was replaced again and the test compounds were added (TPA: 0.01µg/ml; CSC: 32 µg/ml). At different time intervals after start of the exposure three coverslips were washed three times with 0.1 M cacodylate buffer (pH 7.4). Fixation was done in 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) for 30 min followed by treatment with the same solution complemented with either lanthanium nitrate or 0.5-0.8% ruthenium red. All further steps in the fixation procedure contained these tracers. After washing the cover slips three times with the cacodylate buffer, the cells were fixed in 1% osmium tetraoxide for 3 hr. Then the cells were stained with uranyl acetate (0.5%) for 1 hr in the dark and dehydrated in an ethanol series. The dehydrated material was treated twice with propylene oxide and plastic (a mixture of epon: araldite: DDSA= 10:8:24) for 30 min. After impregnation for 30 min with pure plastic, the cells were embedded in fresh plastic containing a small amount of DMP-30. Polymerization took place in a stove at 60° C within 6 hr. The cover slips were removed from the embedded cell material and this was cut into small squares which were glued onto plastic stubs. From this material parallel sections (50-100 nm) were cut with a diamond knife and stained with uranyl acetate (30 min at 40° C) and lead citrate (40 sec at 20o C) in an LKB ultrastainer.

Quantification of results

The sections were examined for cell morphology and the presence of gap junctions. At every time point 75-100 cells with homologous or heterologous neighbouring cells were scored for the presence of gap junctions. The number of gap junctions was expressed as the number of contact zones in 100 tangent planes between two adjacent cells.

Transfer of labeled nucleotides

After isolation the hepatocytes were placed onto 1 cm^2 microscope cover slips together with HGPRT deficient V79 cells in a ratio of 1:10. After 4hr the

medium was replaced to remove dead cell material. The next day [^{3}H] hypoxanthine (10 μCi/ml; specific activity 5 Ci/mmol) was added in a total volume of 2 ml. After 3-4 hr the medium containing the label was removed and the cells were washed four times with Dulbecco's MEM medium containing 0.5 mM hypoxanthine. Prior to fixation the cultures were washed three times with ice-cold trichloroacetic acid for 10 min. Fixation was carried out with an ethanol/acetic acid mixture (3:1) in three steps; 10 min in a 10% fixative solution in Dulbecco's medium; 10 min in a 1:1 mixture of fixative and medium and 10 min in a 3:1 mixture of fixative and medium. Finally the cells were washed with 70% ethanol solution and dried overnight. The cover slips with cells were dipped in the NTB2 emulsion at 42° C, dried for 1 hr in the presence of silicagel and kept at 4° C for 4-5 days. The emulsion was developed (D 19, Kodak) and fixed. Then the cells were washed with water and stained with a toluidine blue solution (0.02% in citrate buffer at pH5) for 10-15 min. The experiments were carried out in triplicate and repeated at least once. For each treatment the number of grains was determined in at least 25 V79 cells.

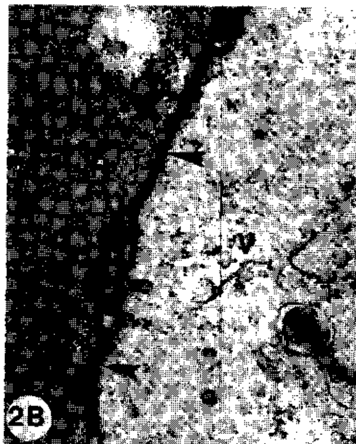
RESULTS

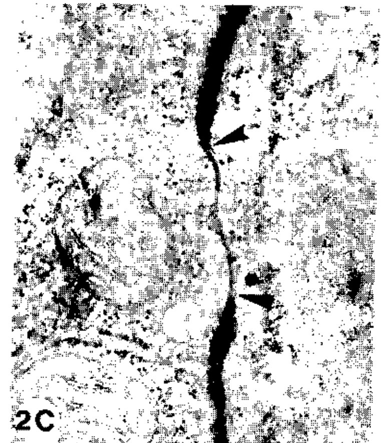
Morphological presence of gap junctions

Transmission electron microscopy on sectioned material: Structure and number of gap junctions between homologous and heterologous cells were investigated. V79 Chinese hamster cells and primary chick-embryo hepatocytes differ in size and ultrastructural details which makes them easy recognizable (Fig I).
Gap junctions were formed in vitro between adjacent hepatocytes (Fig IIa) between primary chick-embryo hepatocytes and V79 Chinese hamster cells(Fig IIb) and between neighbouring V79 Chinese hamster cells (Fig IIc). Generally, the gap junctions between homologous cells were long and the ruthenium red dye was excluded (Figs IIa and IIc); in contrast gap junctions between heterologous cells were very short and could be recognized merely by the closeness of the opposing membranes (Fig IIb).

Figure I V79 Chinese hamster cells (v) and primary chick-embryo hepatocytes in a co-cultivation system. V79 Chinese hamster cells can be recognized by light cytoplasma and by endocytotic vesicles and invaginations of the plasma membrane. The hepatocytes are characterized by dense cytoplasma and by large lipid bodies(1).

Figure II A: Large gap junction (between arrow heads) between two chick embryo hepatocytes. Ruthenium red dye has not penetrated into the junction. 40.000x. B: Contact area between a V79 Chinese hamster cell (V) and a primary chick-embryo hepatocyte (H). Small gap junctions (arrow heads) are present but dye is not excluded. 40.000x. C: Large gap junction (between arrow heads) between two V79 Chinese hamster cells excluding dye from between the lamellae. 40.000x





There were also numerical differences between the gap junctions of the different cell types: gap junctions formed between primary chick embryo hepatocytes were most numerous, followed by the gap junctions formed between V79 Chinese hamster cells and those formed between the primary chick-embryo hepatocytes and the V79 Chinese hamster cells. The number of gap junctions was quantified for the three types at different time points after t=0 both with and without replacement of the culture medium (Table I).

Table I Effect of culture time on the presence of gap junctions in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells.

GAP JUNCTIONS / 100 CONTACT ZONES

time (hr)	not replaced			replaced (*)		
	H-H	V79-V79	H-V79	H-H	V79-V79	H-V79
0	34	19	14	34	19	14
1	34	21	13	31	16	12
12	35	21	13	nt	nt	nt
19	nt	nt	nt	30	16	11
48	35	21	14	29	16	19

H= primary chick-embryo hepatocytes; V79= Chinese hamster cells;
nt= not tested; (*)= the culture medium was replaced every 12 hr.

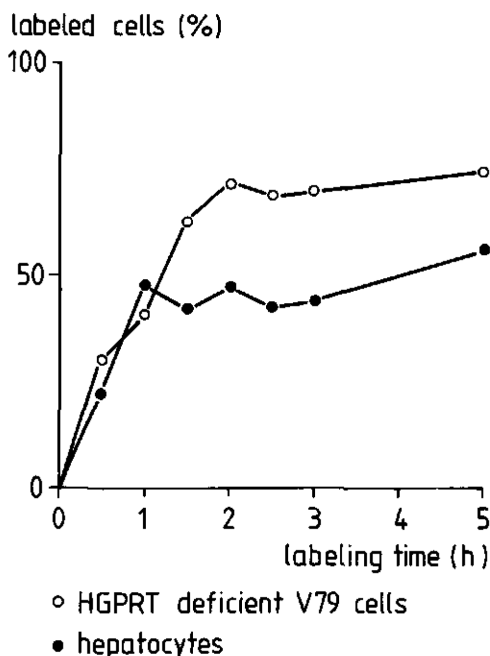
When the culture medium was not replaced the number of gap junctions did not change for at lest 48 hr. However when the medium was replaced every 12 hr a small decrease in the number of gap junctions was observed.

<u>Transfer of labeled nucleotides</u>

To see whether the gap junctions, formed between the primary chick embryo hepatocytes and the V79 Chinese hamster cells were functional in that they would allow the passage of molecules, HGPRT deficient mutants of the V79 cell line were used. These mutants are unable to phosphorylate the hypoxanthine

present in the culture medium. When [^{3}H] hypoxanthine is added to a culture of these mutants no label will be incorporated. Only when these cells, are co-cultured with normal cells and phosphorylated hypoxanthine is transferred into the mutant cells via gap junctions incorporation will occur ultimately resulting in an increased number of grains/cell.
First the concentration of [^{3}H] hypoxanthine necessary to get heavily labeled hepatocytes was studied. In the dose range of 1-10 μCi/ml, using a labeling time of 7 hr, the highest dose gave the best labeling. Next the optimal labeling time was determined. The data are presented in Figure III and show that after 1 hr a steady state situation was reached for the percentage of heavily labeled hepatocytes.

Figure III The relation between labeling time and the percentage of labeled cells in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells.



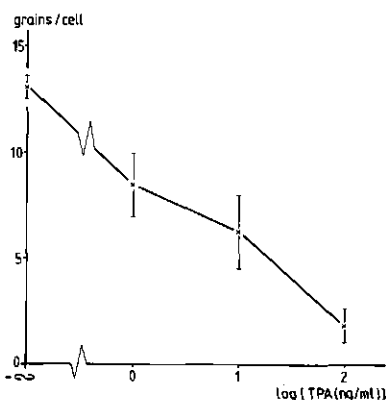
Labeling is with [^{3}H] hypoxanthine (10 μCi/ml; specific activity 5 Ci/mmol). Data are the average value of one experiment carried out in triplicate.

It should be emphasized that only those cells were counted which contained so much label that the number of grains could not be determined. In addition there was always a number of hepatocytes which were labeled less heavily (about 10% of the total population). When the transfer of radioactivity from hepatocytes to V79 mutant cells was studied, the optimal labeling time appeared to be at least 2 h (Fig III). Under those conditions the highest percentage of labeled V79 mutant cells was 75%. Only those cells were considered which were in direct contact with a heavily labeled hepatocyte and a cell was considered to be labeled when the number of grains was at least three times the background value. When [^{3}H] hypoxanthine was added to a monoculture of the V79 mutants, the number of grains never exceeded background values (1-2 grains/cell). When the same cells were co-cultured with primary chick-embryo hepatocytes the number of grains increased to an average value of 13 grains/-cell (Figure IV, control value).

Inhibition of metabolic cooperation

The next objective was to investigate whether known inhibitors of metabolic cooperation were capable of inhibiting the transfer of labeled molecules. Figure IV presents the effects of TPA on the transfer.

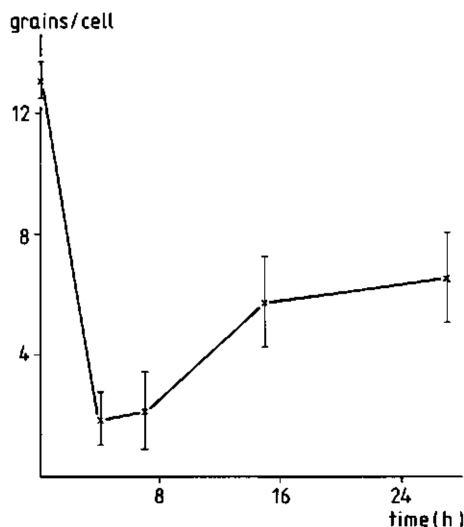
Figure IV **Effect of the phorbolester TPA on the transfer of [^{3}H] hypoxanthine from primary chick-embryo hepatocytes to HGPRT deficient V79 Chinese hamster cells.**



Data are average values of two experiments and are given with their S.D.. Labeling time with [^{3}H] hypoxanthine was 4 hr with 10 μCi/ml. Treatment time with TPA was 4 hr.

Addition of TPA caused a dose related decrease in the number of grains with a maximal inhibition at 100 ng/ml. At this dose the number of grains/cell was reduced to background values. When the effects of different exposure times were studied, inhibition was already maximal after 4 hr (Fig V).

Figure V Effect of culture time on the inhibitory potential of the phorbolester TPA on the transfer of [^{3}H] hypoxanthine from primary chick-embryo hepatocytes to HGPRT deficient V79 Chinese hamster cells.



Data are the average values of two experiments and are given with their S.D.. Labeling time with [^{3}H] hypoxanthine was 4 hr with 10 μCi/ml.

However, after longer exposure times an increase in the number of grains was observed. To exclude the possibility that recovery of metabolic cooperation was due to metabolic breakdown of the TPA, the culture medium was replaced 12 hr after plating and just before the [^{3}H] hypoxanthine was added. When DMBA and CSC were tested both compounds appeared to inhibit metabolic cooperation in the co-cultivation system in a dose related way (Table II). The concentrations applied did not cause a reduction in cell surival, measured as plating efficiency.

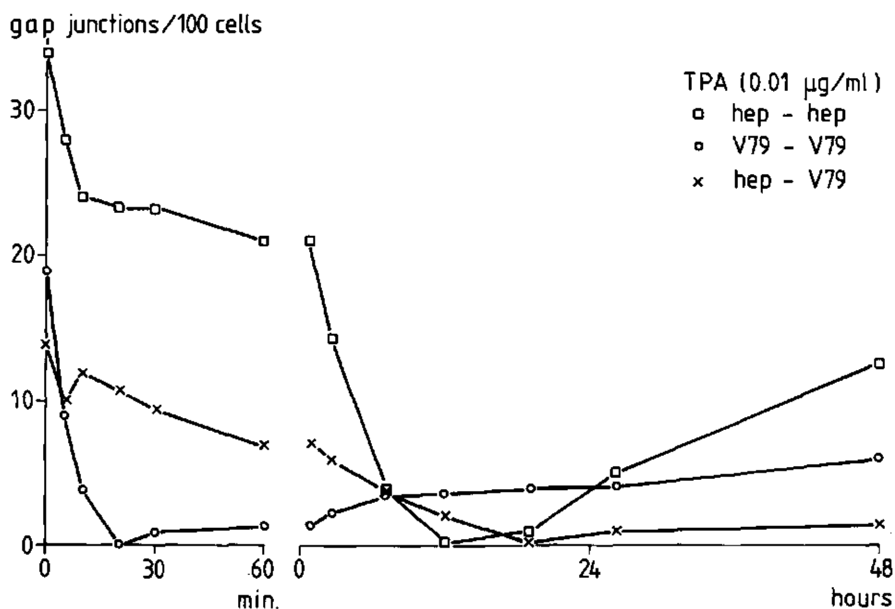
Table II Inhibitory effects of dimethylbenzanthracene (DMBA) and cigarette smoke condensate on the metabolic cooperation between primary chick embryo hepatocytes and V79 Chinese hamster cells.

Exposure	Concentration (μg/ml)	P E (%)	grains/cell
Control	-	95	9.7±1.4
TPA	0.1	95	1.3±0.5
DMBA	2.0	89	5.0±1.2
	4.0	94	1.2±1.4
CSC	8.0	90	8.3±1.8
	16.0	95	3.0±1.4

Data are average values of two experiments carried out in triplicate and are given with their SEM. Labeling time with [^{3}H] hypoxanthine is 4 h with 10 μCi/ml. Exposure time is 4 h. P E = plating efficiency of V79 cells after a 4 hr exposure.

The morphological aspects of exposure to TPA were also studied. In the first experiments the effect of TPA (10 ng/ml) on the number of gap junctions was quantified at different time intervals (Figure VI). A distinction was made between the three possible types of gap junctions occurring. Gap junctions formed between the hepatocytes were present in the highest number. After addition of TPA a fast initial decrease of 30% was observed after 30 min, followed by a slower decrease until no gap junctions were seen after 12 hr. At incubation times longer than 12 hr, a slow but continuous increase was observed. Gap junctions occurring between V79 cells disappeared much faster. After exposure to TPA for 20 min they had completely disappeared. This type of gap junction also reappeared after longer exposure times. Most importantly, heterologous gap junctions, formed between the primary chick-embryo hepatocytes and V79 Chinese hamster cells behaved more or less like homologous hepatocyte gap junctions.

Figure VI Effect of the phorbolester TPA on the presence of gap junctions in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells.



Reappearance of gap junctions in connection with an increased transfer of labeled nucleotides is hypothesized to be related to cell growth and not to adaptation of cells. Therefore the V79 cells were pretreated with mitomycin C, a cross linking agent, which at the concentration applied (25 µg/ml) prohibits cell division but not protein synthesis (Swain, 1977). The results are summarized in Table III.

After longer TPA exposure (20 hr), the transfer of labeled nucleotides is completely inhibited in the mitomycin C pretreated cells whereas the non-pretreated cells show a partially restored transfer after the same exposure time. The amount of grains/cell in the groups treated with mitomycin C without addition of TPA and after 4 hr TPA application does not differ significantly from the control groups. This indicates that there is no effect of mitomycin C treatment on gap junction formation and intercellular communication or an aspecific effect on hypoxanthine metabolism.

Table III **The effect of Mitomycin C treatment on the restore of intercellular communication between primary chick-embryo hepatocytes and V79 Chinese hamster cells during exposure to TPA.**

		Treatment	
Exposure	time (hr)	Mitomycin C (25 μg/ml)	none
Control	4	10.7±0.9	11.7±1.1
	20	11.2±1.3	11.1±1.0
TPA (*)	4	1.7±0.9	2.6±0.8
	20	1.3±0.8	6.1±1.3

Experiments were carried out in triplicate. Data are average values of one experiment and are given with their SEM. (*) Exposure to TPA was 0.1 μg/ml.

Table IV **Effect of cigarette-smoke condensate on the presence of gap junctions in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells.**

	GAP JUNCTIONS / 100 CONTACT ZONES					
time (hr)	Control			CSC (32 μg/ml)		
	H-H	V79-V79	H-V79	H-H	V79-V79	H-V79
0	34	19	14	33	18	14
0.5	nt	nt	nt	32	17	13
1.0	33	21	13	36	16	15
12.0	33	22	13	35	16	15
48.0	33	21	13	32	16	13

H= primary chick-embryo hepatocytes; nt= not tested.

The morphological effects of cigarette-smoke condensate, another inhibitor of metabolic coperation, were also investigated. The sample used in this study originates from the same batch as was used in all other studies. The results

are summarized in Table IV and show that no decrease in the number of gap junctions was observed after exposure to 32 μg/ml.

DISCUSSION

In this chapter data are presented which show that gap junctions are formed between primary chick-embry hepatocytes and V79 Chinese hamster cells and that these gap junctions allow the passage of labeled nucleotides.
Increasing evidence has been obtained that inhibition of intercellular communication between cells is an important determinant of the tumor promoting properties of compounds (Trosko et al., 1983; Yamasaki, 1984). Several test methods have been developed to measure inhibition of metabolic cooperation by means of transfer of poisonous base analogues, electrical coupling and dye transfer (Yotti et al., 1979; Murray and Fitzgerald, 1979; Loewenstein, 1979; Enomoto et al., 1984a, b). However, sofar only very little attention has been paid to the role of drug metabolism in the formation of compounds capable of inhibition of intercellular communication. One example in this category is the indirect acting carcinogen dimethylbenzanthracene (DMBA). When this compound was tested in the co-cultivation system at non-cytotoxic concentrations, inhibition of metabolic cooperation was observed (Table II). This is in agreement with data published by Tachikawa et al. (1986). They reported that exposure to DMBA resulted in a decrease of electrical signal transduction and morphologically the number of gap junctions occurring in primary lingual cells was drastically reduced. In that respect the test system presented in this study has several advantages. Primary chick-embryo hepatocytes are capable of metabolizing a wide range of xenobiotics (van der Hoeven et al., 1984; Bruggeman and van der Hoeven, 1984, 1985; Jongen et al., 1985, 1986). In vitro they maintain their biotransformation capacity for at least 72 hr after plating. Treatment with known enzyme inducers leads to induction rates comparable with in vivo situations (Jongen et al., 1986; Topp and van Bladeren, 1986) which allow also the study of modulating factors. Addition of TPA caused a dose-related decrease in the number of grains in the V79 mutant cells. Morphologically this was accompanied by disappearance of all three types gap junctions. However, there was a remarkable difference in response time between the two cell types. Gap junctions formed between V79 cells responded much

faster than those formed between primary chick-embryo hepatocytes. This is in agreement with the findings of Yancy et al.(1982). The heterologous gap junctions behaved like hepatocyte junctions. When cigarette-smoke condensate was tested no reduction in the number of gap junctions was observed. The concentration applied is toxic, at least for V79 Chinese hamster cells (Jongen et al., 1985). Beside this, the dose used in these experiments inhibits metabolic cooperation between V79 cells (Jongen et al., 1985; Hartman and Rosen, 1984) and the complex mixture also inhibits transfer of labeled nucleotides in the co-cultivation system. This raises the question whether the absence of morphological effects is due to a different mechanism of CSC or that the hepatocytes are capable of detoxifying the complex mixture with respect to this, direct acting, property. An argument in favor of the first hypothesis is provided by the observation that in this co-cultivation system CSC, like TPA, is capable of inhibition of the transport of reactive intermediates from the metabolizing cells to the target cells.

The reappearance of the gap junctions after longer exposure times to TPA parallels the increased transfer of labeled nucleotides. Probably this is not due to metabolic breakdown of the TPA since the compound was replaced in the transfer experiments. This biphasic effect of exposure to TPA has been observed also by several other investigators (Dorman and Boreiko, 1983; Enomoto and Yamasaki., 1985; Kalimi and Sirsat, 1984). Dorman et al. (1983) studied the role of intercellular communication in the promotion of C3H 10T1/2 cell transformation. No correlation was found between the doses of TPA required for promotion and since inhibition of intercellular communication was transient they hypothesized that inhibition of intercellular communication is not a sufficient event for tumor promotion.

The data obtained in this study with mitomycin C suggest that the duration of inhibition of intercellular communication is largely dependent on the growing state of the cells. When the cells do not divide inhibition is continuous. This observation is consistent with the findings of Yamasaki et al., (1984) who reported that inhibition of intercellular communication between cells in culture was transient when the cultured cells were sub-confluent but that inhibition was continuous when confluent cultures were used.

In the previous chapter it was shown that addition of TPA and cigarette-smoke condensate to this co-cultivation system inhibited benzo(a)pyrene induced mutagenicity in the V79 cells. In that paper the conclusion was drawn that these inhibitory effects were due to inhibition of transport of reactive intermediates. Those data, combined with the results presented in this chapter

justify the conclusion that a considerable part of the reactive intermediates is transported from the metabolizing cells to the target cells through gap junctions.

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CHAPTER VI

This chapter is based mainly on the following papers:

1. Jongen, W.M.F., Topp, R.J., Tiedink, H.G.M. and Brink, E.J., (1987), A co-cultivation system as a model for *in vitro* studies of modulating effects of naturally occurring indoles on the genotoxicity of model compounds, Toxic. In Vitro, 1, 105-110.

2. Jongen, W.M.F., Topp, R.J., van Bladeren, P.J., Lapre, J. and Leenen, R., (1987), Modulating effects of indoles stem from a change in the balance between drug-metabolizing enzymes and depend largely on the type of mutagen, submitted for publication.

3. Jongen, W.M.F., Topp, R.J., van Bladeren, P.J., Sandker, G. and Goertz, M., (1987), Inhibitory effects of pretreatment of drug metabolizing cells with B-NF on SCE induction by B(a)P is not related to induction of cytochrome P450 associated enzyme activity, submitted for publication.

THE USE OF THE CO-CULTIVATION SYSTEM TO STUDY MECHANISMS OF CANCER MODULATING COMPOUNDS

A. MODULATING EFFECTS OF NATURALLY OCCURRING INDOLES ON SCE INDUCTION BY MODEL COMPOUNDS

ABSTRACT

The enzyme inducing capabilities of the naturally occurring indoles indole-3-carbinol (I3C) and indole-3-acetonitril (I3A) on cytochrome P450 associated enzymes and conjugating enzymes were studied in cultured primary chick-embryo hepatocytes. In addition the modulating effects of pretreatment with these indoles on the induction of SCEs by mutagens from different chemical classes were investigated. Finally, the effect of pretreatment with I3C on B(a)P metabolism was studied in more detail. At 25 μg/ml I3C induced ethoxyresorufin-O deethylase (EROD) a cytochrome P448 associated enzyme, activity 3.9 fold and ethoxycoumarin-O-deethylase (ETCO), a cytochrome P448/450 associated enzyme, activity 2-fold; UDP-glucuronyltransferase activity (UDPGT) with 3-OH B(a)P as substrate was increased 2-fold. No induction of glutathione-S-transferase was found. I3A showed optimal induction at 35 μg/ml.; EROD activity was increased 1.6 fold and ETCO activity 2-fold. UDPGT was increased 2-fold whereas again no increase was observed for glutathione-S-transferase. The induction ratios between UDPGT activity and cytochrome P450 content was studied after treatment with various inducers. Induction with both I3C and I3A resulted in ratios comparable to the control situation whereas induction with 3-methylcholanthrene resulted in a considerable decrease in this ratio (UDPGT activity/cytochrome P450 content). Kinetic studies showed that both indoles are able to inhibit EROD activity in chick-embryo hepatocyte microsomes pretreated with B-naphtoflavone with I3C having a higher affinity than I3A. Pretreatment with I3C resulted in a 20-40% decrease in SCE induction by benzo(a)pyrene [B(a)P] and dimethylnitrosamine (NDMA). No decrease in SCE induction was observed for 2-aminoanthracene (2AA) and dibromoethane (DBE) and the direct acting alkylating agent ethylmethanesulfonate (EMS). Pretreatment with I3A again resulted in a 20-40% decrease in SCE induction for B(a)P whereas no decrease was observed for DMNA, 2AA and EMS. When a microsomal fraction prepared from I3C-pre-

treated hepatocytes was used, no decrease in B(a)P-induced SCEs was found; in contrast, use of an S-9 fraction prepared from the same cells resulted in a 45% decrease. In addition the profile of metabolite formation from B(a)P was studied. Pretreatment with I3C resulted in a considerable increase in total conversion. When the relative amounts of the individual metabolites were compared with those obtained after 3-MC pretreatment no significant differences could be observed. The data obtained in this study indicate that the protective effects of pretreatment with indoles are not directly correlated with induction of cytochrome P450 associated enzymes. Although both I3A and I3C are good candidates to act as competitive inhibitors for B(a)P metabolism this seems to be of minor importance in this study. Instead the protective effects seem to result from a changed balance of the enzyme systems involved in the indole biotransformation process and point to an essential role of the conjugating enzyme systems.

INTRODUCTION

Epidemiological data indicate that consumption of cruciferous vegetables is associated with a decreased incidence of cancer in human populations (Graham, 1983; Hirayama, 1986). The anticarcinogenic properties of cruciferous vegetables and isolated compounds have been studied in several investigations. When animals were first fed on diets high in cruciferous vegetables and then exposed to various indirect acting carcinogens, tumour yields were lower and survival rates were higher than similarly treated animals on semi-purified diets (Boyd et al., 1982; Stoewsand et al., 1978; Wattenberg, 1983). Subsequently, isolation and identification of the principal compounds involved showed that they were indoles (Loub et al., 1975). When the identified compounds were tested, indole-3-carbinol (I3C), 3,3'-diindolylmethane and indole-3-acetonitrile (I3A) all appeared to inhibit the induction of forestomach neoplasia by benzo(a)pyrene [B(a)P]. I3C and 3,3'-diindolylmethane also inhibited 7,12 dimethylbenzanthracene-induced mammary tumours although I3A did not (Wattenberg and Loub, 1978). The mode of action of this type of anticarcinogenic compounds may be a qualitative and quantitative change in the metabolism of the carcinogens. In contrast to all these findings, Pence et al. (1986) reported recently that indole-3-carbinol enhanced dimethylhydrazine induced tumor formation. Apparently the type of modulation is largely dependent on the choice of the model compound. A number of cruciferous plants, including Brussels sprouts, cabbage, cauliflower and broccoli have been found to induce aryl hydrocarbon hydroxylase (AHH) activity (Wattenberg et al., 1976) and/or the activity of cytosolic glutathione-S-transferase (Sparnins et al., 1982). Moreover, I3C and to a lesser extent, I3A appeared to be significant inducers of hepatic and intestinal monooxygenase (Loub et al., 1975) and glutathione-S-transferase (Sparnins et al., 1982) activities. Sofar *in vitro* data on the protective effects of pretreatment with indoles against the DNA damaging capacity of carcinogens have not been reported. Probably this is due mainly to the fact that in most *in vitro* systems it is not possible to induce biotransformation enzymes. As has been argued in Chapter II the use of *in vitro* cultures of primary chick-embryo hepatocytes offers a potential useful model to study modulating effects of indoles. These hepatocytes maintain their biotransformation capacity in vitro for up to 72 h without loss of activity. Moreover induction with known enzyme inducers leads to enzyme induction comparable with that occurring in *in vivo* situations (Althaus et al.,

1979; Giger and Meyer, 1981; Topp and van Bladeren, 1986). In this study the effects of treating the primary chick-embryo hepatocytes with I3C and I3A on various enzyme systems involved in the biotransformation process were investigated. In addition the protective effects of these compounds against several indirect-acting carcinogens were studied in the co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells.

MATERIALS and METHODS

Materials

Ethoxycoumarin, I3C, I3A, B(a)P and trypsin were all obtained from Sigma Chemical Co. (St Louis, MO, USA).Dimethylnitrosamine (NDMA) and dimethylsulfoxide (DMSO), which was used as solvent, were from E. Merck AG (Darmstadt, FRG). NADP, NADPH and glucose-6-phosphate were obtained from Boehringer Mannheim GmbH (Mannheim, FRG). Ethoxyresorufin was obtained from Pierce Eurochemie BV, Oud-Beijerland.

Methods

Hepatocyte isolation. The isolation of the primary chick-embryo hepatocytes was performed as described in Chapter II.

Mutagenicity assay. From the suspension of hepatocytes, 5 ml was plated in Petri dishes (6-cm in diameter) and incubated for 24 h in a CO_2 incubator. The medium was replaced to remove dead cell material and I3A or I3C was added. At different time points after start of this treatment, the medium was removed and the cells were exposed for a specific time. After removal of the test compound, the cells were washed twice with a physiological salt solution, dissociated with trypsin (0.2%) suspended in Hams F10 medium supplemented with 10% newborn calf serum, penicillin (50 units/ml) and streptomycin (50 µg/ml) plated onto microscope slides (10^5 cells/slide) and incubated for 2 h to allow the cells to attach. The sister chromatid exchange (SCE) assay was performed according to Perry and Wolff (1974) with slight modifications described elsewhere (Jongen et al, 1985). The assay was initiated according to Jongen et al. (1981). In all experiments, control cells were treated with the solvent (DMSO)

in an amount equal to that used for the treated cells. The DMSO concentrations never exceeded 0.2%. All experiments were repeated at least once. The percentage decrease was calculated after subtraction of the control values from the values obtained with the exposed cells.

<u>Preparation of liver homogenate and microsomes</u>. The hepatocyte suspension ($6x10^5$ hepatocytes/ml) was plated in 6-cm diameter Petri dishes (5 ml/dish) and incubated at 37° C in a humidified atmosphere containing 5% CO_2. After 24 h the medium was replaced and appropriate amounts of test compounds were added. At different intervals after addition of the test compounds, the hepatocytes were washed twice with saline phosphate buffer (pH7.4, 0.1 M), scraped off the dishes and suspended in a total volume of 0.4 ml/dish. After homogenization of the suspension in a glass Potter-Elvehjem homogenizer, the homogenate was centrifuged for 15 min at 9,000 g. The supernatant was collected and contained approximately 0.5 mg protein/ml. The S-9 was kept in liquid nitrogen at -196° C and S-9 mix was prepared as described in Chapter II. The microsomal fraction was prepared by centrifuging the S-9 at 105,000 g for 90 min. The supernatant was discarded and the pellet was resuspended in the original volume with 0.1M-phosphate buffer (pH 7.4) containing 0.1 mM EDTA. The microsomal fraction was kept in liquid nitrogen. Previous experiments showed that in a period of at least 1 month, no change in enzyme activity was observed. The mix contained 20% microsomal fraction and was prepared as the S-9 mix except that NADPH was added instead of NADP.

<u>In ovo treatment</u>. 15 day-old chick embryos were injected with a sterile needle through the membrane of the air chamber. Injection was done with 50 µl of a DMSO solution containing the various enzyme inducers. After 22 h the injection was repeated and 22 h later the embryos were killed and the liver material was isolated sterile. Preparation of the respective enzyme fractions was performed as described in Chapter II.

<u>B(a)P metabolite formation</u>. The reaction mixture (total volume 6.0 ml in phosphate buffer; 0.1M;pH 7.4) consisted of the microsomal fraction (3 nmol cytochrome P450), 18 mol $MgCl_2$, 3.36 mol NADPH and 300 nmol B(a)P mixed with 12 µCi [^{3}H] B(a)P (specific activity 7.63 Ci/mmol).
After preincubation of the microsomal fraction and co-factors for 2 min at 37° C the reaction was started by addition of the B(a)P solution (200 µl of a acetone solution). Incubation was done for varying times (5, 10 and 15 min).

At these time-points 2.0 ml of the mixture was mixed with 2.0 ml of acetone to terminate the reaction. To each tube 4.0 ml of ethylacetate was added and the mixtures were extracted for 3 min on a vortex. The organic layer was transferred into a tube containing 1 g of anhydrous Na_2SO_4 and kept overnight at -20° C. The aqueous layer was collected and counted in a liquid scintillation counter. The organic layer was evaporated under nitrogen and the residue dissolved in 100 μl of methanol. This sample was mixed with 20 μl of a standard mixture and injected onto a HPLC column (Lichrosorb 5-RP18; 150x4.6 mm). The metabolites were separated using a water/ methanol gradient program as described by Thakker et al. (1978). Detection was performed with UV at 254 nm. Fractions of 1.2 ml were collected and radioactivity was determined in each fraction with liquid scintillation counting.

Enzyme assays

Protein. The protein content of the subcellular fractions was determined with the biorad method developed by Bradford (1976).

Cytochrome P450. Cytochrome P450 content was determined by its CO-binding spectrum according to the method of Omura and Sato (1964) on a single beam spectrofotometer. Absorption maxima of the Soret bands were determined by applying first derivative spectrofotometry.

Ethoxyresorufin-O-deethylase. The method used to measure EROD activity was essentially that described by Burke and Mayer (1974). The monolayers of primary chick-embryo hepatocytes were washed three times with an ice-cold buffer solution (Tris-HCl, 0.1M. pH 7.8) and the cells were scraped off the dishes and suspended in 0.5 ml buffer.
The suspension was homogenized, as described before, and centrifuged for 30 sec at 14,000 g in a Beckman microfuge. The supernatant was collected and 0.1 ml was transferred into a 1 cm cuvette. After addition of 1.0 ml ethoxyresorufin solution (10 M) in buffer, the mixture was pre incubated for 1 min at 37° C.
After measurement of the endogenous activity, the reaction was started by addition of 1-5 1 of NADPH solution (10mM). The reaction was monitored fluorimetrically using excitation and emission wavelengths of 510 and 586 nm, respectively, and the enzyme activity was calculated from the slope of the

linear part of the activity curve.

Ethoxycoumarin-O-deethylation. The method for measuring ETCO activity was identical to the procedure used for EROD activity but used 7-ethoxycoumarin (1 mM in Tris-HCl) as the substrate. The reaction was monitored fluorimetrically using wavelengths of 360 nm (excitation) and 460 nm (emission).

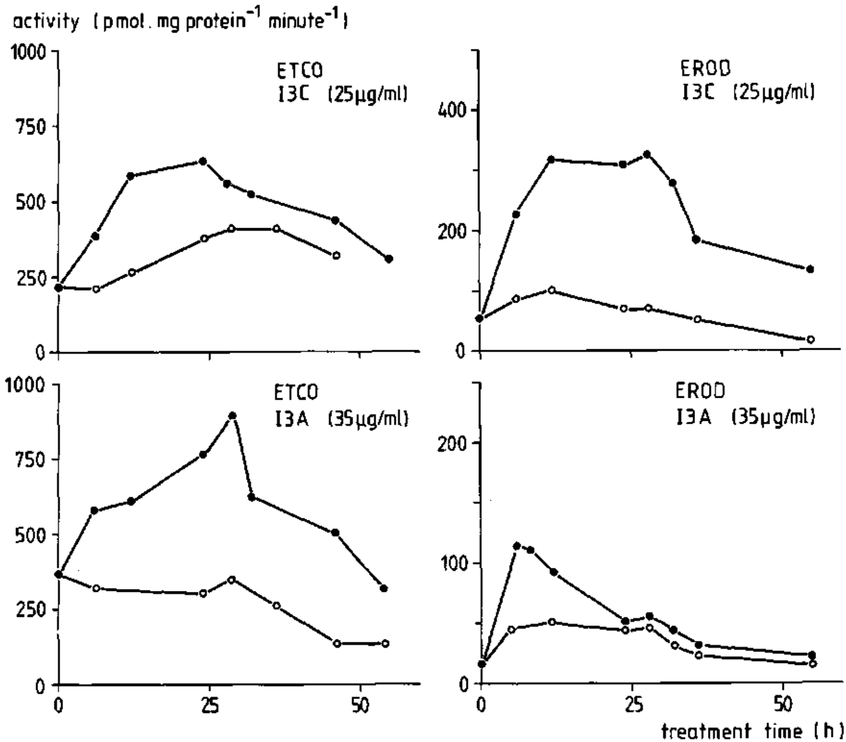
Glutathione-S-transferase. GSH-transferase activity was determined spectrofotometrically according to Baars et al. (1979) using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate. The assay consisted of 1.25 ml 0.13 M phosphate buffer, 3 mM EDTA, pH 8.0; 0.10 ml 100,000 g supernatant containing 15 g protein/ml; Glutathione , 0.1 ml in distilled water (final concentration 1.0 mM); CDNB in 0.05 ml ethanol (final concentration 1.0 mM). The reaction mixture was pre incubated at 37° C for 2 min before the reaction was started by addition of the substrate. Incubation took place in a shaking water bath for 3 min at 37° C. Under these conditions product formation is linear with time and with protein concentration. The reaction is monitored spectrofotometrically at 340 nm. Enzyme activity was calculated from the linear part of the activity curve.

UDP-glucuronyl transferase. Enzyme activity was measured using the method of Singh and Wiebel (1979) with minor modifications using 3-OH-B(a)P as substrate. Isolated primary chick-embryo hepatocytes were cultured for 24 h and subsequently treated with the various enzyme inducers for 22 h. Then the medium was replaced by medium (5.1 ml) containing 0.4% 3-OH-B(a)P (3.1 M) and 2% UDPGA (37.2mM). At various time points after the start of the incubation samples of 0.4 ml of medium were taken, 0.6 ml of water was added followed by extraction with 6 ml of a chloroform/methanol mixture (2:1). After centrifugation for 10 min at 1500 g, 1.0 ml of the aqueous phase was collected and kept at -20° C overnight. After addition of 0.08 ml NaOH (5.0M) to 0.8 ml of the collected aqueous phase the formation of the 3-OH-B(a)P glucuronide was determined fluorimetrically in a 1 cm cuvette using wavelengths of 378 nm (excitation) and 425 nm (emission). At the end of the experiment in each dish the protein content and the cytochrome P450 content was determined.

Enzyme activities

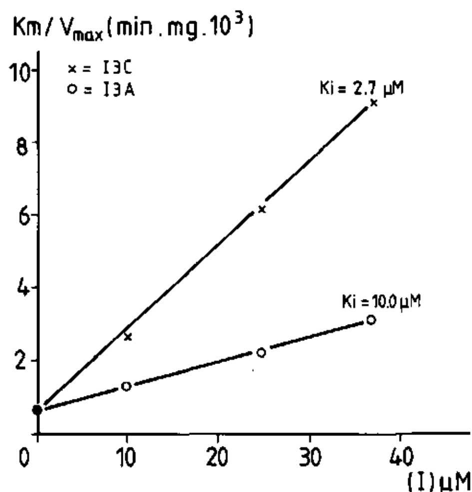
The induction of P450 associated enzymes by treatment of the isolated primary chick-embryo hepatocytes with the two indole compounds was studied at different time intervals and with different concentrations. The results are summarized in Figure I and show that each compound induced both EROD and ETCO activity.

Figure I. Activity of ethoxycoumarin-O-deethylase (a, c) and of ethoxy resorufin O-deethylase (b, d) in control (O) or treated (O) primary chick-embryo hepatocytes, the latter being exposed to either 25 μg indole-3 carbinol/ml (a, b) or 35 μg indole-3-acetonitril/ml (c, d) for 0-50 hr. Data points are means from duplicate experiments.



When various concentrations of the two compounds in the range 5-50 µg/ml were tested, optimal induction was achieved at 25 µg/ml for I3C and at 35 µg/ml for I3A (data not shown).
However the induction kinetics appear to be different for the two compounds. I3C was the better inducer of EROD, with a 3.9 fold increase in enzyme activity after treatment for 20 h at a concentration of 25 µg/ml. I3A gave a much smaller increase (1.6 fold) in enzyme activity, the maximum being reached after treatment for only 6-8 h with 35 µg/ml, the concentration giving the greatest increase. The two compounds showed a greater similarity in their effects on ETCO activity; in both the level of increase and the time to attain optimal induction, there were no major differences, although the concentrations of I3A needed to yield these effects were higher than those of I3C.
A comparative study of in vitro and in ovo induction, which will be published separately (van Bladeren et al., in preparation) showed that no qualitative differences in B(a)P metabolism and metabolite patterns were observable after pretreatment with different enzyme inducers like 3-methylcholanthrene, Aroclor 1254 and B-naphtoflavone. Therefore enzyme fractions prepared from in ovo pretreated material were also used.
Inhibition experiments for both I3A and I3C of EROD activity in microsomes prepared from chick-embryos treated in ovo with B-naphtoflavone showed that both compounds were able to inhibit EROD activity. Determination of the affinity constants showed that both compounds can be classified as competitive inhibitors for EROD activity (Figure II). The better inducing capacity of I3C in the chick-embryo hepatocytes is reflected in a higher affinity constant for EROD activity.
In addition to the effects of pretreatment on P450 associated enzymes the effects on conjugating enzymes were also studied. The data for GSH-S-transferase are summarized in Table I and show that although enzyme activity is measurable no increase in activity is found upon treatment with both indoles.

Figure II. Determination of the affinity constants of indole-3-carbinol (I3C) and indole-3-acetonitril (I3A) for EROD activity in hepatic microsomes prepared from chick-embryos pretreated with B naphtoflavone _in ovo_.



15-day old chick-embryos are injected twice with a 24 hr interval with B-naphtoflavone (0.75 mg/egg).

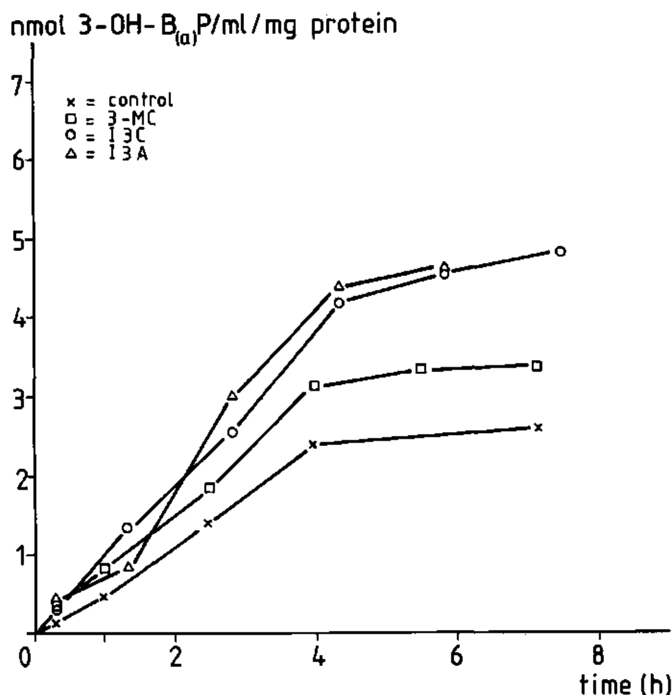
Table I Glutathione-S-transferase activity in cultured primary chick embryo hepatocytes after treatment with naturally occurring indoles.

Treatment	Enzyme Activity (*)
Control	1.28±0.17
I3C (25 μg/ml)	0.87±0.11
I3A (35 μg/ml)	0.98±0.05
Phenobarbital	1.09±0.12

(*) Enzyme activity is expressed as mmol GSH-S-transferase/min/mg protein and is calculated from the slopes of the regression lines.

Phenobarbital which is a known inducer of GSH-S-transferase activity in the rat was used as a positive control. However also for this compound no induction was observed. Apparently this enzyme system cannot be induced under the experimental conditions applied. When the effects of treatment on UDP-glucuronyltransferase using 3-OH-B(a)P as substrate were studied all compounds tested caused an increase in enzyme activity. The data are summarized in Figure III and show that treatment with 3-methylcholanthrene gives a small but consistent increase in UDPGT activity. The effects upon treatment with the indoles were considerable. Both I3A and I3C gave a two-fold increase in enzyme activity.

Figure III. **UDPglucuronyl transferase activity in cultured primary chick-embryo hepatocytes after pretreatment with indole-3-carbinol, indole-3 acetonitril and 3-methylcholanthrene.**



Pretreatment with all compounds was 22 hr (I3C: 25 μg/ml; I3A: 35 μg/ml; 3-MC:6 μg/ml). Activity is expressed as pmol 3-OH-B(a)P/ml/mg protein.

In these experiments cytochrome P450 was determined simultaneously and the inducing capacity of these compounds on cytochrome P450 content was compared with their ability to induce UDPGT activity (Table II).

Table II **Relation between UDPGtransferase (UDPGT) activity and cytochrome P450 content in cultured primary chick-embryo hepatocytes after treatment with various enzyme inducers.**

Ratio UDPGT act./cytochrome P450 (*)

time (min)	treatment			
	control	3-MC	I3C	I3A
0	0	0	0	0
20	2.6	1.3	3.5	3.8
60	9.0	4.3		
80			14.9	10.9
150	27.9	9.1		
170			27.8	25.4
240	46.1	15.8		
260			45.2	37.9
430	50.8	19.4	52.4	44.1

(*) Ratio is expressed as pmol 3-OH-B(a)P/ml/nmol cytochrome P450.

After 3-MC pretreatment the ratio between UDPGT activity and cytochrome P-450 content was three times lower than after pretreatment with both indoles or without pretreatment. This indicates that pretreatment with indoles has a better protective capacity compared with 3-MC pretreatment.

Mutagenicity studies

Because these enzyme studies showed that different types of cytochrome P450 may be involved in the modulating effects of I3C and I3A, compounds from different chemical classes were used to study the effects of enzyme modulation on mutagenicity.

Table III Modulating effect of indole-3-carbinol (I3C) pretreatment on SCE induction by model compounds in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 cells.

Exposure			SCEs/CHROMOSOME		
Compound	Time	Dose	Without I3C	With I3C	Change (%)
B(a)P	24 hr	0.0	0.27±0.02	0.27±0.02	-
(μg/ml)		2.5	0.80±0.10	0.70±0.02	-19*
		5.0	0.96±0.03	0.77±0.02	-28*
		7.5	1.46±0.08	1.07±0.06	-33*
NDMA	4 hr	0.0	0.20±0.01	0.21±0.01	-
(μg/ml)		500.0	0.37±0.04	0.28±0.03	-25*
		1000.0	0.59±0.06	0.41±0.05	-30*
		1500.0	0.91±0.04	0.48±0.07	-46*
		2000.0	1.21±0.06	0.65±0.05	-46*
2-AA	4 hr	0.0	0.21±0.04	0.31-0.03	-
(μg/ml)		10.0	0.68±0.07	0.83±0.02	-
		25.0	0.75±0.02	0.87±0.10	-
		50.0	0.81±0.10	0.87±0.02	-
DBE	4 hr	0.0	0.29±0.03	0.28±0.03	-
(mM)		1.05	0.79±0.11	0.83±0.06	-
		5.26	1.11±0.05	1.45±0.06	+40*
EMS	24 hr	0.0	0.27±0.02	0.27±0.02	-
(μg/ml)		100.0	1.04±0.04	1.02±0.03	-

B(a)P=Benzo(a)pyrene; NDMA=Nitrosodimethylamine; 2AA=2 Aminoanthracene ; DBE=-Dibromoethane; EMS=Ethylmethanesulfonate. Control cells were treated with dimethylsulfoxide. Values given are means ± SEM for one representative experiment carried out in triplicate. Asterix indicates a significant change ($P<0.05$, by Wilcoxon's test) after I3C treatment. I3C pretreatment was with 25μg/ml, for 30 hr for the B(a)P assays and for 24 hr for all other assays.

Table III summarizes the effects of pretreating the primary chick-embryo hepatocytes with I3C on the mutagenicity of different model compounds in the SCE assay in V79 cells. Addition of different concentrations of B(a)P caused a dose-related decrease in the number of SCEs/chromosome. Pretreatment with I3C resulted in a significant decrease at all the dose levels tested. The decrease was proportional to the B(a)P dose applied, the lowest dose giving the smallest decrease. When NDMA was tested the mutagenicity generated by this compound also appeared to be inhibited in a dose-related way by I3C pretreatment. The decrease was comparable to the effects observed with B(a)P. In contrast, pretreatment with I3C did not result in any decrease in mutagenicity for 2-aminoanthracene (2AA). When dibromoethane was tested pretreated co-cultures even exhibited a higher mutagenicity compared with control cultures. As was to be expected, exposure to ethyl methanesulfonate, a directacting compound, generated the same number of SCEs/chromosome with or without pretreatment. The effects of pretreatment with I3A were also studied (Table IV).
Although I3A gave a much smaller increase in EROD activity than I3C, the two compounds gave a similar decrease in the number of SCEs/chromosome generated by exposure to B(a)P. In contrast pretreatment with I3A did not result in any decrease in NDMA generated mutagenicity. Although at the concentrations used both compounds induced ETCO activity to a similar degree, there appears to be no correlation between this effect and inhibition of NDMA mutagenicity in the SCE assay. When different doses of 2AA were examined also pretreatment with I3A did not result in any decrease in mutagenicity. Moreover, in these experiments pretreatment with I3A did not have any effect on the mutagenic activity of ethyl methanesulfonate.
From previous studies it was assumed that longer exposure times gave greater mutagenic effects in this co-cultivation system, facilitating the study of minor changes in mutagenic effects (Bruggeman and van der Hoeven, 1985; Jongen et al., 1985; van der Hoeven et al., 1984). However a theoretical study of modulating factors of the biotransformation process raises the possibility that when longer exposure times are applied, the model compounds used for mutagenicity studies modulate their own biotransformation process and mask effects caused by the pretreatment. Therefore different periods of exposure to B(a)P were also studied (Table V).

Table IV **Modulating effect of indole-3-acetonitril (I3A) pretreatment on SCE induction by model compounds in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 cells.**

	Exposure		SCEs/CHROMOSOME		
Compound	Time	Dose	Without I3A	With I3A	Change (%)
B(a)P	24 hr	0.0	0.34±0.02	0.34±0.02	-
(μg/ml)		3.5	0.84±0.07	0.73±0.05	-22*
		7 5	1.60±0.09	1.23±0.02	-29*
NDMA	4 hr	0.0	0.25±0.01	0.24±0.01	-
(mg/ml)		1.0	1.03±0.07	1.15±0.06	+16*
		1.5	1.20±0.06	1.26±0.13	-
		2.0	1.33±0.06	1.36±0.02	-
2AA	4 hr	0.0	0.26±0.03	0.22±0.04	-
(μg/ml)		10.0	0.61±0.03	0.60±0.07	-
		25.0	0.79±0.05	0.81±0.08	-
EMS	24 hr	0.0	0.34±0.02	0.34±0.02	-
(μg/ml)		100.0	1.07±0.03	1.02±0.03	-

B(a)P=Benzo(a)pyrene; NDMA=Nitrosodimethylamine; 2AA=2 Aminoanthracene EMS=-ethylmethanesulfonate. I3A pretreatment was with 35 μg/ml for 8 hr for the B(a)P assay and for 24 hr in all other assays. Control cells were treated with dimethylsulfoxide. Values are means of one representative experiment carried out in triplicate and asterix indicates a significant change ($P<0.05$, by Wilcoxon's test) after pretreatment.

Table V   **Protective effect of indole-3-carbinol (I3C) pretreatment in a co-cultivation system, consisting of primary chick-embryo hepatocytes and V79 cells exposed to benzo(a)pyrene for different periods.**

Treatment	Dose (μg/ml)	Exposure time (hr)	SCEs/CHROMOSOME Without I3C	With I3C	Decrease (%)
control	0.0	1.0	0.32±0.03	0.33±0.05	-
B(a)P	7.5	1.0	0.83±0.06	0.74±0.06	20*
		2.0	0.94±0.03	0.86±0.07	13*
		4.0	1.24±0.12	0.83±0.09	45*

I3C pretreatment was with 25 μg/ml for 20 hr. Control cells were treated with dimethylsulfoxide. Values are means ± S.D. for two combined experiments each carried out in duplicate and an asterix indicates a significant decrease ($P<0.05$, by Wilcoxon's test) after I3C pretreatment.

Increasing exposure times gave higher mutagenicity, but when the hepatocytes were pretreated with I3C, increasing the exposure time did not increase the mutagenic effect, indicating that the protective effects of pretreatment were greater with longer exposure times, a 45% decrease in mutagenicity being attained with a 4 h exposure.

Because the antimutagenic effects of pretreatment did not correlate with the induction patterns of cytochrome P450 associated enzymes, mutagenicity at the optimal pretreatment time for EROD induction was compared with that after a pretreatment time at which only approximately one third of the maximal increase in EROD activity was observed. The data summarized in Table VI, show that even after a non-optimal pretreatment time of only 5 h, a considerable decrease in mutagenicity was still observed, indicating that factors other than P450 associated enzymes play a significant role.

Table VI **Inhibitory potential on SCE induction by B(a)P of indole-3-carbinol pretreatments of different duration in a co-cultivation system consisting of primary chick-embryo hepatocytes and V79 cells.**

Treatment	Dose (μg/ml)	Duration of I3C pretreatment (hr)	SCEs/CHROMOSOME Without I3C	SCEs/CHROMOSOME With I3C	Decrease (%)
Control	0.0	5	0.32±0.04	0.33±0.03	-
B(a)P	7.5	5	1.52±0.07	1.32±0.07	18**
Control	0.0	20	0.38±0.03	0.36±0.02	-
B(a)P	7.5	20	1.60±0.19	1.23±0.10	30**

Exposure to B(a)P was for 24 hr. Pretreatment with I3C was with 25 μg/ml. Values are means ± SD for five determinations in one representative experiment and asteriks indicate a significant decrease ($P<0.01$, by Wilcoxon's test) after I3C pretreatment.

Therefore experiments were also performed with a pre-incubation assay, in which different enzyme fractions, prepared from non-pretreated hepatocytes were compared with enzyme fractions prepared from hepatocytes that were pretreated with I3C for 20 h. (Table VII).

Table VII Comparison of the induction of SCEs by benzo(a)pyrene [B(a)P] in a pre-incubation assay with V79 cells, using different enzyme fractions prepared from primary chick-embryo hepatocytes pretreated with 25 μg/ml indole-3-carbinol (I3C) for 20 hr.

Treatment	Dose (μg/ml)	Enzyme fraction	SCEs/CHROMOSOME Without I3C	With I3C	Decrease (%)
Control	0.0	S-9	0.34±0.03	0.34±0.03	-
B(a)P	7.5	S-9	0.72±0.02	0.55±0.05	45**
Control	0.0	Microsomes	0.38±0.03	0.36±0.04	-
B(a)P	7.5	Microsomes	0.63±0.02	0.67±0.03	-

Pre-incubation was for 2 hr. Values are means of five determinations per experiment and asteriks indicate a significant decrease ($P<0.01$, by Wilcoxon's test) after I3C pretreatment.

When the effects of pretreatment were studied using an S-9 mix as the metabolizing system, a 45% decrease in mutagenicity was observed, whereas no significant difference was observed when the microsomal fraction was used. These data point to an important role for cytosolic enzymes or cytosolic cofactors, which are present in the S-9 mix but not in the microsomal fraction. The somewhat higher mutagenic effect obtained for B(a)P with S-9 compared with the microsomal fraction may be due to the fact that NADPH was used with the microsomal fraction, whereas in the case of the S-9 mix a regenerative system was used.

B(a)P metabolism

Pretreatment of the hepatocytes with I3C resulted in induction of cytochrome P450 associated enzyme activities which catalyze different reactions. Therefore the effects of pretreatment on microsomal B(a)P metabolism were studied

in more detail. Table VIII shows the data on the formation of B(a)P metabolites in chick-embryo hepatic microsomes which were pretreated in ovo with 3-MC and I3C.

Table VIII **Formation of benzo(a)pyrene metabolites in chick-embryo hepatic microsomes treated in ovo with 3-methylcholanthrene and indole-3 carbinol.**

Treatment	Metabolite formation(*)					
	Dihydrodiols					
	D9,10	D4,5	D7,8	Quinones	9OH	3OH
control	0.073	0.022	0.082	0.13	0.035	0.034
3-MC	0.22	0.027	0.56	0.30	0.34	0.76
induction factor	3.0	1.2	6.8	2.3	9.7	22.4
I3C	0.13	0.030	0.31	0.22	0.25	0.41
induction factor	1.8	1.4	3.8	1.7	7.1	12.1

* Activity is expressed as nmol/min/nmolP450. Treatment with I3C is 3.0 mg/egg and with 3-MC is 0.5 mg/egg. D9,10= B(a)P-9,10-dihydrodiol; D4,5= B(a)P-4,5-dihydrodiol; D7,8= B(a)P-7,8-dihydrodiol.

For both compounds the overall metabolism of B(a)P is considerably increased. However when the profiles of metabolite formation of the two compounds were compared no major differences could be observed in induction ratios. The overall ratio of metabolite formation was 1.6 (3-MC/I3C).The ratios for the various metabolites, with the exception of the B(a)P-4,5-dihydrodiol ranged between 1.4-1.9. Especially formation of the proximate carcinogenic metabolite B(a)P-7,8 dihydrodiol did not differ between the two compounds.

DISCUSSION

Pretreatment of primary chick-embryo hepatocytes in vitro with naturally occurring indoles leads to a reduced induction of SCEs after exposure of the cells to B(a)P. The modulating effects of the investigated compounds differed qualitatively. Pretreatment with I3C leads to reduced genotoxic effects with NDMA whereas no reduction was found with I3A. No inhibitory effects were observed with both indoles when 2AA was tested and when DBE was tested pretreatment with I3C resulted in an increase in the number of SCEs. The latter observation may be explained by the observations of van Bladeren et al. (1980) that DBE is activated into a more potent mutagen mainly by glutathione conjugation and to a lesser extent by microsomal oxydation. Apparently the character of the modulating effects is largely dependent on the choice of the model compounds and the metabolic routes leading to the formation of reactive intermediates formed from them.

Several lines of evidence, presented in this paper indicate that the inhibitory effects of pretreatment with the indoles do not correlate with the induction pattern of cytochrome P450 associated enzymes. (1) Although I3C gave a much higher increase in EROD activity than I3A, the inhibitory effects on B(a)P mediated SCE induction are quantitatively similar. (2) Both indoles give a similar induction of ETCO activity. Nevertheless, their inhibitory effects on NDMA mediated SCE induction are completely different. Pretreatment with I3C gave a reduction in SCEs quantitatively similar to the effects elicited with B(a)P, whereas no reduction was found when the cells were pretreated with I3A (Table III and IV). It is apparent that also in the case of NDMA, the ETCO induction pattern of indole pretreatment is not directly correlated with the protective effects. (3) When the hepatocytes were pretreated with I3C for a period in which induction of EROD activity was approximately one third of the maximum, a considerable decrease in B(a)P-induced SCEs was still observed, indicating the involvement of other enzyme systems having different induction kinetics. (4) The results obtained with enzyme fractions prepared from I3C pretreated hepatocytes showed that the use of the S-9 fraction gave similar results to those obtained with intact cells, but when the microsomal fraction prepared from the same cells was used, the protective effect disappeared. This indicates that cytosolic enzymes play an essential role in the protective effects. (5) When the pattern of metabolite formation of B(a)P after in ovo treatment with I3C was compared with treatment with 3-MC, no major differences

could be observed. The formation of the proximate carcinogenic metabolite B(a)P-7,8 dihydrodiol was increased with a factor 3.8 compared to the value obtained with non-treated hepatic microsomes.

When different periods of exposure to B(a)P are compared, it seems that at the longer exposure time (24 h) the protective effects of pretreatment are smaller (Table III) than with the 4-h exposure period (Table V). Probably, after a longer exposure time, the biotransformation process of the genotoxic agent itself influences the balance of the enzyme systems, giving a decrease in the protective effect. Additional support for this hypothesis was given by the data obtained with the S-9 fraction which showed the same percentage decrease as was obtained with the 4-h exposure to B(a)P (Table VII).

Figure II shows that I3A and I3C are both competitive inhibitors of EROD activity. This implies that they can also act as competitive inhibitor for B(a)P. In theory, pretreatment with the indoles may lead to the formation of an intracellular pool of these compounds. When pretreatment is followed by exposure to B(a)P the intracellular pool of indole can compete with the metabolism of B(a)P resulting in a reduction in the formation of mutagenic compounds. However the data found in the mutagenicity experiments do not support the hypothesis that this effect plays a dominant role in the protective effects observed. Firstly, when the cells were pretreated with I3C, followed by exposure to different doses of B(a)P, the percentage of decrease of the number of SCEs was positively correlated with the dose of B(a)P applied. Secondly, when microsomal fractions were prepared from cells pretreated with I3C no increase in mutagenicity was observed. Apparently the observed increase in metabolite formation after treatment with I3C is not reflected in larger mutagenic effects.

The data presented here indicate that the protective effects of pretreatment with indoles do not correlate with the induction of cytochrome P450 associated enzymes but seem to be more the result of a changed balance in the enzyme systems involved in the biotransformation of these indoles. Support for this hypothesis is given by the finding that the ratio between UDPGT activity and cytochrome P450 content is much higher for 3-MC than for I3C where the increase in UDPGT activity seems to be in balance with cytochrome P450 content. UDPGT activity requires the presence of cytosolic cofactors. Consequently it does not play a role in the effects obtained with microsomal fractions. The use of a microsomal fraction prepared from hepatocytes pretreated with I3C gave the same number of SCEs as the microsomes prepared from non-pretreated cells. The increased potential of pretreated microsomes to metabolize B(a)P is

not reflected in larger genotoxic effects. This points to an important role for the enzyme epoxide hydrolase. This hypothesis is also in agreement with the findings of other authors, who have reported induction of other enzymes, such as glutathione-S-transferase in rats (Sparnins et al., 1982) or epoxide hydrolase in rats (Henrich and Bjeldanes, 1983).

The pretreatment of metabolizing cells with naturally occurring indoles not only modulates the carcinogenic potency of B(a)P in vivo but also has a protective effect on the SCE induction of this compound in vitro. It should be emphasized that the type of effect resulting from indole treatment depends largely on the chemical properties of the mutagenic mutagenic agents.

The co-cultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells offers a promising in vitro model for studying the modulating effects of compounds on the genotoxic activity of indirectly acting xenobiotics.

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B. MODULATING EFFECTS OF B-NAPHTOFLAVONE ON THE INDUCTION OF SCEs BY MODEL COMPOUNDS WITH EMPHASIS ON BENZO(A)PYRENE

ABSTRACT

The inducing capability of the synthetic flavonol B-naphtoflavone on cytochrome P450 content was studied in primary chick-embryo hepatocytes. In addition, the modulating effects of pretreatment with B-NF on the induction of SCEs by mutagens from different chemical classes were investigated. Finally, the effects of pretreatment on B(a)P metabolism were studied more detailed. Pretreatment of cultured primary chick-embryo hepatocytes with B-naphtoflavone resulted in a large increase in cytochrome P450 content (a 2.8-fold increase after 31 hr). Pretreatment with B-NF had no effect on the level of SCEs induced by N-nitrosodimethylamine (NDMA) and 2-aminoanthracene (2AA). Pretreatment with B-NF resulted in a decrease in B(a)P induced SCEs. This inhibitory potential was positively related to the B-NF dose; its magnitude did depend on the dose of B(a)P, at higher doses less inhibition was observed.

When B-NF was applied simultaneously with B(a)P the percentage of decrease was about the same as for pretreatment. Pretreatment with B-NF followed by simultaneous application of B-NF and B(a)P did not result in larger effects.

In addition, subcellular fractions were prepared from chick-embryos pretreated in ovo. The use of the S-9 fraction resulted in a large decrease (80%) in the induction of SCEs by B(a)P whereas the use of the microsomal fraction resulted in a 70% increase in SCE induction, when compared with non-pretreated microsomes. Pretreatment with B-NF in ovo gave rise to a large increase in aryl hydrocarbon hydroxylase (AHH) activity. With all metabolites studied increases in the amounts formed from B(a)P were observed. The formation of one of these metabolites, the proximate carcinogenic and mutagenic metabolite B(a)P-7,8-dihydrodiol was seven-fold increased.

The data strongly suggest that the inhibitory effects of pretreatment of cultured primary chick-embryo hepatocytes with B-NF cannot be ascribed to its inducing capabilities but instead seem to be due to the formation of an intracellular pool of B-NF which acts as a competitive inhibitor for B(a)P metabolism.

INTRODUCTION

Naturally occurring flavonoids are present in many edible plants and their estimated daily intake via the American diet amounts to 1 gram (Brown, 1980). Representatives of this class of compounds like quercetin, rutin, tangeretin and nobiletin have been shown to possess anticarcinogenic properties in experimental animals (Wattenberg et al., 1968; Wattenberg and Leong, 1970). The anticarcinogenic potential of these compounds has been ascribed to their potential to act as enzyme inducers (Wattenberg et al., 1968; Wattenberg and Leong, 1970; Cutroneo et al., 1972; Friedman et al, 1985).
B-Naphtoflavone is a synthetic flavone (5,6-benzoflavone) which is a known potent inducer of cytochrome P448 (Althaus and Meyer, 1981; Nash, 1981; Söderkvist et al., 1982). In several papers it has been reported that B-naphtoflavone has anticarcinogenic properties (Wattenberg et al., 1968; Wattenberg and Leong, 1970; Slaga et al., 1977; Wattenberg, 1978). In mice B-NF inhibited dimethylbenzanthracene (DMBA) induced tumor formation and binding of DMBA metabolites to DNA was decreased (Bowden et al., 1974). Another study reported contradictory effects of B-NF mediated modulation of tumor formation in experimental animals. When a mouse strain, which was not very sensitive to the tumorigenic potential of DMBA, was pretreated with B-NF a good correlation was found between the increase in aryl hydrocarbon hydroxylase (AHH) activity and an increase in tumor formation. When mice were used, which were highly sensitive for the tumorigenic potential of DMBA, the increase in AHH activity was correlated with a decrease in tumor formation and a decrease in the formation of DNA adducts. (Lesca, 1981). These findings suggest that the observed effects, caused by B-NF pretreatment, resulted from a changed balance between activating and detoxifying enzymes.
Recently, Topp and van Bladeren (1986) showed that B-NF was capable of induction of cytochrome P450 in cultured primary chick-embryo hepatocytes. The reported anticarcinogenic properties of this compound in animal studies were suggested to be related to the enzyme inducing capability of this compound. Therefore it was decided to study the effects of pretreatment with B-NF on the mutagenicity of model compounds from different chemical classes, using a cocultivation system consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells. Mutagenicity was determined as the number of sister chromatid exchanges in V79 cells. In addition the mode of action of pretreatment with B-NF on benzo(a)pyrene metabolism was studied in more detail.

MATERIAL and METHODS

Aryl Hydrocarbon Hydroxylase (AHH) Activity

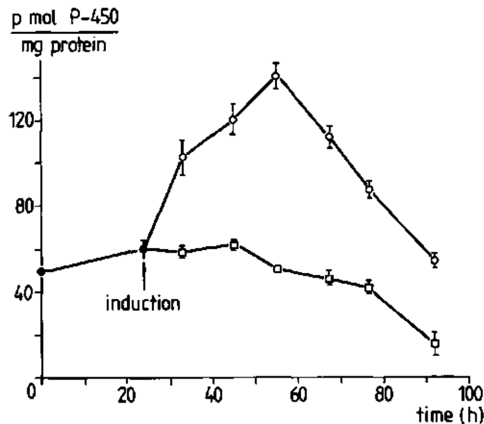
Composition of the incubation mixture and the procedure followed were as described under: B(a)P metabolite formation (Chapter VIa;M&M). Total conversion of B(a)P was measured with [^{3}H] B(a)P in microsomes. AHH activity was determined from regression lines which were calculated from time-course experiments. For practical reasons, in the experiments where the modulating effects of B-NF were investigated a treatment time of 22 hr was applied.

All other material and methods are identical to those used in the experiments with the indoles and are described in chapter VIa. The concentrations of B-NF used are given in legends to figures and tables.

RESULTS

Enzyme induction

Figure I **Effect of B-naphtoflavone on the cytochrome P-450 content in primary chickembryo hepatocytes.**



Treatment with B-NF (o) was with 6 μg/ml. Data represent mean ± SEM of six experiments. Figure is modified from Topp and van Bladeren (1986).

Topp and van Bladeren (1986) studied the induction of cytochrome P450 in cultured primary chick-embryo hepatocytes by B-NF; they found that 6 µg/ml gave maximal induction. A time-course experiment, taken from that study, is depicted in figure I. 24 hr after the hepatocytes were plated, treatment with B-NF was started. Maximal induction was reached at 31 hr after start of the treatment and amounted to a 2.8 fold increase in the cytochrome P450 content.

Mutagenicity studies

Pretreatment with B-NF resulted in a lower frequency of benzo(a)pyrene [B(a)P] induced SCEs (Table I).

Table I Inhibitory effects of pretreatment of primary chick-embryo hepatocytes with B-naphtoflavone (B-NF) on the mutagenicity of benzo-(a)pyrene [B(a)P] after co-cultivation with V79 Chinese hamster cells.

TREATMENT			
B-NF(µg/ml)	B(a)P(µg/ml)	SCEs/CHROMOSOME	Decrease (%)
0	0	0.22±0.03	-
6.0	0	0.20±0.02	-
0	7.5	0.88±0.05	-
2.0	7.5	0.80±0.04	9(*)
4.0	7.5	0.73±0.06	20(*)
6.0	7.5	0.64±0.05	33(*)

Treatment time with B-naphtoflavone was 22 hr. Exposure time for B(a)P was 4 hr. Data ±SEM are average values of one representative experiment carried out in four-fold. Asteriks indicate a significant decrease ($P<0.05$, by Wilcoxon's test).

This reduction was related to the dose of B-NF applied and amounted to a 33% decrease at 6 µg/ml. In table II the results of experiments with model compounds from different chemical classes are summarized.

Table II **Inhibitory effects of pretreatment of primary chick-embryo hepatocytes with B-NF on the mutagenicity of model compounds from different chemical classes after co-cultivation with V79 Chinese hamster cells.**

Exposure	Dose (μg/ml)	SCEs/chromosome Without B-NF	With B-NF	Decrease (%)
Control		0.20±0.01	0.20±0.02	-
B(a)P	2.5	0.47±0.03	0.35±0.04	44(*)
	5.0	0.59±0.04	0.45±0.04	36(*)
	7.5	0.84±0.05	0.70±0.03	22(*)
DMNA	1000.0	1.21±0.05	1.25±0.07	-
	1500.0	1.68±0.05	1.64±0.05	-
2AA	5.0	0.47±0.03	0.51±0.03	-
	10.0	0.62±0.04	0.63±0.06	-

B-NF= B-naphtoflavone; B(a)P=benzo(a)pyrene; NDMA= dimethylnitrosamine; 2AA= 2-aminoanthracene. Exposure to the model compounds was 4 hr. Pretreatment with B-NF was with 6 μg/ml for 22 hr. Data ± SEM are average values of one representative experiment carried out in fourfold. Asteriks indicate a significant decrease ($P<0.05$, by Wilcoxon's test).

Pretreatment with B-NF was with 6 μg/ml in these experiments. When different doses of B(a)P were tested, the percentage of inhibition, caused by B-NF pretreatment, decreased with increasing B(a)P exposure. In contrast to the data obtained with B(a)P, no inhibitory effects of pretreatment were observed for NDMA and 2AA. In addition to pretreatment with B-NF the effects of simultaneous exposure to B(a)P and B-NF were studied also (Table III).

Table III **Comparison of different treatment procedures on the inhibitory potential of B-naphtoflavone (B-NF) on benzo(a)pyrene [B(a)P] mediated mutagenicity in a co-cultivation system.**

B-NF treatment		Exposure		Decrease
pretreatment	simultaneous	[B(a)P]	SCEs/chromosome	(%)
-	-	0	0.20±0.02	-
+	-	0	0.21±0.01	-
-	+	0	0.23±0.02	-
-	-	7.5	0.84±0.05	-
+	-	7.5	0.70±0.03	23(*)
-	+	7.5	0.65±0.04	30(*)
+	+	7.5	0.67±0.08	28(*)

Exposure to B(a)P was for 4 hr. Treatment with B-NF was with 6 μg/ml. Pretreatment was for 22 hr, simultaneous treatment was for 4 hr. Data ± SEM are average value of one representative experiment carried out in four-fold. Asteriks indicate a significant decrease ($P<0.05$, by Wilcoxon's test).

Pretreatment of the primary chick-embryo hepatocytes gave the expected decrease in the number of SCEs/chromosome. Simultaneous application of B-NF and B(a)P gave a slightly larger decrease than did pretreatment. However when the hepatocytes were pretreated with B-NF and this compound was also present during B(a)P exposure no additional effect of simultaneous treatment was observable.

To find out which mechanisms are responsible for these effects of B-NF, enzyme fractions were prepared from in ovo induced chick-embryos.

A comparative study of in vitro and in ovo induction, which will be published separately, showed that no qualitative differences in B(a)P metabolism and metabolite patterns were observable following pretreatment with different types of inducers like 3-methylcholanthrene, and Aroclor 1254 (van Bladeren et al., in preparation).

Firstly, experiments were done with different subcellular fractions summarized in table IV.

Table IV Comparison of the genotoxic effects of benzo(a)pyrene [B(a)P] in a pre-incubation assay with V79 Chinese hamster cells using different hepatic enzyme fractions prepared from chick-embryos pretreated with B-naphtoflavone (B-NF).

Treatment	Dose (μg/ml)	Enzyme fraction	SCEs/chromosome Without B-NF	SCEs/chromosome With B-NF	Change (%)
Control	0	S-9	0.24±0.01	0.27±0.01	-
B(a)P	7.5	S-9	1.16±0.02	0.47±0.04	-78**
Control	0	Microsomes	0.22±0.01	0.23±0.03	-
B(a)P	7.5	Microsomes	0.72±0.04	1.46±0.02	+68**

Pre-incubation was for 2 hr. Values are means ± SEM of five determinations per experiment and asterisks indicate a significant change ($P<0.01$, by Wilcoxon's test) after B-NF pretreatment.

In these experiments NADPH was used as cofactor for both enzyme fractions. When the S-9 fraction was used, pretreatment with B-NF resulted in a 78% reduction in B(a)P mediated mutagenicity. This effect is much larger than that observed after in vitro pretreatment. However when the microsomal fraction was used the opposite phenomenon was observed. Pretreatment with B-NF resulted in an increase in the induction of SCEs by B(a)P with 68%.

B(a)P metabolism

Secondly the metabolism of B(a)P was studied in more detail. Table V summarizes the effects of pretreatment with B-NF on induction of aryl hydrocarbon hydroxylase (AHH) activity.

Table V **Induction of aryl hydrocarbon hydroxylase (AHH) activity in chick embryo hepatic microsomes, treated *in ovo* with 3-methylcholanthrene (3-MC) and B-naphtoflavone (B-NF).**

AHH ACTIVITY

	mg protein*		nmol cytochrome P450**	
Treatment	activity	I.F.	activity	I.F.
Control	0.040	-	0.412	
3-MC	0.821	20.5	2.55	61.9
B-NF	0.860	21.5	3.32	80.6

(*) Enzyme activity is expressed as nmol 3-OH-B(a)P/min/mg protein. (**) Enzyme activity is expressed as nmol 3-OH-B(a)P/min/nmol cytochrome P450. I.F.=induction factor. 15-Day old chick-embryos were injected twice with a 24 hr time interval with 3-MC (0.5 mg/egg) and B-NF (0.75 mg/egg).

A comparison was made between the effects of B-NF and those of 3-MC. The data show that both compounds give a considerable increase in AHH activity both when expressed per mg protein and when expressed per nmol cytochrome P450 with B-NF giving the largest increase. In the same enzyme fractions the pattern of metabolite formation from B(a)P was studied (Table VI). Treatment with both inducers leads to considerable increases in the amount of all metabolites formed. When the patterns of metabolite formation were compared no major differences were observable between 3-MC treatment and B-NF treatment. Both compounds gave a seven-fold increase in the amount of the proximate carcinogenic and mutagenic metabolite B(a)P-7,8-dihydrodiol (D7,8). The only clear difference between the two inducers is that the formation of the B(a)P-4,5 dihydrodiol is much more increased after B-NF treatment than with 3-MC.

Table VI Formation of benzo(a)pyrene metabolites in chick-embryo hepatic microsomes pretreated *in ovo* with 3-methylcholanthrene (3-MC) and B-naphtoflavone (B-NF).

METABOLITE FORMATION*

Treatment	Dihydrodiols D9,10	Dihydrodiols D4,5	Dihydrodiols D7,8	Quinones	9OH	3OH
Control	0.073	0.022	0.082	0.13	0.035	0.034
3-MC	0.22	0.027	0.56	0.30	0.34	0.76
I.F.	3.0	1.2	6.8	2.3	9.7	22.4
B-NF	0.30	0.093	0.55	0.51	0.31	1.03
I.F.	4.1	4.2	6.7	3.9	8.9	30.3

* Activity is expressed as nmol/min/nmol cytochrome P-450. D9,10- B(a)P9,10-dihydrodiol; D4,5- B(a)P-4,5-dihydrodiol; D7,8- B(a)P-7,8-dihydrodiol. 15-Day old chick-embryos were injected twice with a 24 hr time interval with 3-MC (0.5 mg/egg) and B-NF (0.75 mg/egg). I.F.- induction factor.

DISCUSSION

When the effects of pretreatment with B-NF on SCE induction by various model compounds were compared no decrease was observed for NDMA and 2-AA. Obviously the modulating effects of B-NF are restricted to premutagens which are activated via specific biotransformation routes.

Pretreatment of primary chick-embryo hepatocytes with B-NF leads to reduced genotoxic effects after exposure of the cells to B(a)P. When the effects of pretreatment were compared with simultaneous exposure the reduction in genotoxicity was approximately the same for both types of treatment. The observation that pretreatment followed by simultaneous application of B-NF did not result in a larger decrease than either one of them, combined with the observed decrease in inhibition with increasing B(a)P exposure, raised the

question whether it is justified to ascribe the inhibitory potential of B-NF to its inducing capabilities. Several lines of evidence argue against this explanation. Pretreatment with B-NF leads to a large increase in AHH activity, the enzyme responsible for the activation of B(a)P into its mutagenic metabolites. This is confirmed in experiments where the metabolite pattern was studied. For example, the amount of the proximate carcinogenic and mutagenic metabolite D7,8 was increased seven-fold. Also when mutagenicity experiments were performed with the microsomal fraction pretreatment with B-NF gave a large increase in the number of SCEs compared with non-pretreated microsomes.

A possible explanation for the way B-NF exhibits its inhibitory potential would be the presence of residual B-NF either in the intact cells or in the S-9 fraction after pretreatment. As was seen in the experiments on B(a)P metabolism, B-NF specifically induces AHH activity to a large extent. Obviously, AHH is the enzyme responsible for the metabolism of B(a)P into its mutagenic intermediates. Consequently, B-NF can be considered a competitive inhibitor of B(a)P metabolism. The presence of B-NF in the intact cells would then lead to a reduced conversion of B(a)P compared with a situation where no residual B-NF is available. This explanation would also clarify why the dose-response relationship for B(a)P is inversely related with the percentage of inhibition. Increasing the dose-level of B(a)P would cause a shift in the competition between the metabolism of B(a)P and B-NF favoring B(a)P metabolism. In addition it would clarify why, in the experiments with subcellular fractions, the use of an S-9 fraction gives a large decrease in mutagenic effects whereas the use of a microsomal fraction gives a considerable increase in the number of SCEs compared with non-pretreated microsomes. When S-9 fractions are used residual B-NF would still be present whereas the microsomal fraction would not contain residual B-NF. Finally it would explain why the effects of pretreatment and simultaneous exposure are comparable and that combined treatment does not give a larger decrease in SCE induction.

In conclusion, the data presented in this study strongly suggest that the inhibitory effects of pretreatment of cultured primary chick-embryo hepatocytes with B-NF cannot be ascribed to its inducing capabilities but instead seem to result from the formation of an intracellular pool of B-NF which acts as a competitive inhibitor for B(a)P metabolism.

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CHAPTER VII

SUMMARY AND CONCLUDING REMARKS

Epidemiological data show that in western societies 1 out of 3 persons gets cancer and 1 out of 4 persons dies of cancer. This makes cancer, next to heart and vascular diseases, a major cause of death. There are three major factors contributing to the cancer death rate in humans:

1. ionizing radiation
2. certain viruses
3. chemicals

Intensive research efforts for many decades have led to the development of a generalized concept which implies that the process of tumor formation proceeds stepwise. This stepwise development includes both genetic and epigenetic factors.

In this thesis studies are described which were aimed at the development of an in vitro system enabling the study of the effects of compounds on various important aspects of the cancer process. In the first chapter a description is given of the cancer process as it emerges from epidemiological data and data from studies with experimental animals. The process can be divided in three more or less distinct phases: the initiation phase, the promotion phase and the progression phase. Within the concept presented, the role of intercellular communication was emphasized and inhibition of intercellular communication has been described as an essential step in tumor promotion. In addition the role of drug metabolism in the formation of reactive intermediates is described briefly and the advantages of the principle of using intact cells, compared with subcellular fractions is discussed. Finally the objectives of the investigations are presented.

In chapter II the principles of the test methods used in the investigations are described, including methods determining interactions of chemicals with DNA of cells as well as methods designed to study effects of compounds on intercellular communication.

Chapter III deals with the relation between the sensitivity of the co-cultivation system, the genetic endpoint and the type of xenobiotic. It was shown that the use of primary chick-embryo hepatocytes as an activation system showed the best performance compared with subcellular fractions from chick-embryo liver and rat liver pretreated with Aroclor 1254. Induction of SCEs in V79 Chinese hamster cells is no good endpoint to determine the potential of cigarette-smoke condensate (CSC) to interact with DNA. The small but consistently

observed increase in the number of SCEs after exposure could not be ascribed to a reaction of CSC with DNA of V79 cells. In contrast, the complex mixture was clearly positive in the test on forward mutation at the HGPRT locus.
When a co-cultivation system is used to study the interactions of reactive intermediates with DNA of cells, the transport of reactive intermediates is of vital importance for an adequate measurement of these interactions. Any factor disturbing the transport from drug metabolizing cells to target cells influences the outcomtive intermediates. From the data obtained with time-course studies and those obtained with different mutagens it was concluded that this type of transport is not restricted to specific types of ultimate mutagens but is a phenomenon of a more general nature. If this transport occurs in vivo this might have consequences as to which cells in a given tissue are at risk to become neoplastic upon exposure to mutagens. For example, drug metabolizing cells like the primary chick-embryo hepatocytes are protected more efficiently against toxic intermediates than non metabolizing cells like the V79 cells.
Definitive evidence that functional heterologous gap junctions are formed between the two cell types used in the co-cultivation system is given in chapter V.
The purpose of the studies presented in this chapter was twofold. The first aim was to investigate whether functional gap junctions are really formed between the two cell types and the second aim was to study the possibility to develop a test system measuring inhibition of metabolic cooperation using the co-cultivation system. The presence of gap junctions was visualized with transmission electron microscopy. The formation of heterologous gap junctions was difficult to determine. In contrast to homologous gap junctions, the heterologous gap junctions were very small and could be recognized merely by the closeness of the opposing membranes. Studies on the transfer of labeled nucleotides showed unequivocally that the heterologous gap junctions were functional. Addition of the phorbolester TPA inhibited this transfer, which was accompanied by the disappearance of all gap junctions. Upon longer treatment times transfer was partly restored and also morphologically the gap junctions reappeared. It was shown that this adaptation is associated with the mitotic index of the cells.
A model system was developed, enabling the study of the role of drug metabolism in the formation of compounds with the potential to inhibit intercellular communication. This model is based on the use of a mutant ($HGPRT^-$) V79 cell line which lacks the capacity to phosphorylate hypoxanthine. These mutant V79 cells are co-cultured with primary chick-embryo hepatocytes in the presence of

[^{3}H] hypoxanthine. The HGPRT$^-$ V79 cells are not capable of phosphorylating this hypoxanthine and cannot incorporate the label in the genetic material. Only when the hypoxanthine is phosphorylated by the primary chick-embryo hepatocytes and is transported through gap junctions to neighbouring HGPRT$^-$ V79 cells, incorporation will occur. Incorporation of label is visualized with autoradiographic methods and the accurate number of grains can be determined by counting them under the microscope. When TPA and CSC were tested with this method, both were able to inhibit this transfer. When the effect of CSC on disappearance of gap junctions was investigated at cytotoxic concentrations, no decrease in the number of gap junctions was found. In the same system the complex mixture inhibits metabolic cooperation. Therefore the conclusion was drawn that inhibition of intercellular communication can be accomplished along different metabolic pathways.
When dimethylbenzanthracene, an indirect acting carcinogen was tested at non-cytotoxic concentrations, it inhibited the transfer of labeled nucleotides completely. No effect was found when the compound was tested in the assay using the transfer of a poisonous base analogue between two types of V79 cells. Since V79 cells lack the capacity for drug metabolism it was assumed that not the parent compound but one or more of its metabolites is responsible for the observed effects. This compound is the first example of an indirect acting inhibitor of intercellular communication which emphasizes the importance of the use of adequate drug metabolizing systems.
Chapter VI deals with studies on cancer modulating effects of various compounds. These studies comprize investigations towards enzyme inducing capabilities and the effects of pretreatment on SCE induction by mutagens from different chemical classes. In addition the effects of pretreatment on benzo-(a)pyrene "B(a)P" metabolism were studied in detail.
In the first part the effects of two naturally occurring indoles, indole-3-carbinol (I3C) and indole-3-acetonitril (I3A) were examined. Both compounds induced the cytochrome P450 associated enzymes EROD and ETCO in cultured primary chick-embryo hepatocytes. Induction was observed also for UDPglucuronyltransferase whereas no induction of glutathione-S-transferase was found. I3C was a better inducer than I3A; the increases in enzyme activity were larger and obtained at a lower concentration than that of I3A.
The effects of pretreatment with the indoles on SCE induction by mutagens from different chemical classes were studied. Pretreatment with I3C resulted in a decrease in SCE induction for B(a)P and dimethylnitrosamine (NDMA). No difference was found for 2-aminoanthracene (2-AA) and the direct acting mutagen

ethylmethanesulfonate (EMS). On the other hand an increase in SCE induction was found with dibromoethane (DBE).
Pretreatment with I3A resulted in a decrease in SCE induction by B(a)P whereas no decrease was observed for NDMA, 2-AA and EMS. Apparently there are also qualitative differences between the modulating effects of I3C and I3A. Another conclusion is that the choice of the mutagen is crucial. The question whether pretreatment of metabolizing cells leads to decreased or enhanced effects on induction of SCEs, largely depends on the metabolic pathways involved in the biotransformation of the mutagens selected. As was shown, pretreatment with I3C leads to a reduced SCE induction by B(a)P whereas an increase was observed for DBE.
The modulating effects of pretreatment with I3C on B(a)P-induced SCEs were studied in detail. The observation that both I3C and I3A are competitive inhibitors for B(a)P metabolism could imply that the observed inhibitory effects on SCE induction are, at least partly, due to the presence of residual I3C or I3A inside the cells at the time of exposure to B(a)P. Although this cannot be ruled out completely, several lines of evidence argue against this possibility. First, the observed decrease in B(a)P induced SCEs is proportional to the dose applied. If competitive inhibition would play a significant role, increasing doses of B(a)P would decrease the protective effects of pretreatment. Secondly, when microsomes prepared from pretreated chick-embryo hepatocytes were used, the increase in the formation of mutagenic intermediates from B(a)P is not reflected in an increase in SCE induction. This suggests that other enzyme systems play an essential role in the observed effects. Thirdly, the modulating effects of I3C are not restricted to a specific type of mutagen but comprizes mutagens from different chemical classes which are activated via different metabolic pathways. Especially in the case of dibromoethane pretreatment with I3C leads to an increase in SCE induction.
Therefore it seems probable that competitive inhibition does not play a significant role in the observed effects. Instead the modulating effects of pretreatment results from a changed balance between the enzyme systems involved in the biotransformation of the mutagens aplied and, in the case of B(a)P, point to an essential role of conjugating enzymes.
In the second part of this study the modulating effects of B-naphtoflavone (B-NF), a synthetic flavonol with reported anti carcinogenic properties, were examined. B-NF treatment of cultured primary chick-embryo hepatocytes led to a large increase in cytochrome P450 content. No effect of pretreatment with B-NF on SCE induction by 2-AA and NDMA were observed. In contrast, a decrease in

the number of SCEs, induced by B(a)P was found. Detailed studies with subcellular fractions, prepared from chick-embryo liver pretreated in ovo, showed that when an S9 fraction was used a large decrease in the number of SCEs was found whereas when a microsomal fraction was used, a large increase was found. Combined with the observed large increase in aryl hydrocarbon hydroxylase activity and the increase in formation of the proximate carcinogenic metabolite B(a)P-7,8 dihydrodiol, this has led to the conclusion that enzyme induction by B-NF is not associated with a protective effect. Instead it was postulated that the observed decrease in SCE induction by B(a)P in intact cells, stems from the formation of an intracellular pool of B-NF which acts as a competitive inhibitor for B(a)P metabolism.
In conclusion it can be stated that the studies presented in this thesis illustrates that the combined use of different cell types with specific characteristics enables the study of various important aspects of the cancer process in vitro The results show that the co-cultivation system, consisting of primary chick-embryo hepatocytes and V79 Chinese hamster cells is a promising tool in cancer research.

CHAPTER VIII

SAMENVATTING EN DISCUSSIE

Epidemiologische gegevens laten zien dat in westerse maatschappijen 1 van elke 3 personen kanker krijgt en dat 1 van elke 4 personen aan kanker overlijdt. Kanker is dus naast hart- en vaatziekten een belangrijke doodsoorzaak. Er zijn drie belangrijke factoren bekend die bijdragen aan kanker als doodsoorzaak bij mensen:

1. ioniserende straling
2. bepaalde virussen
3. chemische stoffen

Intensief onderzoek gedurende tientallen jaren heeft geleid tot het formuleren van een algemeen aanvaarde hypothese die ervan uitgaat dat het proces van tumorvorming een meerstaps proces is. Deze stapsgewijze ontwikkeling wordt veroorzaakt door genetische en epigenetische factoren.

In dit proefschrift worden experimenten beschreven die erop gericht waren een in vitro model te ontwikkelen dat het mogelijk maakt diverse belangrijke aspecten van het kanker proces nauwkeurig te bestuderen.

In het eerste hoofdstuk wordt een beschrijving gegeven van het proces van tumor vorming zoals het naar voren komt uit epidemiologische gegevens en gegevens afkomstig uit studies met proefdieren. Tijdens het proces kunnen drie min of meer aparte fasen onderscheiden worden: de initiatie fase, de promotie fase en de progressie fase. In het beschreven model is de rol van intercellulaire communicatie benadrukt en remming van intercellulaire communicatie wordt beschouwd als een essentiele stap in de tumor promotie. Bovendien wordt aandacht besteed aan de rol van biotransformatie bij de vorming van reactieve intermediairen en de voordelen van het gebruik van intacte cellen versus subcellulaire fracties worden bediscussieerd. Tenslotte worden de doelstellingen van het onderzoek beschreven.

In hoofdstuk II worden de principes van de gebruikte testsystemen behandeld. Deze omvatten zowel de methoden die zijn gebruikt om interacties van stoffen met het DNA van cellen vast te stellen als ook methoden die zijn ontwikkeld om effecten van stoffen op intercellulaire communicatie te meten.

Hoofdstuk III gaat over het verband tussen de gevoeligheid van het gebruikte co-cultivatie systeem, de verschillende genetische eindpunten en de effecten van diverse chemische stoffen. Uit de resultaten bleek dat het gebruik van primaire, embryonale kippe-hepatocyten als activerend systeem de beste resultaten gaf vergeleken met subcellulaire fracties afkomstig van embryonale kippe

lever en ratte lever, voorbehandeld met Aroclor 1254. Inductie van SCEs (homologe uitwisselingen tussen zusterchromatiden) in V79 Chinese hamster cellen is geen geschikt eindpunt om het vermogen van cigarette-rook condensaat (SRC) om met DNA te reageren te onderzoeken. De kleine maar voortdurend waargenomen verhoging van het aantal SCEs na blootstelling kon niet worden toegeschreven aan een reactie van SRC met het DNA van V79 cellen. In tegenstelling hiermee vertoonde het complexe mengsel een duidelijk positief effect in de test op voorwaartse mutaties op het HGPRT locus.

Wanneer een co-cultivatie systeem wordt gebruikt om interacties van reactieve intermediairen met het DNA van cellen te onderzoeken is een adequaat transport van de reactieve intermediairen van vitaal belang voor het meten van dit type interacties. Elke factor die het transport van de cellen met biotransformatie capaciteit naar de doelcellen verstoort of beinvloedt heeft een beslissende invloed op de uitkomsten van de experimenten. In hoofdstuk IV is het transport van reactieve intermediairen van de primaire, embryonale kippe-hepatocyten naar de V79 Chinese hamster cellen bestudeerd. In principe kan dit transport langs twee verschillende routes plaatsvinden, namelijk dwars door de cellulaire membranen en door de intercellulaire ruimte en via cel-cel kontakt waarbij gap junctions als een sluis functioneren. Als eerste werd aangetoond dat voor een adequaat transport van reactieve intermediairen de beide celtypen in ieder geval heel dicht tegen elkaar aan moeten liggen. Wanneer de cellen werden behandeld met SRC of de phorbolester TPA, voordat blootstelling aan de mutagene stoffen plaatsvond, werden de mutagene effecten met ongeveer 50% gereduceerd. Hierbij werd geen verschil gevonden voor de verschillende eindpunten. De phorbolester TPA is een bekende remmer van intercellulaire communicatie en behandeling van cellen met deze verbinding maakt elk transport door gap junctions onmogelijk. Deze waarnemingen suggereerden dat in dit co-cultivatie systeem heterologe gap junctions worden gevormd tussen de primaire, embryonale kippe-hepatocyten en de V79 Chinese hamster cellen en dat er transport van reactieve intermediairen door deze gap junctions plaats kan vinden. Daarnaast suggereerden deze gegevens dat SRC mogelijk ook een remmer van intercellulaire communicatie is. Toen SRC werd getest in een methode die gebruik maakt van de overdracht van giftige base-analogen tussen twee typen V79 cellen werd inderdaad gevonden dat het complexe mengsel metabole cooperatie tussen deze V79 cellen remde. De hypothese dat transport van reactieve intermediairen ook door heterologe gap junctions plaatsvond werd ook bevestigd met behulp van een mutante V79 cellijn die metabole cooperatie deficient is (MC^{-}-27). Toen deze cellijn werd gebruikt waren de gegevens die ermee werden verkregen volle-

dig vergelijkbaar met de gegevens die waren verkregen in aanwezigheid van TPA. De resultaten laten dan ook duidelijk zien dat in het gebruikte co-cultivatie systeem bij het transport van reactieve intermediairen routes van intercellulaire communicatie een belangrijke rol spelen. Uit experimenten met verschillende mutagenen en studies naar effecten van kweektijden kan worden afgeleid dat dit type van transport niet beperkt is tot specifieke vormen van reactieve intermediairen maar een verschijnsel van algemene aard is. Indien deze vorm van transport ook _in vivo_ een rol van betekenis speelt kan dat gevolgen hebben voor de vraag welke cellen het grootste risico lopen om geinitieerd te raken na blootstelling aan een mutagene stof. Bijvoorbeeld, cellen met biotransformatie capaciteit zoals de primaire, embryonale kippe-hepatocyten zijn beter uitgerust om zichzelf te beschermen tegen toxische intermediairen dan cellen, zoals de V79 cellen, die geen biotransformatie capaciteit hebben. Het definitieve bewijs dat functionele heterologe gap junctions worden gevormd tussen de beide cel typen zoals ze in het co-cultivatie systeem worden gebruikt wordt gepresenteerd in hoofdstuk V.

De studies die in dit hoofdstuk worden gepresenteerd hadden een tweetal doelstellingen. Allereerst is nagegaan of er daadwerkelijk functionele gap junctions kunnen worden gevormd tussen de beide cel typen en de tweede doelstelling was om na te gaan of het mogelijk was, gebruik makend van het co-cultivatie systeem, een model te ontwikkelen waarmee remming van intercellulaire communicatie kan worden bepaald.

De aanwezigheid van gap junctions werd zichtbaar gemaakt met transmissie electronenmicroscopie. De vorming van heterologe gap junctions was moeilijk vast te stellen. In tegenstelling tot homologe gap junctions bleken heterologe gap junctions zeer klein te zijn en hun aanwezigheid kon eigenlijk alleen maar worden afgeleid uit het tegen elkaar aan liggen van de beide tegenover elkaar liggende celmembranen. Experimenten waarbij gebruik werd gemaakt van de overdracht van radioactief gelabelde nucleotiden toonden echter ondubbelzinnig aan dat de heterologe gap junctions functioneel waren. Na toediening van de phorbolester TPA trad er volledige remming van deze overdracht op. Wanneer gekeken werd naar de morfologische aanwezigheid van gap junctions bleek dat de remming gepaard ging met het verdwijnen van alle gap junctions. Wanneer langere blootstellings tijden werden toegepast bleek het transport via gap junctions gedeeltelijk te zijn hersteld en ook morfologisch bleken de gap junctions weer te verschijnen. Het kon worden aangetoond dat dit adaptatie verschijnsel samenhangt met de delingssnelheid van de cellen.

Een model systeem kon worden ontwikkeld dat het mogelijk maakt om de rol van

biotransformatie, bij de vorming van verbindingen die intercellulaire communicatie kunnen remmen, te bestuderen. Dit model is gabaseerd op het gebruik van een mutante (HGPRT deficiente) V79 cellijn die het vermogen mist om hypoxanthine te phosphoryleren. Deze mutante V79 cellen worden samen gekweekt met de primaire, embryonale kippe-hepatocyten in de aanwezigheid van [^{3}H] hypoxanthine. De HGPRT deficiente V79 cellen kunnen dit hypoxanthine niet phosphoryleren en vervolgens incorporeren in het genetisch materiaal. Pas wanneer het hypoxanthine is gephosphoryleerd door de primaire, embryonale kippe-hepatocyten en vervolgens door de heterologe gap junctions naar aangrenzende HGPRT deficiente V79 cellen wordt getransporteerd zal inbouw in het genetische materiaal plaatsvinden. Deze inbouw wordt dan zichtbaar gemaakt met behulp van autoradiografie en het precieze aantal korrels kan worden bepaald door ze onder een licht microscoop te tellen. Toen de effecten van TPA en SRC werden onderzocht met deze methode bleken beide de overdracht van radioactiviteit te remmen. Toen de effecten van SRC op het verdwijnen van gap junctions werd onderzocht bij cytotoxische concentraties, werd geen afname van het aantal gap junctions waargenomen terwijl het complexe mengsel in hetzelfde systeem wel de intercellulaire communicatie remt. De conclusie lijkt dan ook voor de hand te liggen dat remming van intercellulaire communicatie via verschillende mechanismen veroorzaakt kan worden.

Toen dimethylbenzanthraceen, een indirect werkend carcinogeen werd onderzocht bij niet-cytotoxische concentraties, bleek ook deze verbinding in staat de overdracht van gelabelde nucleotiden volledig te remmen. Toen de verbinding werd onderzocht in het testsysteem waarbij gebruik wordt gemaakt van de overdracht van giftige base analogen tussen twee typen V79 cellen werd geen remming van metabole cooperatie waargenomen. Aangezien V79 cellen geen biotransformatie capaciteit hebben ligt het voor de hand te veronderstellen dat niet de oorspronkelijke stof maar een of meer van zijn metabolieten verantwoordelijk is voor de waargenomen effecten. Deze verbinding is waarschijnlijk het eerste voorbeeld van een indirect werkende remmer van intercellulaire communicatie hetgeen het belang onderstreept van het gebruik van adequate biotransformatie systemen.

Hoofdstuk VI handelt over onderzoek dat is verricht naar de modulerende effecten van diverse verbindingen. Deze studies omvatten onderzoek naar het vermogen van deze verbindingen om enzyminductie te veroorzaken als ook onderzoek naar de effecten van voorbehandeling met deze verbindingen op het vermogen van mutagenen van verschillende chemische klassen om SCEs te induceren. Bovendien werden de effecten van voorbehandeling op het metabolisme van benzo(a)pyreen

[B(a)P] in detail bestudeerd.

In het eerste gedeelte werden de effecten van de van nature voorkomende indolen, indol-3-carbinol (I3C) en indol-3-acetonitril (I3A), onderzocht. Beide verbindingen bleken de cytochroom P450 geassocieerde enzymen EROD en ETCO in gekweekte primaire, embryonale kippe-hepatocyten te verhogen. Ook het enzym UDPglucuronyltransferase werd geinduceerd terwijl geen effect werd waargenomen voor glutathion-S-transferase. I3C gaf grotere effecten dan I3A: de waargenomen verhogingen in enzymactiviteit waren groter en werden verkregen bij lagere concentraties. De effecten van voorbehandeling met beide indolen op het vermogen van diverse mutagenen om SCEs te induceren werd ook onderzocht. Voorbehandeling met I3C resulteerde in een afname van SCE inductie door B(a)P en dimethylnitrosamine (NDMA). Geen verschil werd waargenomen voor 2-aminoanthraceen (2-AA) en het direct werkende ethylmethaansulfonaat (EMS). Aan de andere kant werd een toename gevonden in het aantal SCEs wanneer blootstelling plaatsvond met dibromo-ethaan (DBE).

Voorbehandeling met I3A resulteerde in een afname van SCE inductie door B(a)P terwijl geen afname werd gevonden voor NDMA, 2-AA en EMS. Blijkbaar zijn er ook kwalitatieve verschillen tussen de modulerende effecten van I3C en I3A. Een andere conclusie die getrokken kan worden is dat de keuze van het type mutagene verbinding van cruciaal belang is. De vraag of voorbehandeling van metaboliserende cellen leidt tot verminderde of verhoogde effecten hangt grotendeels af van de omzettingsroutes die betrokken zijn bij de biotansformatie van de betreffende verbindingen. Zoals is aangetoond leidt voorbehandeling met I3C bij B(a)P tot een verminderd effect terwijl voor DBE een verhoging wordt waargenomen. De modulerende effecten van voorbehandeling met I3C op de inductie van SCEs door B(a)P is in detail bestudeerd. De waarneming dat zowel I3C als I3A competitieve remmers zijn van het B(a)P metabolisme zou kunnen inhouden dat de waargenomen remmende effecten op SCE inductie, tenminste gedeeltelijk, verklaard zouden kunnen worden door de aanwezigheid van I3A en I3C in de primaire, embryonale kippe-hepatocyten ten tijde van de blootstelling aan B(a)P. Ofschoon deze mogelijkheid niet geheel kan worden uitgesloten zijn er diverse argumenten die tegen deze hypothese pleiten. Allereerst is de waargenomen afname in inductie van SCEs door B(a)P proportioneel met de toegepaste doses. Als competitieve remming een significante rol zou spelen zou een toename van de dosis B(a)P een afname van het beschermende effect van voorbehandeling te zien geven. Ten tweede, toen microsomen, afkomstig van voorbehandelde primaire, embryonale kippe-hepatocyten, werden gebruikt leidde de waargenomen toename in de vorming van mutagene intermediairen niet tot een toename

in het aantal SCEs. Dit suggereert dat andere enzymen een essentiele rol spelen bij de waargenomen beschermende effecten. Ten derde zijn de waargenomen modulerende effecten van I3C niet beperkt tot een speciaal type mutageen maar wordt de werking van mutagenen afkomstig uit diverse chemische klassen beinvloedt. Speciaal in het geval van DBE leidt voorbehandeling tot een toename van het aantal SCEs. Daarom lijkt het waarschijnlijk dat competitieve remming niet een belangrijke rol speelt bij de waargenomen effecten. In plaats hiervan lijken de modulerende effecten van voorbehandeling toegeschreven te kunnen worden aan een verschuiving in de balans tussen de verschillende enzymen die betrokken zijn bij de biotransformatie van de betreffende mutagenen en, in het geval van B(a)P, wijzen op een essentiele rol van conjugerende enzijmen.

In het tweede gedeelte van deze studie zijn de modulerende effecten van B-naphtoflavon (B-NF) bestudeerd. B-NF is een synthetisch flavonol waarvan anticarcinogene eigenschappen zijn gerapporteerd in de literatuur. Behandeling van gekweekte primaire, embryonale kippe-hepatocyten leidde tot een grote toename van het cytochroom P450 gehalte van deze cellen. Voorbehandeling met B-NF had geen effect op de inductie van SCEs door 2-AA en NDMA. In tegenstelling hiermee werd wel een reductie van het aantal SCEs, veroorzaakt door B(a)P, gevonden. Gedetailleerde studies met subcellulaire fracties, afkomstig van _in ovo_ voorbehandelde embryonale kippe lever, toonden aan dat wanneer een S-9 fractie werd gebruikt een grote afname in het aantal geinduceerde SCEs werd gevonden terwijl bij het gebruik van een microsomale fractie een grote toename werd gevonden. Gecombineerd met de waargenomen grote toename in AHH activiteit en de gevonden toename in de vorming van de 'proximate' carcinogene en mutagene metaboliet B(a)P-7,8-dihydrodiol leidde dit tot de conclusie dat door B-NF veroorzaakte toename in enzym activiteit niet is geassocieerd met de beschermende effecten. In plaats hiervan werd geconcludeerd dat de waargenomen afname in SCE inductie door B(a)P afkomstig is van de vorming van een intracellulaire pool van B-NF die als competitieve remmer optreedt van het B(a)P metabolisme.

Samenvattend kan gesteld worden dat de studies die in dit proefschrift worden gepresenteerd laten zien dat het gecombineerd gebruik van cellen met specifieke eigenschappen het mogelijk maakt om diverse belangrijke aspecten van het proces van tumor vorming _in vitro_ te bestuderen. De resultaten laten zien dat het co-cultivatie systeem, bestaande uit primaire, embryonale kippe-hepatocyten en V79 Chinese hamster cellen een veelbelovend onderzoeksmodel is in het kanker-onderzoek.

CURRICULUM VITAE

Wim M.F. Jongen werd geboren te Heerlen op 2 december 1949, en behaalde in 1968 het diploma HBS-B aan het Eykhagencollege te Schaesberg (gemeente Landgraaf). In 1972 werd het diploma HBO-B analytisch-chemisch behaald aan de Zuidlimburgse Laboratoriumschool te Sittard.
Vanaf september 1972 tot december 1975 was hij als analist werkzaam op het toenmalige Laboratorium voor Insecticiden Onderzoek, waar hij betrokken was bij onderzoek naar de ontwikkeling van scheidingsmethodieken ten behoeve van bestrijdingsmiddelenonderzoek.
Vanaf januari 1976 is hij werkzaam op de vakgroep Toxicologie van de Landbouw Universiteit waar hij aanvankelijk als analist werd ingeschakeld bij het onderzoek en onderwijs in de chemische mutagenese. Met ingang van 1 januari 1985 is hij door een daartoe door het college van bestuur in het leven geroepen commissie benoemd tot universitair docent in de toxicologie. Het promotieonderzoek waarvan de resultaten zijn beschreven in dit proefschrift is grotendeels na die datum tot stand gekomen.

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