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Review Article

Lung carcinogenesis: Pivotal role of metals in tobacco smoke

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Abstract

Although significant progress has been made in unraveling the molecular mechanisms responsible for tobacco smoke toxicity and carcinogenicity, only limited information is available concerning the mechanisms by which tar particles and the gaseous phase constituents of tobacco smoke participate and contribute to carcinogenic processes in lung cancer.

The present review critically evaluates how metals contained in the tar particles and the gaseous phase of tobacco smoke play a leading role in the carcinogenic process, taking into consideration the physiology and pathophysiology of the bronchial epithelium. Overwhelmingly, the published data indicate that the bronchopulmonary epithelial cells may represent the first and most critical line of defense against cigarette smoke.

Keywords: Metals; Genotoxicity; Critical mass; Pre-neoplasia; Field cancerization

Contents

Abbreviations: AP-1, Activator protein-1; CREB, c-AMP-response element binding protein; CS, Cigarette smoke; ERK, Extracellular signal regulated kinase; GAAD45, Growth arrest and DNA damage 45; GSH, Glutathione; GSSG-R, Glutathione reductase; HSP, Heat shock protein; IARC, International Agency for Research on Cancer; IERGs, Immediate and early response genes; IGFBP, Insulin-like growth factor binding protein; JNK, c-Jun N-terminal kinase; MDM2, Murine double minute 2; mPK, Mitochondrial protein kinases; NAD(P)H, Nicotinamide adenine dinucleotide phosphate; NF-κB, Nuclear factor kappa B; p38 MAP kinase, p38 mitogen activated protein kinase; PAHs, Polycyclic aromatic hydrocarbons; ROS, Reactive oxygen species; $\Delta\psi_m$, Mitochondrial membrane potential; 8-OHdG, 8-hydroxy-deoxyguanosine.

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Introduction

Cigarette smoke (CS) can be divided into two phases: the gaseous phase and particulate matter (tar). Both phases are harmful, containing high concentrations of toxic and carcinogenic compounds [1] and are both associated with diverse pulmonary disorders, including cancer. The metabolic activation and detoxification mechanisms of these compounds have been extensively studied [2]. Although it is well established that tar contains a large number of carcinogens [3], more recent publications suggest that chemicals in the gaseous phase of tobacco smoke are of major importance in the cytotoxic and carcinogenic effects of tobacco smoke on the bronchopulmonary epithelial cells [4–6]. At present, it is well known that for these lesions to occur, both phases of tobacco smoke are required [7]. After decades of intensive research, it has become apparent that, although there is unlikely to be a single mechanism involved in tobacco toxicity and carcinogenicity, some general principles have emerged [2].

Cilia as a pulmonary defence mechanism

In cigarette smoke, four biological processes characterize the kinetics of toxic substances: uptake, distribution, metabolic transformation and elimination. Uptake means that the chemicals enter the lung by inhalation; they are then quickly distributed among the major organs [8]. From the 500 ml of inhaled air during each inhalation, which contains 35 ml of tobacco smoke, 350 ml are exhaled and 150 ml are entrapped inside the bronchial tree. This entrapped air, mixed with tobacco smoke, is further diluted 14 times with the inhaled air, each time with 500 ml of air, in about one minute, until the next puff. This results in an extreme dilution of the constituents of the tobacco smoke in the inhaled air.

Daily, people inhale approximately 10^{10} of dust particles [9]. This figure increases significantly in smokers, because of the inhalation of a large number of tar particles. Tar particles of 0.2–5 μm in diameter are deposited on the mucous covering the cilia of the bronchopulmonary epithelium. The mucous, which is an important source of antioxidants, protects the air-ducts from the trachea to the end of the bronchioli. Each bronchoepithelial cell has 200 cilia. These cilia exhibit continual movement, which is directed from the end of the bronchus towards the larynx, pushing the mucous (covering the cilia) outwards [9]. The storing of tar particles on the layer of the mucous is accompanied by an increase in mucous production by the goblet cells and activation of the purification mechanism. When the bronchial epithelial cells are stripped of their defense mechanism against inhaled particles they become the target of the toxic and potentially carcinogenic effects of these particles.

The toxic effect of tobacco smoke on the bronchopulmonary epithelium

The role of the gas phase components

At present, there are no clues as to which constituent(s) of the gas phase of tobacco smoke is/are responsible for its carcinogenic action. There is strong evidence that metabolically-activated or direct action genotoxic components and inhibitors of DNA repair in the gaseous phase of tobacco smoke may contribute to DNA damage and to smoking-associated cancers of the upper aerodigestive tract [10]. The gas phase contains several groups of toxic compounds, most of which are well known as animal carcinogens and/or possibly human carcinogens. These compounds listed in Table 1, are all able to induce damage to the bronchoepithelial cells in humans and in experimental animals [11,12]. Metabolites of two of these compounds represent good examples to annotate and discuss. Benzene, for example, has been classified as a “Group 2A carcinogen” by the International Agency for Research on Cancer (IARC) [13,14]. It is a clastogenic carcinogen, which can induce DNA strand breaks, chromosomal mutations and aneuploidy in mammalian cells [11]. It can also induce a high frequency loss of heterozygosity on chromosome 11 in the $p53^{+/-}$ of strain A/J mice [15]. Benzene must be metabolized in vivo, to cause chromosomal changes, suggesting that the metabolites of benzene are responsible for any cytogenetic changes [16,17]. 1,3-Butadiene is also known to be a human carcinogen, based on substantiated evidence of carcinogenicity in studies in humans and experimental animals (rats and mice); these studies indicate a causal relationship between exposure to

Table 1
Major groups of toxic and carcinogenic chemicals in the gas phase of tobacco smoke

1. Hydrocarbons
Methyl-propane, Methyl-butane, Hexane, Ethylene, Acetylene, Propylene, Butadiene, Isoprene, Pentadienes, Methyl-pentadienes, Ethyl-pentadienes
2. Aldehydes and Ketones
Formaldehyde, Acetaldehyde, Acrolein, Crotonaldehyde, Methacrolein, Propionaldehyde, Isobutyraldehyde, Acetone, Propanone, Butanone
3. Nitriles and Amines
Methyl nitrile, Ethyl nitrile, Acetonitrile, Acrylonitrile, Benzonitrile, Methacrylonitrile, Ethylamine, Benzylamine, Phenylamine
4. Aromatic Hydrocarbons
Benzene, Toluene, Ethyl-benzene, Xylenes, Styrene
5. Heterocyclic compounds of oxygen
Furan, Methyl furans, Dimethyl furans
6. Other volatile non-organic compounds
Hydrogen sulfide, Methyl mercaptan, Ethyl mercaptan, Sulfur dioxide, Hydrogen cyanide, Ammonia, Nitrogen dioxide, Nitric oxide, Carbon dioxide, Carbon monoxide

1,3-butadiene and excess mortality from lymphatic and/or hematopoietic cancers [18,19]. 1,3-Butadiene has been shown to be metabolized to mutagenic and carcinogenic epoxides (epoxybutene and diepoxybutane) [20–22].

The gaseous phase components of CS are transported freely during inhalation, by simple diffusion, towards the broncho-pulmonary epithelial cells. Their toxic activity on the cells begins immediately, with the first puff, simply following the laws of gases. The toxic components of the gaseous phase harm the cilia of the bronchial epithelium and impair the movement of the mucous by altering its viscosity and by disturbing or paralyzing the ciliary beat [23–25]. Acrolein and nitrogen dioxide are known to induce the loss of cilia in the rodent respiratory tract [23,26]. At first the mobility of the cilia is impaired, later they become inert and finally their structure is destroyed. This results in the layer of the mucous covering the cilia not being able to move normally towards the exterior (the larynx) and it becomes stagnant in the alveoli, in the bronchioli and on the walls of the large bronchi. Eventually, the mucous remains in the interior of the bronchial tree, and in combination with goblet cell hyperplasia, it can be an important factor in causing obstruction of the small airways [27].

The role of tar

The inhaled particles of tar are deposited on the lung tissue in two distinct stages: 1) In the initial stage of smoking, when anatomical/functional alterations do not exist in the lung tissue, the very small particles of tar are deposited in the sub-epithelial layer, in the lung interstitium and beyond the ciliated airways [28] and, 2) in the long term, tar particles are deposited in the area of ciliated airways where the mucociliary escalator is destroyed by the gaseous phase constituents (acetaldehyde, ammonia, hydrogen cynide and SO_2) of tobacco smoke. If this happens, the tar particles become continually entrapped in the mucous which stagnates inside the bronchoalveolar space, enriched with toxic/carcinogenic material [28].

For tar to exert its toxic and carcinogenic activity, prior destruction of the bronchopulmonary protective mechanism by the gaseous phase components seems to be necessary. Then, the tar can cause lung damage at the point when, by chance, an appropriate critical mass of carcinogenic elements is created, mainly of some of the trace elements of heavy metals (hexavalent chromium [29], arsenic [30], lead [31], mercury [32], nickel [33,34] and in particular cadmium in ionic form [35–43]). However, the extremely low concentrations of the toxic metals in tobacco smoke (in ng per cigarette: Hg 5.4, Ni 11.95-75, Pb 12.6, Cd 116, Cr 4.5) and their thinning out in an extremely large volume of atmospheric air during inhalation (7L/min), result in a tremendous dispersion of these molecules on a huge surface of lung tissue ($\sim 70 m^2$). Yet, if mucous enriched with tobacco smoke constituents (mainly with metals and Poly-cyclic Aromatic Hydrocarbons (PAHs)) stagnates inside the bronchoalveolar spaces, and an appropriate quantity of carcinogenic molecules accidentally becomes concentrated in a certain area of the lung tissue then, the adoptive and repairing capacities of the lung epithelial cells fail, ensuring a suitable environment for the carcinogenic process to begin. The continuous enrichment of the mucous with the carcinogenic compounds of tobacco smoke can be followed by the progressive accumulation of multiple genetic changes which underline the multi-step nature of tumorigenesis [44]. It has been proposed that the total accumulation of genetic alterations rather than their relative order, is more important for carcinogenesis [45,46], since the potency of a genotoxic compound depends not only on its capability to cause DNA damage, but also on the cell's capacity to repair the specific damage [47].

The role of metals

Certain metals which exist in cigarette smoke and thus are inhaled during smoking can cause serious diseases, including lung cancer [48–51] Recent studies have shown that carcinogenicity due to metals is, in general, the result of the production of the reactive oxygen species [52–54].

Inhaled metals are not biodegradable and as a result they are deposited and remain for long periods in various areas of the pulmonary tissue. Some metals, including zinc, copper, iron and calcium actively participate in diverse, important cellular activities, such as the control of gene transcription, neural conductivity, oxygen uptake from the lungs and transfer to peripheral tissues, various enzymic functions, cellular respiration, oxidation-reduction activities and the control of cellular apoptosis. Some toxic metals are able to mimic the functions of "useful" bio-metals and as a consequence, to substitute them into the various cellular processes, thus causing serious malfunctions to different vital cellular activities. In this way, toxic metals are able to activate and/or deactivate the cellular functions controlled by other non-harmful metals which are useful to life. In some cases, biologically useful metals could, under certain conditions, become toxic.

Toxicity of chromium

Chromium salts, among which is chromium dichromate ($K_2Cr_2O_7$), comprise an example of mutagenicity, genotoxicity and carcinogenicity [55,56]. Cr(VI) as chromate salt ($Cr^{VI}O_4^{2-}$) easily penetrates the cell membrane, probably through the ion channels [57]. Intracellular chromium reduction is considered as a necessary step for the carcinogenic action of the ion metal. The intracellular reduction product of Cr(VI) is Cr(III) [58]. The intermediate products of the biological reduction of Cr(VI) include Cr(V), Cr(IV), sulfur radicals ($RS^{\bullet}$), carbon centered radicals ($R^{\bullet}$) and reactive oxygen species (ROS) [59,60]. Low molecular weight cellular constituents reduce Cr(VI) in vitro at normal pH. These constituents are glutathione (GSH) [61,62], cysteine [63], lipoic acid [64], molecules that contain diols such as NAD(P)H, ribose, fructose and arabinose [65,66] as well as ascorbate [67]. Among these components, ascorbate and GSH are the best non-enzymic candidate compounds. The reduction of Cr(VI) by GSH creates glutathionyl radicals ($GS^{\bullet}$) [61,62] and Cr(V) and Cr(IV) complexes [68]. These two stable compounds Cr(V) and Cr(IV) are used as models for the study of the role of intracellular Cr(IV) and Cr(V) in the mechanisms of carcinogenicity by Cr(VI). Cr(V) and Cr(IV) react with H_2O_2 and produce hydroxyl radicals ($^{\bullet}OH$) [67]:

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + {}^{\bullet}OH + OH^-$$
$$Cr(IV) + H_2O_2 \rightarrow Cr(V) + {}^{\bullet}OH + OH^-$$

The glutathionyl radical may react with other thiol molecules to generate $O_2^{-\bullet}$ radicals:

$$RS^{\bullet} + RSH \rightarrow RSSR^{-\bullet} + H^+$$
$$RSSR^{-\bullet} + O_2 \rightarrow RSSR + O_2^{-\bullet}$$

The formation of $O_2^{-\bullet}$ could then lead to the formation of H_2O_2.

Enzymic factors may also function as Cr(VI) reductants, as for example, glutathione reductase (GSSG-R) [69–71], lipoyl-dehydrogenase and $NADP^+$ ferredoxin oxidoreductase [72,73]. In the presence of NAD(P)H, glutathione reduces Cr(VI) to Cr(V) which is identified as the Cr(V)-NADPH complex. In vivo, it is likely that NADPH flavoenzymes and not GSH or ascorbate are the major one-electron Cr(VI) reductants [74]. Cr(IV) is a potent intermediate that can produce $^{\bullet}OH$ radicals from H_2O_2 through a Fenton-type reaction as shown above [67].

Chromium (IV) inside the cells is reduced to Cr(III). Cr(III) inside the cell can produce hydroxyl radicals from H_2O_2 in a pH-dependent process.

$$Cr(III) + H_2O_2 \rightarrow Cr(IV) + {}^{\bullet}OH + {}^{\bullet}OH$$

The role of free radicals in Cr(VI)-induced carcinogenesis: DNA damage

When DNA is incubated with Cr(VI) and ascorbate, a significant number of DNA strand breaks is produced. The addition of H_2O_2 effectively promotes DNA damage. The number of DNA fragments is directly related to the number of free radicals. The latter react with guanine at various positions. Among them the best studied is 8-hydroxy-deoxyguanosine (8-OHdG) [75]. The formation of this adduct is considered as an indicator of ROS implication in the carcinogenic mechanism. It has been shown that hydroxyl radicals ($^{\bullet}OH$) created by Cr(V) and Cr(IV) cause the hydroxylation of 2-deoxyguanosine (dG) and the formation of 8-OHdG [67,76].

NF-kB and AP-1 activation

Cr(VI) can cause NF-kB activation in Jurkat cells [77]. $^{\bullet}OH$ radicals produced during the Fenton-type reaction from Cr(V) and Cr(IV) play an important role in the activation mechanism of NF-kB by Cr(VI). Cr(VI) may also cause the expression of the c-myc oncogene through NF-kB activation. The ROS are used as an activation signal which initiates the activation of AP-1 and NF-kB in response to the Cr(VI) stimulus, while p38 and JNK act as executing kinases for the activation of AP-1 and NF-kB respectively.

p53 activation

The tumor-suppressor protein p53 plays an important role in protecting cells from oncogenic damage. Cr(VI) may activate p53 in human epithelial cells [60]. Superoxide dismutase which produces H_2O_2 from $O_2^{-\bullet}$, increases p53 activity [78]. Catalase, which consumes H_2O_2 limits the formation of peroxide-driven oxidants and represses p53 activation. NADPH which accelerates the one-electron reduction during transition from Cr(VI) to Cr(V) and increases the production of $^{\bullet}OH$ radicals, induces p53 activation. Consequently, the $^{\bullet}OH$ radicals produced during the reduction of Cr(VI) are responsible for p53 activation (Fig. 1).

Apoptosis

It has been proven that Cr(VI) can cause apoptosis [79]. Cr-induced apoptosis depends on the p53 state, since mutations in the p53 gene facilitate the development of resistance to apoptosis and increased survival of the damaged cells. The ROS produced by Cr(VI) play a dual role in the mechanism of carcinogenesis: they cause genetic damage and apoptosis. Cr(VI)-induced carcinogenesis depends on the balance between these two processes (Fig. 2). Apart from Cr (VI), other carcinogenic metals can cause apoptotic cell death [80,81].

Termination of the cell cycle

The cell cycle controls the initiation of DNA division and mitosis, in order to assure that the genome remains intact. Lack of fidelity during DNA division, which may arise from a mutation, can lead to cell death or cancer. It has been verified that 1) Cr(VI) can cause cell cycle arrest at the G2/M phase in human lung epithelial cells A549 [82], 2) while Cr(VI) at relatively low concentrations causes cell cycle arrest, at high concentrations it causes apoptosis and, 3) the ROS produced after cell exposure to Cr(IV) are involved in this cell cycle arrest. Hydrogen peroxide is a key molecule in this process.

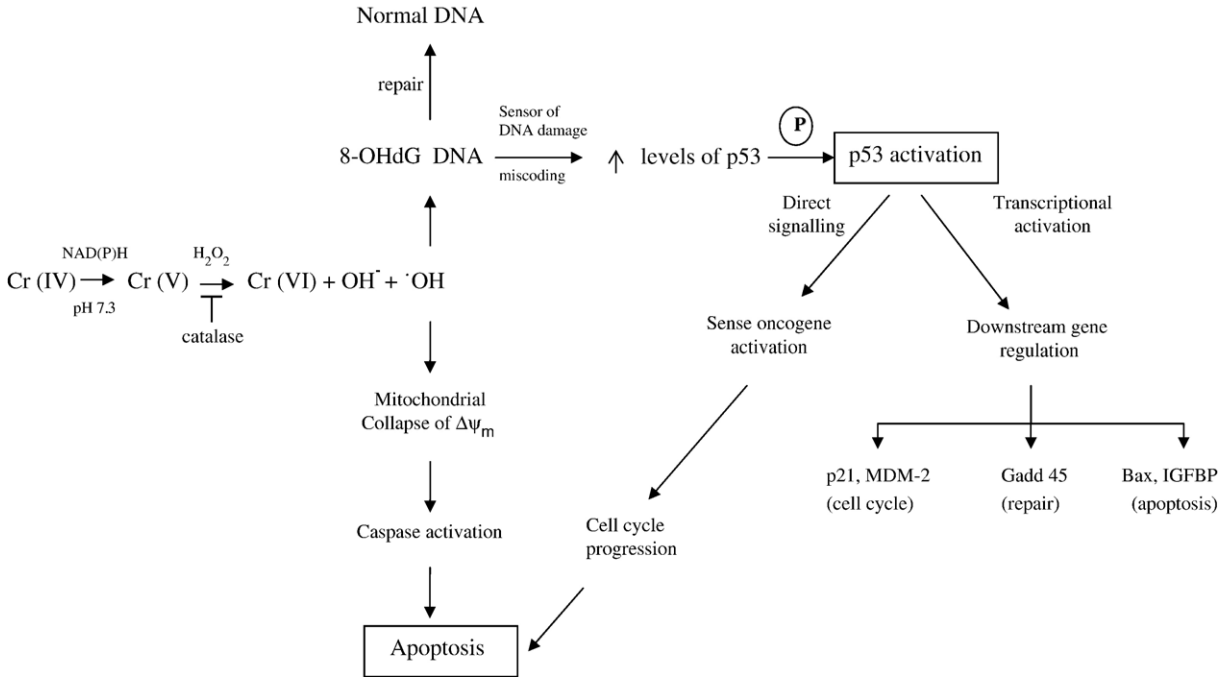

Fig. 1. Molecular carcinogenic effects of chromium. Chromium (VI) is reduced *in vitro*, at normal pH in the presence of low molecular weight cellular constituents such as NAD(P)H. The reduction of Cr(VI) creates two stable compounds, Cr(V) and Cr(IV) which react with H_2O_2 and produce hydroxyl radicals ($^{\cdot}OH$). Hydroxyl radicals induce DNA damage, recognized by a 'sensor' molecule that identifies a specific type of lesion, possibly by the p53 protein. The sensor modifies p53 by phosphorylation. A modified p53 and an allosteric change in its molecule permit DNA binding to a specific sequence regulating several downstream genes (p21, MDM2, GADD45, Bax, IGFBP). Two signaling pathways for cellular apoptosis are possible: one recruiting transcription and one inducing direct signaling with no transcription of downstream genes required.

Toxicity of cadmium

Cadmium is among the most toxic compounds in cigarette smoke. The daily Cd intake by the smoker is dose-dependent on the number of cigarettes smoked [83]. Cadmium levels in the lipoid tissue of smokers are four times higher than that of non-smokers (10 ng/g) [84]. Cadmium levels of 3.0 μg/g dry tissue have been found in the pulmonary tissue of smokers compared to 1.1 μg/g in non-smokers. This finding can be used as an indicator of pulmonary damage [85], since the life span of Cd in human lungs is calculated to be equal to 9.4 years.

Cadmium genotoxicity

The basic mechanisms involved in carcinogenesis due to exposure to cadmium are gene deregulation, oxidative stress, E-cadherin dysfunction and the inhibition of DNA repair and its contribution to apoptosis (Fig. 2).

Gene deregulation and information transfer

The deregulation of gene expression is considered to be the most important factor in a multi-stage model of chemical carcinogenesis. In particular, the induction of cellular proto-oncogenes [86] and the stimulation of cellular proliferation [87], play a very important role in the proliferation process stage, after an initial mutagenic incident.

The early and immediate response genes (IERGs) are primary oncogenes which undergo early transcriptional activation when resting cells are exposed to mitogenic compounds, such as cadmium. They code for transcriptional factors and play an important role in chemical carcinogenesis. The products of IERGs constitute mitogenic growth signals that stimulate the proliferation of cells and they are considered to be important factors in a multi-stage carcinogenic model [86]. Cell exposure to cadmium induces the expression of many "stress" genes, such as the genes that code for metallothioneine for heat-stress proteins (HSPs), those that participate in oxidative stress response or those that participate in glutathione synthesis (GSH). Inside the cell, cadmium induces the production of denatured or pathologic proteins by reacting with adjacent thiolic groups or by substituting zinc (Zn) in protein molecules that contain zinc (HSP induction signal) [88]. Cell exposure to cadmium results in the significant induction of genes HSP10, HSP32, HSP40, HSP60, HSP70, HSP89, HSP90 and HSP110.

Genes that control glutathione and thiol proteins

Glutathione and other proteins that contain thiol groups are key players in cellular defense against cadmium toxicity and carcinogenicity. The ionic cadmium (Cd^{2+}), which is considered responsible for its toxicity and carcinogenicity, is eliminated by glutathione and thus its reaction with important cellular targets is prevented. The GSH reducing cycle which induces glutathione peroxidase and glutathione reductase renders cadmium-induced ROS atoxic. Cell exposure to cadmium induces the genes for γ-glutamino-cysteine synthase (γ-GCS), glutathione-S-transferases (GST-α and GST-π) as well as increased glutathione production, which all quickly and effectively eliminate Cd^{2+} toxicity [89,90]. Frequent exposure to cadmium, however, may overcome the beneficial effects of

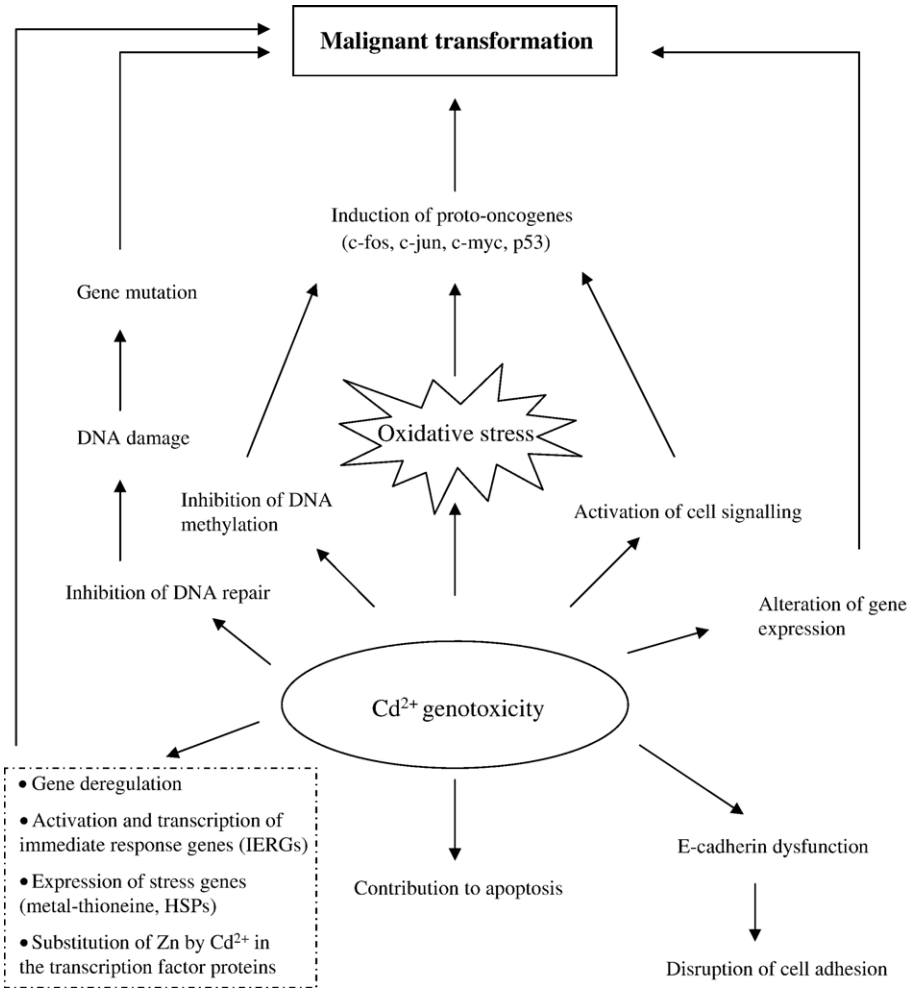

Fig. 2. Molecular carcinogenic effects of cadmium. Immediate biochemical effects of cadmium involve inhibition of DNA methylation, activation of cell signaling, E-cadherin dysfunction, alteration of gene expression and participation in the malignant transformation processes, such as gene deregulation, activation and transcription of immediate response genes (IERGs), as well as substitution of Zn by Cd^{2+} in the transcription factor proteins. Cadmium may also contribute to apoptosis, thus diminishing the number of cells committed to neoplastic transformation; this, however, may give rise to a cadmium-adapted cell fraction in order to escape death.

glutathione and related defense mechanisms and cause toxicity and carcinogenicity [91]. In general, the expression of antioxidant genes such as those that code for superoxide dismutase and catalase, is suppressed by cadmium [92–94].

Suggested genotoxicity mechanism

Cadmium activates the expression of many proto-oncogenes, including c-fos, c-jun and c-myc as well as the tumor suppressor gene, p53 [95–101]. Today, it is believed that the mechanism of cadmium action is indirect, possibly acting through metalloproteins [102], or through the substitution of Zn^{2+} by cadmium in transcription factor proteins. Another suggested mechanism is the induction of proto-oncogenes through the mobilization of intracellular calcium [103,104]. In human cancer cells, gene shifting is observed. The shifting mechanism remains unknown. It is supposed that the shifting creates a transformed phenotype through the expression of the corresponding gene. Both c-myc and c-jun RNA are cell nucleus transcription factors necessary for cell transition from the rest stage (G0) to the proliferation state (G1), and consequently, their overexpression alters the cell cycle.

Mechanisms which alter gene expression

Many mechanisms that involve secondary messengers such as the ROS and the intracellular Ca^{2+} are considered to be responsible for cadmium-induced deregulation of gene expression. Cadmium exposure results in increased intracellular Ca^{2+} levels [104,105]. Ca^{2+} directly deregulates gene expression by reacting with specific response elements such as the CREB factor (c-AMP-response element binding protein) found in the promoter region of these genes [106]. Alternatively, cadmium effects can be mediated indirectly through the activation of protein kinases which cause overexpression by phosphorylation of the various transcription factors [107]. In addition, cadmium activates calmodulin-dependent target genes by its ability to mimic calcium ions [108]. The kinases that are activated during the cell's exposure to cadmium include: protein kinase C [109–111], stress-activated protein kinase [112], tyrosine kinase, casein kinase-II [113] and three more kinase types that are activated by the mitogens of the mPK family, such as the protein kinase that is activated by extracellular ERK signals, JNK and p38 MAP kinase [114,115].

Disruption of E-cadherin-mediated cell adhesion

According to Pearson and Prozialesk [116], cadmium carcinogenesis involves E-cadherin, a molecule that regulates cellular permeability and polarity [117]. E-cadherin is a Ca^{2+}-associated transmembranic glycoprotein which participates in calcium-dependent cell-to-cell adhesion. The intracellular part of the E-cadherin molecule is bound to the actin of the cell skeleton through molecules called catenins. The extracellular part of E-cadherin has Ca^{2+} binding sites as well as an area through which the molecule adheres to the adjacent cell. Cd^{2+} linking to E-cadherin reduces the flexibility of the protein molecule and limits the space available for uniform cell-to-cell adhesion [118]. The disassociation of cell adhesion through E-cadherin signals the activation of genes through β-catenin, which functions at the early stages of tumor initiation. β-catenin modifies the expression of many genes, including c-myc and c-jun.

Cadmium and apoptosis

Studies have shown that the exposure of primary lung epithelial cells to cadmium induces the anti-apoptotic protein Bax [119], suggesting that cadmium is capable of triggering apoptosis via a mitochondrial-dependent pathway.

Suppression of DNA repair by cadmium

The inhibition of DNA repair has been suggested as a mechanism that contributes to cadmium genotoxicity [120]. Low cadmium concentrations suppress base excision DNA repair [121] and the binding of the repair proteins to the DNA, while this suppression is reversed by addition of Zn. Cadmium reduces the cell's ability to repair the mis-pairing dGTP oxidation yields of 8-oxo-dGTP which are mistakenly incorporated into the DNA and cause AT → CG transitions. The cells are protected from 8-oxo-dGTP by 8-oxo-dGTPases. The 8-oxo-dGTPases are inhibited by cadmium, thus providing an additional mechanism which contributes to the mutagenic and carcinogenic potential of this metal [122].

ROS and cellular antioxidant system

The carcinogenic action of cadmium is related to the production of the ROS. Cadmium produces hydroxyl radicals [123], superoxide radicals, nitric oxide and hydrogen peroxide [124,125]. It reduces intracellular glutathione and significantly inhibits the activities of superoxide dismutase, glutathione peroxidase and catalase [126].

Toxicity of Nickel

The amount of nickel that the tobacco plant absorbs form the ground is quite significant. As a result, tobacco leaves contain large amounts of nickel [127], with 0.64 and 1.15 μg/g per dry leaf. During smoking, tobacco smoke contains approximately 75 ng per cigarette [127]. Other studies have shown a higher Ni content in inhaled smoke. Epidemiological studies, as well as experiments in rats, have shown that the exposure of humans and laboratory animals to an environment with nickel-containing particles results in lung damage [128–130]. Following inhalation of these particles, the accumulation of neutrophilic granulocytes increased protein concentration [131], as well as prostaglandin and cytokine production. The response to these processes is the development of oxidative stress [132,133]. Nickel (as well as other metals), catalyzes the formation of the ROS that cause lipid peroxidation. Carbonyl compounds, one of the lipid peroxidation byproducts, are produced from the lipids [134] and proteins [135] of the cell membrane. Some carbonyl compounds such as acetaldehyde (CH_3CHO), participate as mediators to biological response (which is the prostaglandin synthesis from the airway epithelial cells) [136]. It has been shown that histones in the cell nucleus are targets for Ni(II) ions. Nickel (II) binds histones, inducing sequence-specific histone hydrolysis and the resultant complex mediates oxidative damage to the nuclear DNA [137].

Pre-neoplasia: a question of the first magnitude

The results of several studies have shown that cigarette smoking is associated with the development of pre-neoplastic changes in the human lung [138] and lung cancer [139]. However, only a small number of the pre-neoplastic lesions progresses to invasive cancer whereas the majority may remain stationary or even regress [140]. Little is known about the biological and molecular genetic events responsible for these pre-neoplasias [141]. There is increasing evidence that the progressive accumulation of multiple genetic changes underlies the multi-step nature of tumorigenesis [140]. Wistuba *et al* [142] suggest that "the development of epithelial cancers requires multiple mutations and stepwise accumulation, which may represent a mutator phenotype". Thus, it is possible that those pre-neoplastic lesions that have accumulated multiple mutations are at higher risk for progression to invasive cancer [142]. In addition, the same authors have detected allele-specific mutations in smoking-related damaged epithelium; the mechanism by which this phenomenon occurs is unknown.

Pre-neoplasia can be identified in the bronchial epithelium which appears to be morphologically normal in smokers [143,144]. This phenomenon is possibly due to the increased survival of the epithelial cells because of increased resistance to apoptosis [145,146]. Such genetic changes are not found in the bronchial epithelium of lifetime non-smokers [144]. These premalignant alterations are often extensive and focalized and they occur throughout the whole extent of the respiratory tract. This is of extreme importance since it appears to be related to the development mechanisms of lung cancer in smokers; this is referred to as field cancerization [147]. Berenblum referred to "two sequential stages of carcinogenesis: the initiation stage, which is completed within a short time, and the promoter action stage which develops slowly, requiring protracted contact with the carcinogen" [148]. While the "two sequential stages of carcinogenesis" is a hypothesis, we could assume that a protracted contact can be assured only when there is, for example, a steady source of ROS production (i.e. metals such as cadmium, chromium, nickel in a certain area of the lung tissue). Some authors estimate that "although the dose of each carcinogen per cigarette is quite small, the cumulative dose in

a lifetime of smoking is substantial" [139]. It appears that a cumulative dose can be also assured only when there is a steady source of carcinogens in some areas of the lung and when other quantities of carcinogenic compounds such as metals, together create the critical mass which in turn is capable of triggering the carcinogenic process.

The rationale behind the creation of the critical mass

The rationale behind the creation of the critical mass of carcinogenic compounds which is necessary for the carcinogenic process to proceed, has become apparent in numerous pioneer studies over the last fifty years. The aim of these studies was to identify the carcinogenic properties of certain components of tobacco smoke, such as the PAHs, especially benzo[a] pyrene, as well as some other compounds in the solid and gaseous phases of tobacco smoke.

The mechanisms by which lung carcinogenesis was extensively studied in the past were based on *in vivo* experiments, such as intratracheal instillation or implantation after thoracotomy of the carcinogenic material [149–156], or on *in vitro* experiments based on cultured bronchoepithelial cells exposed to carcinogens present in mainstream tobacco smoke (performed through a puffing mechanism to generate tobacco smoke-bubbled phosphate buffered saline extracts or condensates) [157–166]. These exposure methodologies which were applied in order to bring the bronchoepithelial cells in contact with the carcinogenic material, gave rise to conditions which were not relevant to exposure in humans. However, it is worth noting that the comparison between laboratory processes used to expose cells to the carcinogenic material of cigarette smoke and normal smoking conditions, is of paramount importance. In the laboratory process, there is the application of a large quantity of carcinogenic substances (metals, PAHs) in a certain area of the lung tissue of laboratory animals, and the addition of a large quantity of suspensions of tar particles and tobacco smoke condensate in cell cultures or in test tubes. This creates a direct, local, appropriate critical mass of carcinogenic substances, so that, on the one hand, the defense mechanisms of the cells are depleted in a short time period and on the other hand, the repair mechanisms of cell DNA cannot respond to the increased demands which have been created. Such cumulative conditions with large quantities of carcinogenic substances in a part of the lung tissue could occur only with exceptional difficulty during normal smoking. It is, however, possible to amass a great quantity of carcinogenic material in the respiratory tract when special conditions of stagnation of the mucous are created, due to destruction of the pulmonary defense mechanisms by the gaseous phase of cigarette smoke and the concentration of carcinogenic substances in the mucous due to continued smoking. The opinion that it is extremely important that there should be a concentration of a specific quantity of carcinogenic substances (critical mass) in a certain area of the lung in order to create appropriate circumstances for the development of cancer, indirectly, but clearly supports the excellent in vitro study of Yoshie and Oshima [164]. These researchers demonstrated that low concentrations of Cu^{2+} or Fe^{2+} do not result in the breakage of DNA by the components of the tar, whereas high concentrations do. When there is no tar or tobacco smoke condensate, high concentrations of metals only do not produce breakage of the DNA strand [164]. Metals are insufficient in order to begin the process of mutation followed by carcinogenesis, but the presence of tar is necessary.

Speculation and future directions

The following questions remain to be answered: Why do some premalignant lesions progress to invasive cancer, while others remain unchanged for a long period of time? What are the additional genetic changes required for the development of an invasive cancer? In addition, i) why does only a very small percentage (<20%) of heavy smokers get lung cancer, while their lungs in all cases show pre-neoplastic alterations in the DNA? ii) why do so many years (20–40) have to go by before cancer develops in smokers? [167], and iii) how do the same alterations exist in the healthy cell area of the lungs of a cancer patient as well as in a heavy smoker who, however, does not have cancer?

Along with the knowledge that metals may cause lung cancer when inhaled in cigarette smoke, we have come to understand that the answers to the above questions can be based on some logical assumptions. One serious assumption would perhaps be the fact that the necessary critical mass of carcinogenic metals does not develop in some area of the lungs of the smokers by chance. Additionally, as it is often reported in the literature, it is highly possible that both the defensive mechanisms of the cells and the rate of repairing the DNA damage are more active whereas in heavy smokers who get lung cancer, the repair systems are not efficient enough. But even then, why does cancer only develop at one site of the lung tissue, when most of the lung epithelial cells have already undergone mutational changes which are widespread in all regions of the bronchial epithelium of heavy smokers?

Progress in this area could be made if we could follow step-by-step development of all pre-neoplastic mutations of the DNA for 10–20 years in order to measure the number of mutations over a long time period. It is already known that in each cell, and everyday, a steady state of human DNA lesions occurs, by internal oxidation (ROS) in the cell (130.000 bases in nuclear DNA and 8.000 in mitochondrial DNA) which are continuously repaired [168–174]. Knowing this, one would expect to see perhaps a yearly evolving number of mutations of multiple sizes which peak at the final aim, which is cancer. Today, it is believed that mutational alterations in smokers do not remain steady, but increase progressively with time [139]. Both the duration and the extent of the increase in mutations have, however yet to be understood.

We assume that the inhaled metals are not biodegradable and for this reason they are deposited in different areas of the lung interstitium, fatty tissue and lung epithelial cells and they stay there. For example, cadmium, which is thought to be one of the most genotoxic metals in cigarette smoke [42,175–180], is deposited in the lung tissue where it remains for a long time (a half life cycle of 10 years) [181,182]. The level of cadmium in

the lung fatty tissue of smokers is four times higher than that in non-smokers (10 μg/g). Cadmium was also increased up to 3.0 μg/g in the dry lung tissue of smokers, versus 1.1 μg/g in non-smokers. Also, the corresponding values for chromium were 4.3 μg/g dry wt lung tissue for smokers, versus 0.4 μg/g dry wt lung tissue of non-smokers [181]. Furthermore, the insoluble particles of nickel provide a continuous source of Ni (II) ions, which induce cancer, in a process that takes many years [182–184]. We suggest that cancer still occurs when, in a certain part of the lung, there is accidental (random) concentration of critical quantities of carcinogenic substances, especially metals [140]. The creation of a critical mass of metals in some area of the lung tissue will have, as a result, the production of strong oxidants through metal catalysis [182,185] and the surrounding biomolecules will be attacked [186–188].

It appears that the interspersing of carcinogenic substances in the lung tissue happens by chance and the future (by chance) accumulation of these substances in a certain part of the lung tissue could create damage to genomic DNA which the cells will no longer be able to repair.

Summary and conclusion

The normal bronchial epithelium that lines the airways of the lungs provides a barrier to the external environment by using the pulmonary clearance system which involves the cilia of the bronchoepithelial cells and the mucous blanket which covers the cilia. Normally, the tar particles in tobacco smoke are deposited on the mucociliary escalator and are continually expelled outwards by the movement of the cilia. The cytotoxic potential of the gaseous phase of tobacco smoke paralyses this function and finally destroys the structure of the cilia. During the initial stages, the lung epithelial cells are exposed to low concentrations of carcinogens (metals) which are dispersed in a myriad of small particles throughout the lung tissue and deposited in the lung interstitium and fatty tissue. The lung epithelial cells are then exposed to low concentrations of carcinogens (metals). The intracellular redox status of the epithelial cells can regulate their antioxidants by shifting the oxidation to a reducing condition [189]. Also, the expression of a great number of DNA repair enzymes is upregulated [190]. Oxidized bases are continually repaired by single nucleotide repair mechanisms (90%) and by long-patch base excision repair (10%).

In the long term, when the cilia are destroyed by the gaseous phase of cigarette smoke, the mucous becomes stagnant in the interior of the bronchial tree, enriched by the tar particles (as long as the smoker continues to smoke), causing the obstruction of the small airways. Then, an appropriate critical mass of carcinogenic material (metals) is created by chance in a certain area of the lung tissue. The metal-induced catalysis of the formation of strong oxidants at this site of the tissue exceeds the protective (antioxidative) and DNA-repairing capacities of the cells; this ensures the progressive accumulation of multiple genetic changes which underline the multi-step nature of carcinogenesis and facilitate an appropriate environment for causing cancer.

In conclusion, carcinogenesis in the lungs of heavy smokers is mainly due to the oxidative process caused by metals. The tar particles of CS play a critical role in this process. Further studies are required in order to investigate 1) the course of pre-neoplastic lesions progressing to invasive cancer and 2) the biological and molecular genetic events responsible for these pre-neoplasias. Progress in this area could be made if we could follow the step-by-step development of mutations in the nuclear and mitochondrial DNA over a long period of time focusing on the mechanisms related to resistance to apoptosis and on the increased survival of a small population (15–25%) of genetically damaged cells which manage to avert apoptosis.

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