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Review

DNA damage and alterations of gene expression in chronic-degenerative diseases★✪

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Chronic-degenerative diseases (CDD) recognise a variety of exogenous and endogenous risk factors interacting with the organism for many years before disease onset. We applied genomic and postgenomic molecular analyses in experimental models characterised by different contribution of exogenous and endogenous CDD risk factors.

Exposure of mice to halogen light for 28 days resulted in induction of cyclobutane dimers and oxidative DNA damage in the skin. Evaluation of postgenomic alterations by cDNA arrays revealed upregulation of DNA repair pathways, increased cell division rate and protooncogenes transcription, resulting in skin tumors, 1 year later.

Exposure of *p53*–/+ mutant mice to cigarette smoke (CS) for 28 days induced DNA adducts formation in the lung. Postgenomic alterations included decreased apoptosis and increased cell division, as compared to CS-exposed wild type mice. These phenomena resulted in lung tumors, 9 months later.

Transplacental exposure of mouse foetuses to cigarette smoke induced DNA adduct formation in the liver. cDNA arrays analyses demonstrated decreased cell division, apoptosis increase, and tissue hypoxia. These phenomena resulted in growth retardation of the whole organism.

Molecular alterations were investigated in human trabecular meshwork, the non-replicating ocular epithelia involved in the pathogenesis of chronic degenerative glaucoma. Results indicate increased oxidative DNA damage in glaucoma patients as compared to unaffected controls.

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Abbreviations: CDD, chronic-degenerative diseases; HTM, human trabecular meshwork; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; CS, cigarette smoke; POAG, primary open angle glaucoma.

These four experimental studies suggest that DNA damage may result in different CDD (cancer, growth retardation, glaucoma) depending on the replication rate of the target cell population.

Chronic-degenerative diseases (CDD) are a variety of pathological situations characterised by a long latency period and the occurrence of cellular degenerative phenomena. CDD include age-related pathologies such as cancer, cardiovascular diseases, and neuro-ophthalmic diseases, such as dementia and glaucoma.

Our previous studies suggested that genotoxic damage is a common phenomenon occurring in these situations (De Flora *et al.*, 1996) and demonstrated that DNA alterations are associated not only with cancer, but also with human atherosclerosis (De Flora *et al.*, 1997) and age-related brain and heart degeneration in mice (Izzotti *et al.*, 1999). These findings indicate that DNA alterations detected in CDD are related to both exogenous and endogenous causes. As an example, it was established that the amount of DNA adducts induced by exposure to genotoxic agents is affected by metabolic gene polymorphism assets not only in cancer (Tanningher *et al.*, 1999), but also in human atherosclerosis (Izzotti *et al.*, 2001).

As a matter of fact, CDD recognise a great variety of risk factors, of both exogenous and endogenous origin, interplaying in an intricate network to determine the risk of an organism to develop the disease. Nowadays, the balance of this interplay may be evaluated in a healthy organism before the onset of the CDD during its long latency by using suitable molecular biology tools.

At the genomic level, the formation of nucleotide alterations, as resulting from environmental risk factors and endogenous processes, may be detected with great sensitivity by ^{32}P postlabelling methods (Izzotti *et al.*, 1998). At the same level, the occurrence of gene polymorphisms affecting the rate of DNA damage may be detected by restriction fragment length polymorphism/polymerase chain reaction (RFLP/PCR) methods.

In recent years, the development of microarray technology for multigene expression analysis resulted in a dramatic improvement of our ability to collect data at the postgenomic level, by examining the transcriptional activity of hundreds or thousands of genes in the same experimental model. This approach results in a detailed molecular painting of postgenomic changes induced in the organism by administering DNA damaging agents, as we demonstrated for hexavalent chromium (Izzotti *et al.*, 2002).

We applied genomic/postgenomic analyses in various experimental models to shed light on the interplay between endogenous and exogenous risk factors associated with CDD development. The obtained results indicate that DNA damage may result in different CDD depending on the replication rate of the target cell population.

EXPOSURE OF SKH-1 HAIRLESS MICE TO HALOGEN LIGHT

Light is one of the main cancer risk factors naturally present in our environment. To evaluate its effects at the molecular level, we exposed SKH-1 hairless mice to light containing UV-A/B but not UV-C wavelengths, as emitted by halogen lamps. In a first group of mice the exposure was performed for 28 days to evaluate molecular end-points and, in a parallel group of mice, for additional 18 months to evaluate tumour development.

Light altered the following genomic molecular end-points in the skin: (*a*) appearance of DNA cyclobutane dimers-related photoproducts, as detected by ^{32}P postlabelling following digestion to trinucleotides (Bykov & Hemminki, 1995; Izzotti, 1998); (*b*) 8-hydroxy-2′-deoxyguanosine (8-OH-dG, 1.94-fold increase), as detected by ^{32}P postlabelling and formic acid thin-layer chromatography

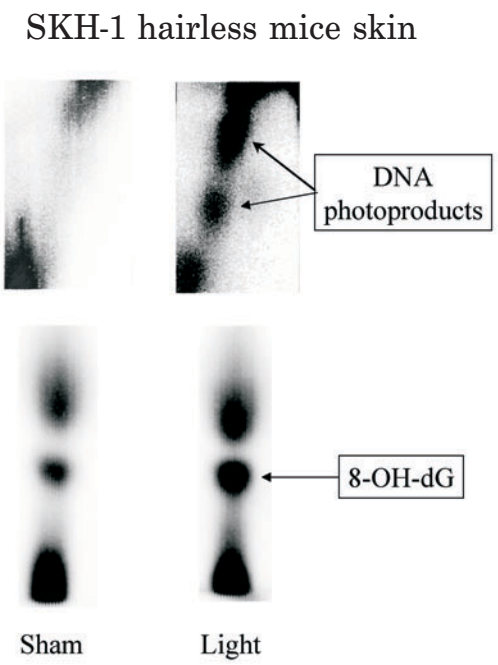

Figure 1. Detection of nucleotide alterations in the skin of SKH-1 mice sham-exposed or exposed for 28 days to halogen light containing UV-A/B, but not UV-C.

Nucleotide alterations, as detected by ^{32}P postlabelling methods, include DNA photoproducts related to the formation of cyclobutane thymine dimers (upper panels), and 8-OH-dG (lower panels).

(Devanobyina & Gupta, 1996; Izzotti *et al.*, 1999) (Fig. 1). In the same animal postgenomic analyses evaluating the expression of 597 genes by cDNA arrays (Atlas^{TM}, Mouse Expression) (Clontech, Palo Alto, CA, U.S.A.) detected upregulation of various genes (Fig. 2), mainly belonging to the following functional categories: (*a*) increase of cell division rate (cyclins); (*b*) response to oxidative stress (e.g., glutathione cycle); (*c*) immunosuppression (e.g., interleukins 10, 12, T cell death associated protein); (*d*) cytokeratins 1, 14, 18, and 19; (*e*) DNA repair pathways, including nucleotide excision repair (e.g., RAD23A, 23B, 50, 51, 52, XPG, XPAC, XPBC), base excision repair (e.g., XRCC1, apurinic/apyrimidinic endonuclease, uracil-DNA glycosylase), mismatch repair (MSH2, MSH6); (*f*) apoptosis induction; (*g*) protooncogenes (e.g., *p53*, c-*fos*, H-*ras*, *jun*-B, and c-*myc*). The increase of cell division (2.92-fold), apoptosis (4.68-fold), and p53 protein accumulation in the nuclei (18.14-fold) were confirmed in skin slides by microscope determination of proliferating cell nuclear antigen (PCNA), TUNEL assay, and immunohistochemistry, respectively.

These data indicate that, after only 28 days of exposure, light induces in the skin a cancer prone asset. As demonstrated by monitoring animals for the following 18 months, this situation evolves in the appearance of skin cancers.

Molecular analyses suggest that some phenomena occurring in light-exposed skin, i.e., immuno-suppression and oxidative damage, could also affect other distant organs. An increased formation (3.04-fold) of lipophilic bulky DNA adducts, as detected by ^{32}P postlabelling after butanol enrichment (Gupta, 1985; Izzotti, 1998), was demonstrated in the bone marrow of light exposed animals. These adducts could be related to the increased rate of cytogenetic alterations, including micronuclei formation in blood normochromatic erythrocytes (1.80-fold as compared to unexposed mice), micronuclei formation in bone marrow polychromatic erythrocytes (1.56-fold), and alteration of the polychromatic/normochromatic ratio in bone marrow (28% increase).

These findings suggest that exposure to the UV-A/B containing halogen light could contribute not only to the development of skin cancer, but also of other systemic diseases, as already established for viral infections (e.g. type 1 herpes simplex virus), or, more recently, proposed for lymphoid malignancies by epidemiologic (Levi *et al.*, 1997) and experimental studies in *p53*$^{+/-}$ mice (Jiang *et al.*, 2001).

EXPOSURE OF *p53* MUTANT MICE TO CIGARETTE SMOKE

Although cigarette smoke (CS) is an established carcinogen for humans, it is very diffi-

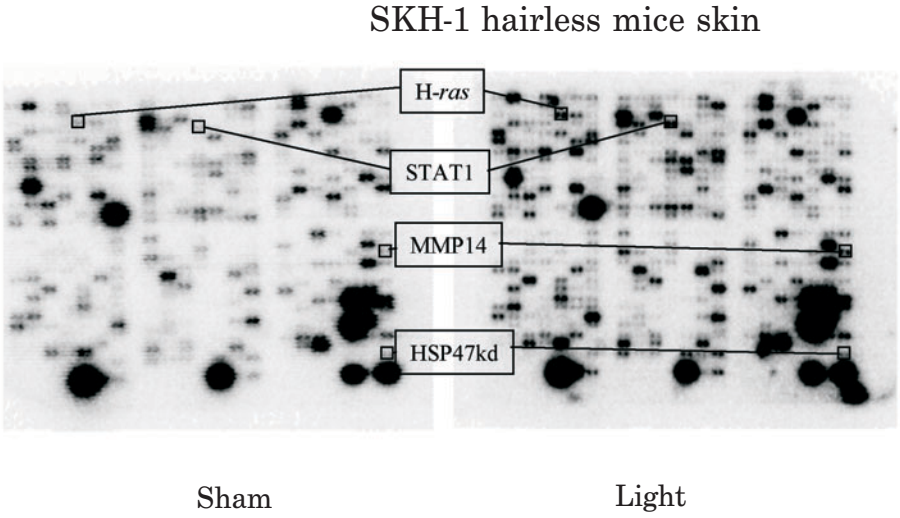

Figure 2. Multigene expression analysis as evaluated for 597 genes by nylon cDNA membrane array (Clontech, Atlas™ Mouse Expression), in the skin of SKH-1 mice sham-exposed or exposed for 28 days to halogen light containing UV-A/B, but not UV-C.

Boxes highlight some examples of genes whose expression was enhanced following light exposure.

cult to reproduce CS tumorigenicity in animal models. To set up an experimental model of increased endogenous susceptibility to CS carcinogenic effects, we compared wild type A/J mice (*p53*+/+) with transgenic A/J mice carrying a dominant negative *p53* mutation (*p53*–/+), following either sham- or environmental CS-exposure for up to 9.5 months.

In unexposed animals an age-related increase of lipophilic bulky DNA adducts was detected by ^{32}P postlabelling since 1 month to 9.5 months in the lung (2.9-fold increase) and heart (3.0-fold) of *p53*–/+ but not of *p53*+/+ mice. Similarly, a more consistent age-related increase of micronuclei in blood normochromatic erythrocytes was detected in *p53*–/+ *vs.* *p53*+/+ (32% *vs.* 16%) male mice. Interestingly, the same end point was not modified in female mice, irrespective of the *p53* status. These findings suggest a possible role of *p53* in age-related endogenous DNA alterations, as recently proposed by using *p53* mutant mouse (Tyner *et al.*, 2002).

In *p53*–/+ mutant mice exposed to CS, DNA adduct levels in the lung were 1.0 ± 0.20 (mean ± SE) (unexposed), 5.7 ± 1.13 (1 month exposure), 9.5 ± 1.17 (9.5 month exposure), and 9.3 ± 0.91 (9.5 months + 1 week of exposure discontinuation).

In *p53*+/+ wild type mice exposed to CS, DNA adducts levels in the lung were 1.8 ± 0.37 (unexposed), 3.3 ± 0.41 (1 month), 6.6 ± 1.03 (9.5 months), and 7.0 ± 0.62 (9.5 months + 1 week exposure discontinuation).

Therefore, accumulation of CS-related DNA adducts was significantly ($P < 0.05$) higher in *p53*–/+ *vs.* *p53*+/+ mice, both after 1 and 9.5 months of exposure.

Again males were more sensitive than female mice to DNA alterations, their increase of CS-related DNA adducts in the lung after 28 days being in *p53*–/+ 7.1-fold (male) *vs.* 2.9-fold (female), and in *p53*+/+ 3.3-fold (male) *vs.* 1.9-fold (female).

As compared to wild type mice, CS-exposed male mutant mice underwent a lower induction of apoptosis in bronchial epithelium (2.5-fold in *p53*–/+ *vs.* 3.33-fold in *p53*+/+) and a more intense micronuclei formation in pulmonary alveolar macrophages (1.52-fold *vs.* 1.40-fold) and peripheral blood normochromatic erythrocytes (1.63-fold *vs.* 1.33).

Multigene expression analysis of 1185 genes was performed by nylon membrane cDNA ar-

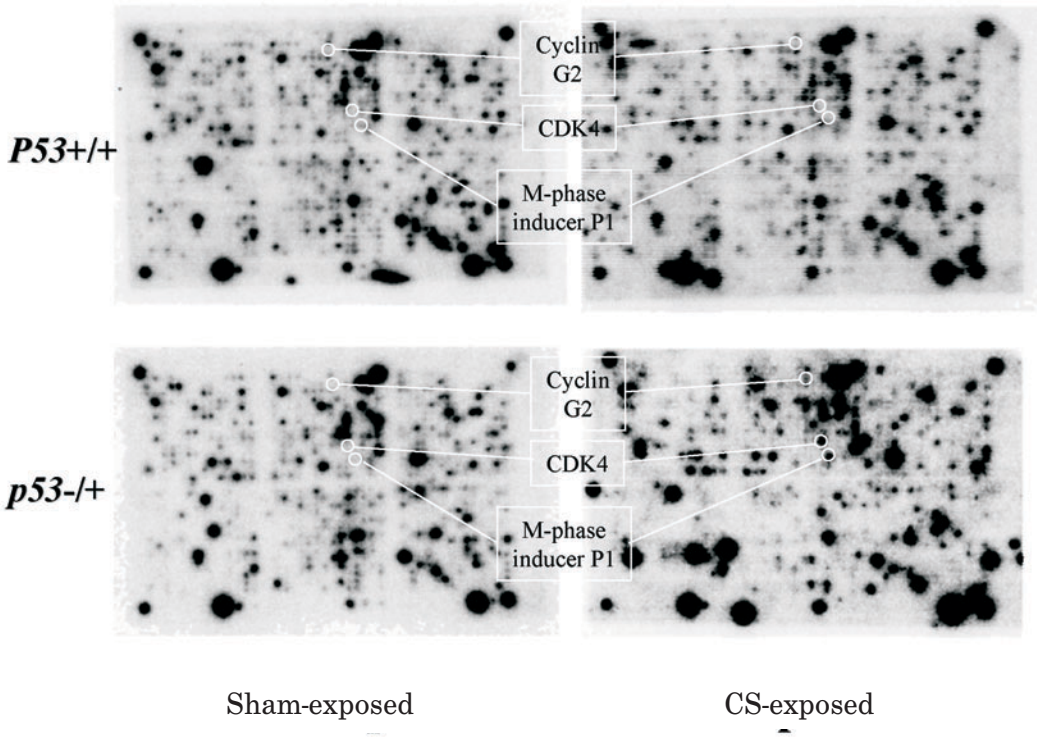

Figure 3. Evaluation by nylon-membrane cDNA-array (Atlas^{TM} Mouse 12 Array) of expression of 1185 genes in the lung of wild type (upper panels) and *p53*–/+ mutant (lower panels) (UL53-3 X A/J)F_1 mice, either sham-exposed (left panels) or exposed to cigarette smoke (CS) for 28 days (right panels).

Boxes highlight some examples of genes whose expression was enhanced following CS exposure in *p53*–/+ but not in *p53*+/+ mice.

rays (Clontech Atlas^{TM} Mouse Cancer) (Fig. 3) after 28 days of exposure. A different gene expression panel in response to CS in the lung was detected comparing mutant and wild type mice, these differences mainly affecting genes involved in apoptosis and cell cycle control. Pro-apoptotic genes (e.g., TNF receptors and inducers, and *p53*) were overexpressed in wild type but not in mutant mice. Cell cycle inducers (e.g., G2/M cyclins, cell division kinases, M phase inducers) were upregulated in mutant mice only, while cell cycle negative regulators (e.g., cell division kinase inhibitors) were upregulated in wild type mice only. In addition, a 1.56-fold increase in the expression of K-*ras* was detected only in mutant mice, as confirmed by reverse transcriptase/polymerase chain reaction (RT/PCR).

These data suggest that wild type mice respond to CS exposure by increasing apoptosis and decreasing cell cycle rate, while mutant mice lack the protective effect of apoptosis and are more sensitive to the promotive stimuli of CS.

These molecular findings indicate that mutant mice possess several mechanisms making them more susceptible, as compared to wild type animals, to the carcinogenic activity of CS. Accordingly, significant ($P < 0.05$) increases of lung tumour incidence (67.5% *vs.* 32.3%) and multiplicity (0.95 *vs.* 0.3 per animal) were detected in mutant as compared to wild type mice, after 9.5 month exposure to CS.

TRANSPLACENTAL EXPOSURE OF MICE TO CIGARETTE SMOKE

CS is involved in the arising of various CDD in adult organism. A particular problem is represented by the effect of CS exposure during pregnancy, an endogenous asset increasing susceptibility to genotoxic agents.

To explore this situation at a molecular level, we exposed pregnant Swiss albino mice to environmental CS throughout pregnancy, since conception to the 18th day, when the animals were sacrificed and foetuses collected. Molecular analyses were performed in foetuses liver, this organ possessing during the intrauterine life not only metabolic but also haematopoietic activity.

CS exposure increased formation of: (*a*) lipophilic bulky DNA adducts, as detected by ^{32}P postlabelling following butanol enrichment (6.3-fold increase as compared to sham exposed animals); (*b*) 8-OH-dG, as detetected by ^{32}P postlabelling (4.6-fold increase).

Multigene expression of 746 genes was tested by cDNA arrays (Atlas^{TM} Mouse Stress, Atlas^{TM} Mouse Expression) (Clontech). The expression of 116 genes (15.5%) was increased more than two-fold in the liver as a consequence of CS exposure. The upregulated genes fall in the following functional categories: xenobiotic metabolism, glutathione metabolism, response to oxidative damage, stress response, DNA repair, protein folding and removal, apoptosis inducers, cell cycle inhibitors, haematopoietic function.

These findings indicate that CS induces metabolic activities not yet active in unexposed foetus, hence resulting activation of CS components to genotoxic metabolites. However, DNA repair activities are poorly inducible in foetal liver. Therefore the defensive mechanism against genotoxic damage in the foetus is mainly achieved by decreasing cell division rate, thereby increasing the chances of performing efficient DNA repair. In addition, apoptosis, which is already physiologically active and highly inducible in the foetus, is furthermore increased. These defensive phenomena result in a decreased cell division rate and in the occurrence of growth retardation, as confirmed by the significant decrease of body mass gain (–16.4%) in CS-exposed as compared to sham exposed foetuses. These conclusions support at the molecular level epidemiological data suggesting that growth retardation diseases are associated with transplacental CS exposure. These diseases include: (*a*) delivery of small for date newborns (Perera *et al.*, 1998); (*b*) retardation in bone development and oral clefts (van Rooij *et al.,* 2001); (*c*) retardation in the development of respiratory centres in the central nervous system resulting in an increased risk of sudden infant death syndrome (Cooke, 1998).

Another CS effect detected by postgenomic analysis in our study was the induction of tissue hypoxia. This phenomenon is related to the abundant presence in CS of a specific inhibitor of the mitochondrial respiratory chain, i.e., hydrogn cyanide, one cigarette containing 400–500 μg of this toxic compound (IARC, 1986). Hydrogen cyanide is selectively detoxified by conjugation with thiosulfate, as catalysed by thiosulfate sulfurtransferase (rhodanese). This gene was the most consistently upregulated gene (4.37-fold) in the liver of CS exposed foetuses. In addition, CS induces formation of carbon monoxide-hemoglobin, another potent inducer of tissue hypoxia. Foetal haemoglobin (HbF) is by far more capable than maternal haemoglobin (HbS) of binding carbon monoxide, this resulting in an increased susceptibility of foetal organs towards CS-induced hypoxia. Multigene analyses indicate that the foetus counteracts this situation by increasing erythrocyte production in the liver, as suggested by the increased expression of genes coding for (*a*) erythropoietin receptor precursors; (*b*) transferrin and low density lipoprotein receptors, uptaking red blood components, i.e. iron and cholesterol; (*c*) blood vessel growth, i.e., vascular endothelial growth factor precursor and angiogenin; (*d*) transcription of haematopoietic domains, i.e. CACCC box binding protein. These data explain the finding of increased haematocrits in the blood of foetuses undergoing maternal smoking (Bush *et al.*, 2000).

CS induced in the mouse foetus upregulation of several genes involved in proliferation and differentiation of leukocytes, such as c-*myb*,

c-*myc*, L-*myc*, c-*rel*, c-*fos* and *lfc* (lymphoid blast crisis), most of them being leukemia-lymphoma associated protooncogenes. It is noteworthy that this overexpression occurs in the These data support at the molecular level the epidemiologic finding that these childhood tumours may be associated with maternal smoking during pregnancy (Boffetta *et al.,* 2000).

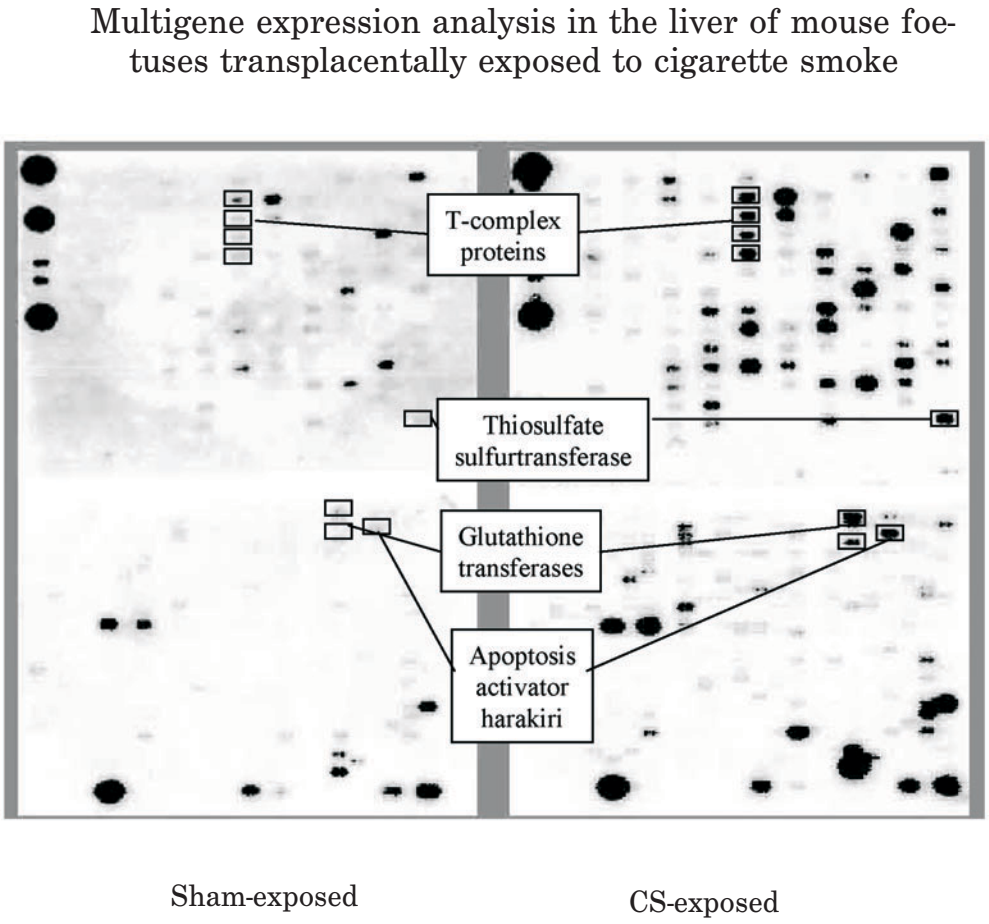

Figure 4. Multigene expression as evaluated for 746 genes by nylon-membrane cDNA-array (Atlas™ Mouse Expression, Atlas™ Mouse Stress) in the liver of Swiss albino mouse foetuses either sham-exposed (left panel), or transplacentally exposed to environmental cigarette smoke (CS) since conception to the 18th day of pregnancy (right panel).

Boxes highlight some examples of genes whose expression was enhanced following transplacental CS exposure.

presence of DNA damage, as demonstrated not only by the formation of nucleotide alterations (DNA adducts, 8-OH-dG), but also by the occurrence of cytogenetic damage resulting in an increase (2.1-fold) of micronucleated polychromatic erythrocytes in foetal liver of CS exposed foetuses. Therefore, molecular analyses suggest that transplacental exposure to CS could be associated with an increased risk of developing leukemias and lymphomas.

HUMAN CHRONIC-DEGENERATIVE GLAUCOMA

Primary open angle glaucoma (POAG) is a CDD representing the main cause of irreversible blindness affecting more than 90 million people worldwide (Goldberg, 2000). POAG pathogenesis implies a progressive age-related cellular loss in the human trabecular meshwork (HTM), the non proliferating dif-

ferentiated epithelia of the ciliary body regulating the aqueous humour outflow from the eye anterior chamber. HTM ipoplasia results in increased intraocular pressure, optic nerve damage and progressive irreversible blindness.

To shed light on the cause of HTM damage in POAG, we collected HTM specimens form 45 POAG patients undergoing therapeutical eye surgery (trabeculectomy) and 46 unaffected controls. Oxidative DNA damage was evaluated by ^{32}P postlabelling, the only method able to detect 8-OH-dG in the very minute specimens (<1.5 mg) made available by ocular surgery. 8-OH-dG was increased (3.6-fold, $P < 0.0001$) in POAG patients as compared to controls. Oxidative DNA damage was significantly associated with intraocular pressure (maximum values and fluctuation) and with visual field defects, as detected by computerised ocular field analysis. No anamnestic variables, such as exposure to cigarette smoke, sex or age, were associated with 8-OH-dG levels.

The only parameter affecting oxidative damage was the polymorphisms for the glutahione *S*-transferase M1 gene, as detected by polymerase chain reaction (Izzotti *et al.*, 2001). POAG patients having GSTM1 deletion had 2.2-fold higher 8-OH-dG amounts than GSTM1 positive subjects. In addition, GSTM1 deletion was significantly more frequent in POAG patients than in unaffected controls (95.8% *vs*. 60.8%, $P < 0.05$).

To confirm these findings at the postgenomic level, we are performing multigene expression analyses in HTM biopsies. Due to the very low amount of tissue available, we set up a method employing high performance RNA extraction, mRNA reverse transrciption to first strand DNA, first strand DNA amplification by polymerase chain reaction, complementary radioactive probe synthesis and cDNA hybridisation on nylon membrane array. Preliminary results indicate that GST transferase genes are expressed in HTM in a physiological situation. Further studies are ongoing to evaluate whether or not their expression is modified in POAG patients.

The obtained results support the hypothesis that oxidative damage is associated with POAG (Alvarado *et al.,* 1984) and explain the recent finding that oxidative stress response pathways (i.e., ELAM-1/IL-1/NF-kB) are activated in the HTM of POAG patients (Wang *et al.,* 2001).

CONCLUSIONS

The herein reported studies indicate that DNA damage may contribute to the occurrence of different CDD depending on the cellular target affected (Table 1). Whenever genotoxic damage occurs in dividing cell, such as skin or respiratory epithelia, the consequent disease is cancer. In this view, we demonstrated that light is able to induce skin cancer in SKH-1 hairless mice, and CS is able to induce lung cancer in *p53* mutant mice. Whenever DNA damage affects a developing organism, as it occurs in transplacental exposure, the main consequence is a significant decrease of cell division rate resulting in growth retardation, as we demonstrated in mouse foetuses exposed to environmental CS. Whenever oxidative DNA damage occurs in a non dividing perennial cell population, such as HTM, the consequence is degeneration, cell loss and tissue ipoplasia, resulting in POAG.

All mentioned CDD are characterised by a long latency before the clinical onset. During this period, the use of postgenomic analyses revealed that the existing DNA damage has resulted in serious consequences for the affected tissue and also indicated the attributes of the incoming diseases. Interestingly, these indications were obtained in healthy organisms during the CDD latency period. In fact, molecular alterations were detected in light exposed mice after 28 days of exposure, while skin cancers appeared only after further additional 12 months. Again, genomic and postgenomic changes were only detected in

Table 1. Effects and contribution of exogenous and endogenous risk factors to the development of chronic-degenerative diseases in various experimental studies.

DNA damage may result in different diseases depending on the replication rate of the target cell population.

Species/ Organ	Exposure	Contribution of risk factors		Cell division rate	Effects	Consequent disease
		Exogenous	Endogenous			
Hairless mice/skin	Halogen light	High	Intermediate	High	DNA damage Cell proliferation Protooncogene expression	Cancer
p53–/+ mice/lung	Cigarette smoke	High	High	Intermediate	DNA damage Decreased apoptosis Cell proliferation	Cancer
Mouse foetus/liver	Transplacental cigarette smoke	High	High	High	DNA damage Cell cycle arrest Apoptosis	Growth retardation
Human eye/trabecular meshwork	None	Low	High	Null	DNA damage	Degenerative glaucoma

CS exposed *p53* mutant mice after 28 days, while consequent tumours were only detected 9.5 months later. Therefore, the reported experimental models suggest that molecular analyses at the genomic and postgenomic levels may represent an useful tool in CDD prevention for the early identification of molecular situations oriented towards these pathologies in still healthy organisms.

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