NITRIC OXIDE SYNTHASES AND REACTIVE OXYGEN SPECIES DAMAGE IN PLEURAL AND LUNG TISSUES AND NEOPLASIA

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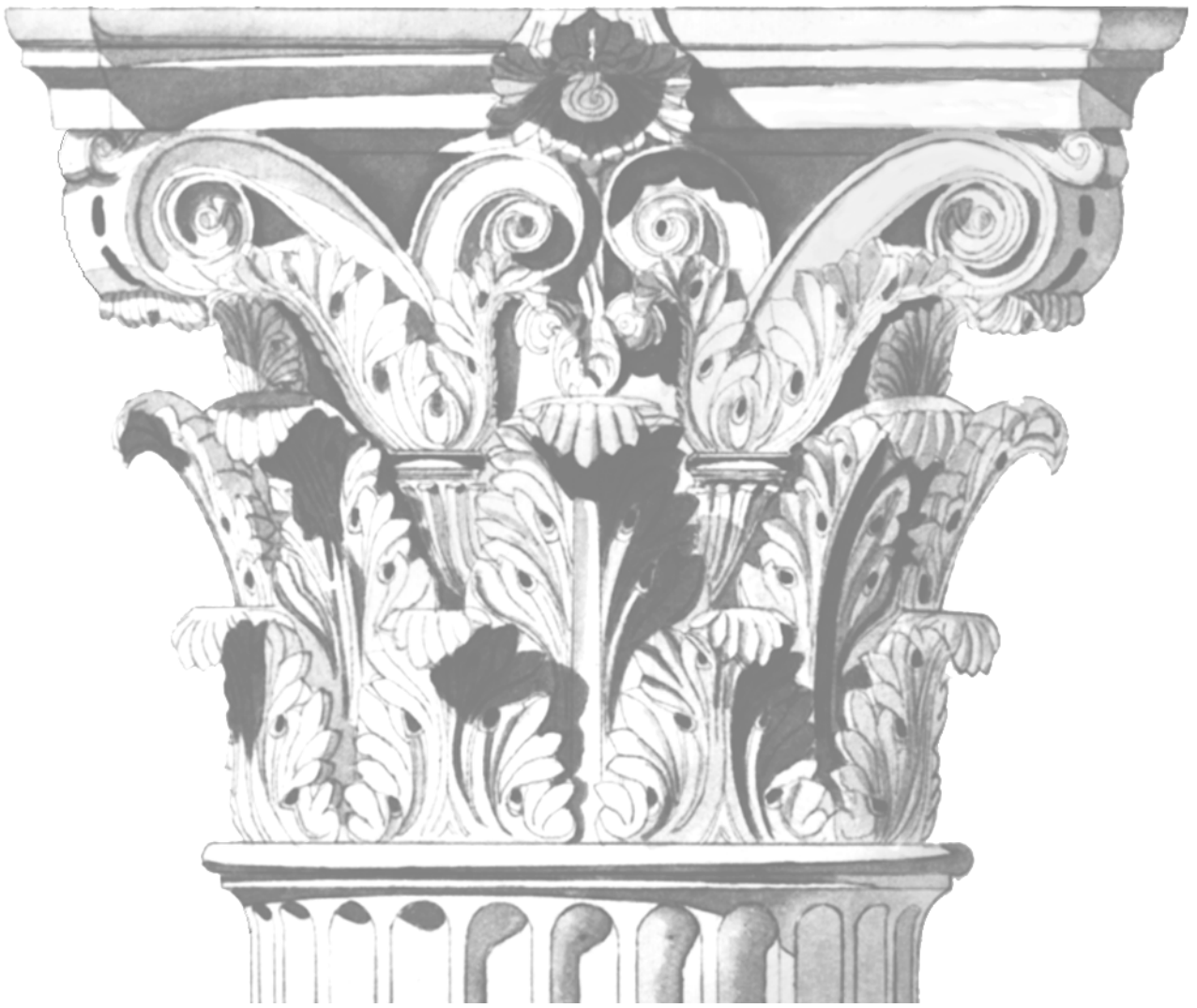

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Abstract

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) have been linked with the pathogenesis of lung malignancies and chronic obstructive pulmonary disease (COPD). *In vitro* studies indicated that mesothelioma and lung carcinoma cell lines synthesize nitric oxide synthases (NOS) mRNA. The Comet-assay indicated that asbestos fibers caused DNA single -strand breaks in mesothelial cells, and this effect was enhanced by glutathione depletion. The use of FPG in the Comet assay indicated that the asbestos induced DNA strand breaks were oxidant mediated.

In vivo non-neoplastic pleura was mostly negative for inducible NOS (iNOS), while inflamed pleura was positive. The immunohistochemical expression of iNOS was detected in 74% and 96% of malignant mesotheliomas and metastatic pleural adenocarcinomas, respectively. Epithelial and mixed mesotheliomas expressed more often intense iNOS immunoreactivity compared to the sarcomatoid subtype.

Normal mesothelial cells showed occasional positivity for endothelial NOS (eNOS), but reactive mesothelial cells were strongly stained. eNOS was found in 89% of mesotheliomas. Vascular endothelial growth factor (VEGF) was identified in 47%, a VEGF receptor FLK1 in 69% and the VEGF receptor, FLT1, in 71% of mesotheliomas. FLK1 or FLT1 immunoreactivities were more often seen in epithelioid and biphasic mesotheliomas than in sarcomatoid mesotheliomas.

In lung samples of non-smokers, smokers and COPD patients, the levels of nitrotyrosine were higher in alveolar macrophages of smokers and COPD patients than in the non-smokers and in the alveolar epithelium of smokers and COPD patients than in the non-smokers. The iNOS expression was weak in the bronchial and alveolar epithelium in all groups but eNOS was most prominently expressed in alveolar macrophages while neuronal NOS (nNOS) was negative in all of the major cell types of the lung. Bronchial metaplasia-dysplasia-sequence was clearly positive for iNOS, nNOS and nitrotyrosine. Thus, smoking can cause protein nitration also in normal lung. Prominent iNOS and nNOS immunoreactivity in metaplasia-dysplasia-lesions suggests a divergent role of NOSs in carcinogenesis and destruction of alveolar epithelium in emphysematous lung.

In lung cancer samples, iNOS was detected in 40% cases, while 89% and 81% cases were positive for eNOS and nNOS, respectively. Intense eNOS staining was seen more often in adenocarcinomas than in squamous cells carcinomas, and iNOS immunoreactivity was seen more often in grade I-II tumors than in grade III tumors. The patients with tumors showing high expression of iNOS, eNOS and nNOS, exhibited better survival, but this was not an independent prognostic factor.

Keywords: chronic obstructive pulmonary disease, mesothelioma, nitric–oxide synthase, non-small-cell lung cancer

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Abbreviations

Ab	Antibody
AM	Alveolar macrophages
AP	apurinic/apyrimic
BAL	Bronchoalveolar lavage
BSO	Buthionine sulfoximine
cGMP	Cyclic guanosine 3', 5'-monophosphate
cNOS	Constitutive nitric oxide synthases
COPD	Chronic obstructive pulmonary disease
COX2	Cyclooxygenase 2
DCDHF-DA	2'7'-dichlorodihydrofluorescein-diacetate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLK1	Vascular endothelial growth factor receptor FLK1
FLT1	Vascular endothelial growth factor receptor FLT1
FLT4	Vascular endothelial growth factor receptor FLT4
FVIII	Factor VIII
FPG	Formamidopyrimidine glycosylase
γ-GCS	gamma-glutamylcysteine synthase
γ-GCSh	gamma -glutamylcysteine synthase heavy subunit
γ-GCSl	gamma -glutamylcysteine synthase light subunit
GPx	Glutathione peroxidase
GRO-α	Growth related oncogene alpha
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HIF	Hypoxia inducible factor
HOCL	Hypochlorous acid
HPF	High power field
IL	Interleukin

iNANC	Inhibitory non-adrenergic non-cholinergic
iNOS	Inducible nitric oxide synthase
L-NAME	L-arginine-methyl ester
LTB_4	Leucotriene-B_4
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NADPH	Reduced nicotinamide dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOSs	Nitric oxide synthases
NSCLC	Non-small cell lung cancer
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide anion
$^1O_2$	Singlet oxygen
$OH^{\bullet}$	Hydroxyl radical
8-OHgua	8-hydroxyguanosine
$ONOO^{\bullet}$	Peroxynitrite
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PDE	Phosphodiesterase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SCC	Squamous cell carcinoma
SCLC	Small cell lung carcinoma
SOD	Superoxide dismutase
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TUNEL	3'-end labeling of fragmented DNA
VEGF	Vascular endothelial growth factor
XTT	The microculture tetrazolium dye colorimetric assay

List of original articles

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I Puhakka A, Ollikainen T, Soini Y, Kahlos K, Säily M, Koistinen P, Pääkkö P, Linnainmaa K and Kinnula VL (2002) Modulation of DNA single-strand breaks by intracellular glutathione in human lung cells exposed to asbestos fibers. Mutat Res 514:7-17.

II Soini Y, Kahlos K, Puhakka A, Lakari E, Säily M, Pääkkö P and Kinnula VL (2000) Expression of inducible nitric oxide synthase in healthy pleura and in malignant mesothelioma. Br J Cancer 83:880-6.

III Soini Y, Kahlos K, Puhakka A, Lakari E, Säily M, Pääkkö P and Kinnula VL (2000) Endothelial nitric oxide synthase is strongly expressed in malignant mesothelioma but does not associate with vascular density or the expression of VEGF, FLK1 or FLT1. Histopathology 39:179-86.

IV Puhakka A, Harju T, Pääkkö P, Soini Y and Kinnula VL. Nitric oxide synthases are associated with bronchial dysplasia. Submitted.

V Puhakka A, Kinnula V, Näpänkangas U, Säily M, Koistinen P, Pääkkö P and Soini Y (2003) High expression of nitric oxide synthases is a favorable prognostic sign in non-small cell lung carcinoma. APMIS 111:1137-46.

Contents

1 Introduction

Oxygen is an essential element of life. During normal aerobic cell metabolism, oxygen is reduced to water, and as a byproduct of this reaction, endogenous reactive oxygen species (ROS) are generated (Halliwell & Gutteridge 1989). ROS include species such as singlet oxygen, superoxide anion, hydrogen peroxide, hydroxyl radical and nitric oxide. Although these compounds are potentially toxic to all aerobic organisms, they are nonetheless crucial for many physiologic processes (Dalton *et al.* 1999). Exogenous ROS are provided for example in ambient air, pollutants and cigarette smoke.

There are several nonenzymatic and enzymatic systems in the body that can scavenge free radicals. Nonenzymatic systems include endogenous or exogenous antioxidants, for example tocopherols, ascorbate, retinoic acid, sulfhydryl-containing compounds, such as cysteine and glutathione; and serum proteins, such as albumin, ceruloplasmin and transferrin. The antioxidant enzymes include superoxide dismutases, catalase and glutathione peroxidases (Halliwell & Gutteridge 1989). If the production of ROS exceeds the antioxidative capacity, oxidative stress occurs.

The lungs are a vital organ; they have one cardinal function, the exchange of gases between inspired air and the blood. By virtue of their continuous contact with the environment via inhaled air, compared with other organs the lungs are exposed to a higher amount of microbes and particles, and also higher oxygen pressure. Thus the lungs are at increased risk of oxidative stress and inflammatory processes.

Cigarette smoke is the main causative agent of lung cancer and chronic obstructive pulmonary disease (COPD), two disease groups responsible for substantial morbidity and mortality worldwide (American Thoracic Society 1995, Tyczynski 2003). The reason why not all smokers develop these diseases is still a matter of intense debate (Bascom 1991, Simonato *et al.* 2001, Siafakas *et al.* 1995).

COPD is an obstructive lung disease characterized by chronic bronchitis, irreversible bronchial obstruction and destruction of alveolar walls leading to emphysema. Lung cancer is a malignancy which arises mainly from the cells of the bronchial tree (Cotran *et al.* 1994). Up to 95% of lung tumors are carcinomas (Cotran *et al.* 1994). About 2-5% of lung tumors are of mesenchymal origin. Malignant mesothelioma arises from either visceral or parietal pleura, and in most cases its development is associated with an

occupational asbestos exposure. Common features to all these diseases are poor treatment results and poor overall prognosis.

Since the discovery that endothelium derived relaxing factor was nitric oxide in 1987 (Ignarro *et al.* 1987, Palmer *et al.* 1987), a huge amount of research has revealed that NO acts as a mediator in a wide variety of physiologic and pathophysiologic conditions such as inflammatory diseases and cancer (Moncada *et al.* 1991, Wink *et al.* 1998, Davis *et al.* 2001). NO is produced by three nitric oxide synthase (NOS) enzymes. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed, while inducible NOS (iNOS) expression is enhanced by various inflammatory cytokines (Nathan & Xie 1994). The excessive production of NO by iNOS acts as a host defense, but it may lead to direct tissue injury or cause damage via formation of reactive nitrogen species (RNS). NO also up-regulates the synthesis of vascular endothelial growth factor (VEGF), an important endothelial cell mitogen needed during tumor angiogenesis to support tumor progression. ROS and RNS are known to be associated with the pathogenesis of many respiratory diseases including asthma, COPD, parenchymal diseases and malignancies (Kharitonov *et al.* 1994, Rahman & MacNee 1996, Saleh *et al.* 1997, Fujimoto *et al.* 1997, Fujimoto *et al.* 1998, Ambs *et al.* 1998 a, b & c, Kumar-Singh *et al.* 1999, Marrogi *et al.* 2000 a & b, Lakari *et al.* 2002, Maestrelli *et al.* 2003). Nonetheless, many questions still remain, e.g. little is known on the systematic expression of all three NOS subtypes in pulmonary malignancies and their association with prognosis.

The present study was performed to investigate the expression of nitric oxide synthases, markers of tissue injury and tumor angiogenesis associated factors in bronchial precancerous lesions, pulmonary malignancies and COPD. Also the cellular mechanisms of ROS/RNS mediated lung cell injury caused by asbestos were investigated in bronchial and mesothelial cells and pleural tissue samples.

2 Review of the literature

2.1 Reactive oxygen species and antioxidants

A free radical is by definition an atom or a molecule which contains an unpaired electron in its outer orbit (Halliwell & Gutteridge 1989). Radicals are therefore highly reactive and can consequently cause harmful effects on cell structures. Free radicals and other oxygen-related reactive compounds, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) are collectively termed reactive oxygen species (ROS) (Halliwell & Gutteridge 1989). Although $^1O_2$ and H_2O_2 are not radicals, the oxidizing ability of $^1O_2$ is increased due to its altered spin, whereas H_2O_2 can form ROS in the presence of transition metals (Fenton reaction). Oxides of nitrogen, i.e. nitric oxide (NO) and nitrogen dioxide (NO_2) molecules fall into the definition of free radicals, since they possess odd numbers of electrons (Halliwell & Gutteridge 1989).

Reactive oxygen species are generated during normal aerobic metabolism in the reaction where molecular oxygen (O_2) is reduced to water (H_2O) (Fig. 1). The intermediates of this reaction are superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide H_2O_2 and hydroxyl radical $OH^{\bullet}$. The most reactive and harmful form of these oxygen radicals is the hydroxyl radical (Halliwell & Gutteridge 1989).

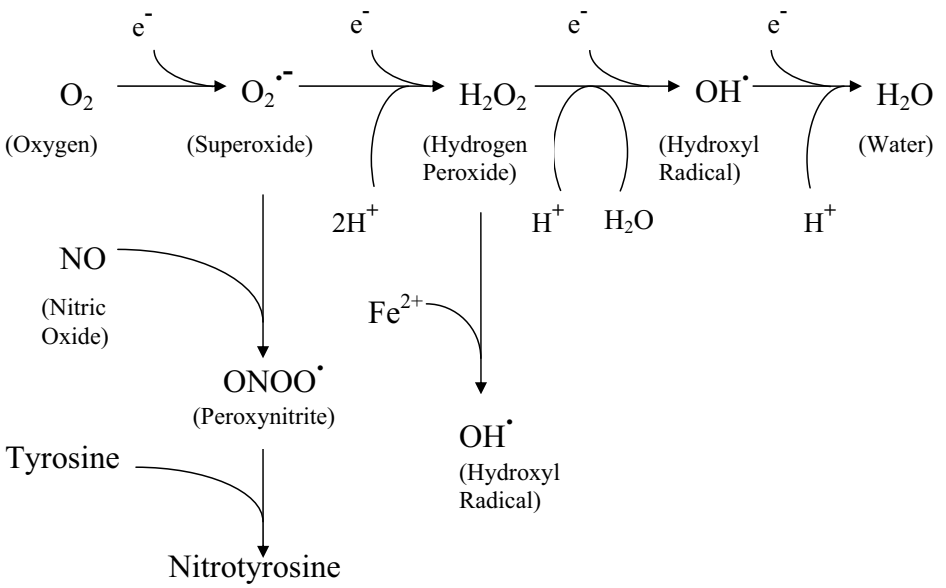

Fig. 1. The formation of reactive oxygen species and nitric oxide chemistry. Modified from Halliwell & Gutteridge 1989 and Davis *et al.* 2001.

The cells may be exposed both to endogenous and exogenous ROS. Inside cells, ROS are formed as by-products of normal cellular metabolism. Stimulated production of ROS by the enzyme NADPH-oxidase, i.e. "the respiratory burst", in neutrophils and macrophages is essential for their bactericidal action (Babior 1978). These cells use also the myeloperoxidase (MPO) H_2O_2/Cl^- system to generate hypochlorous acid (HOCl) for use in the host defense (Halliwell & Gutteridge 1989). At low concentrations, HOCl rapidly inactivates α_1-antitrypsin, the main inhibitor of proteolytic enzymes (Halliwell & Gutterige 1989).

Most of the ROS produced in the lung tissue comes from neutrophils, alveolar macrophages and eosinophils, but also bronchial and alveolar epithelial cells and endothelial cells are capable of producing ROS (Kinnula *et al.* 1995a). Exogenous exposure to ROS/reactive nitrogen species (RNS) in lung cells occurs for example via inhalation of tobacco smoke and pollutants. Exogenous ROS in turn can enhance the formation of intracellular ROS (Hoidal *et al.* 1981, Bridges *el al* 1985). In addition to phagocyte-derived oxidants, also oxidants carried by tobacco smoke, such as NO and peroxynitrite, are able to inactivate the α_1-antitrypsin (Pryor & Stone 1993).

The harmful and toxic effects of ROS are now well known. These include the capacity of ROS to cause damage to lipids, proteins and DNA (Freeman & Crapo 1982, Hensley *et al.* 2000). In recent years, the role of ROS in normal cellular physiology has become a focus of interest. Low levels of ROS act in signal transduction, are intracellular messengers during cell growth and differentiation, and are regulators of gene expression

(Dalton *et al.* 1999, Finkel *et al.* 2000, Hensley *et al.* 2000, Sauer *et al.* 2001, Thannickal *et al.* 2000, Forman *et al.* 2002).

Elimination of excessive ROS is critical for cell survival and an imbalance between low antioxidative capacity and excessive ROS production can lead to oxidative stress (Thannickal *et al.* 2000) and predispose a cell to oxidative damage. There are several antioxidant enzymes present in the cells which can scavenge ROS. Superoxide dismutase (SOD) converts $O_2^{\cdot-}$ to H_2O_2, which in turn is removed by catalase, glutathione (GSH) and glutathione peroxidase (GPx) (Halliwell & Gutteridge 1989). In addition, GSH is a scavenger of hydroxyl radicals and singlet oxygen. The rate limiting enzyme of GSH synthesis is γ-glutamylcysteine synthase (γ-GCS). The γ-GCS protein consists of two subunits, i.e. heavy (γ-GCSh) and light (γ-GCSl) chains. Both subunits are induced by inflammatory cytokines such as TNF-α, oxidants and cigarette smoke (Rahman & MacNee 2000a & b). In addition to enzymes, some vitamins, small molecules such as uric acid and proteins can act as antioxidants (Halliwell & Gutteridge 1989, Dalton *et al.* 1999, Hensley *et al.* 2000). Vitamins harboring antioxidant capacity include ascorbic acid, α-tocopherol and retinoic acid (vitamin C, E and A, respectively). Binding the transition metals by proteins such as ferritin and transferrin is a part of extracellular antioxidant defense. Intracellularly, metal ions such as Zn^{2+}, Cu^{+}, Cd^{2+}, and Hg^{2+} are stored in a non-toxic form by metallothionein proteins (Halliwell & Gutterige 1989).

2.2 Nitric oxide synthases and chemistry of NO

NO is a free radical gas which has a major role as a signaling molecule in a variety of physiological effects. Cellular production of NO from L-arginine is catalyzed by three nitric oxide synthase (NOS) enzymes; inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS), encoded by distinct genes (Nathan & Xie 1994). Two of the NOS enzymes, eNOS and nNOS, are constitutively expressed and their activities are regulated by intracellular calcium concentrations via calmodulin (Davis *et al.* 2001). Expression of iNOS is enhanced by immunological or inflammatory stimuli including endotoxins and proinflammatory cytokines such as bacterial lipopolysaccharide, TNF-α IL-1 and IFN-γ (Nathan & Xie 1994). The inducible enzyme form, iNOS is not Ca^{2+}-dependent, and it generates a large amount of NO for a prolonged time compared with the constitutive NOSs (cNOS) (Moncada *et al.* 1991, Al-Ali *et al.* 1998).

NO has many functions; it acts as a regulator of vascular tone, as an inhibitor of platelet adhesion, as an effector molecule in immunological reactions and as a neurotransmitter (Ignarro *et al.* 1987, Palmer *et al.* 1987, Moncada *et al.* 1991). NO is involved in the in pathophysiology of several disease groups, such as inflammatory diseases, diabetes and cancer (Davis *et al.* 2001). The role of NO in carcinogenesis is controversial, as NO has both anti-tumor and tumor promotive properties (reviewed in Wink *et al.* 1998 and in Ricciardolo *et al.* 2004). NO derived from macrophages, natural killer cells, and endothelial cells participate in tumoricidal activities against many tumors. This can be mediated by inhibition of tumor cell respiration and/or the induction of apoptosis within the growing tumor (reviewed in Wink *et al.* 1998 and in Ricciardolo *et*

al. 2004). On the other hand, tumor –promoting effects of NO include participation in the initiation of cancer by causing neoplastic transformation (reviewed in Wink *et al.* 1998 and in Ricciardolo *et al.* 2004). NO has also increased a metastatic capacity in tumor cells (Edwards *et al.* 1996). In the experimental setting, iNOS deficient mice developed fewer lung tumors than wild-type mice, and also the VEGF content of these tumors was reduced (Kisley *et al.* 2002). This suggests that under certain conditions NO promotes primary tumor growth this being at least partly related to angiogenesis of the tumor.

In physiologic conditions, lung cells capable of generating NO include neutrophils, macrophages, mast cells, non-adrenergic non-cholinergic inhibitory neurons, fibroblasts, vascular endothelial and smooth muscle cells, and pulmonary epithelial cells (Gaston *et al.* 1994). The effects of NO can be either direct or indirect. The concentration, and therefore the source of NO are the major determinants of its biological consequences.

The direct and normal physiological effects of NO are mediated mainly by interactions with cGMP. As NO is a small lipophilic readily diffusible gas, it is an ideal intra- and intercellular messenger. It diffuses into the effector cell and binds to the haem iron complex of soluble guanylate cyclase (sGC), leading to the formation of increased amounts of cyclic guanosine monophosphate (cGMP) (Al –Ali *et al.* 1998). This leads to activation of the cGMP –dependent protein kinase, the mediator of smooth muscle relaxation and in lung this is reflected as pulmonary vaso- or bronchodilatation (Gaston *et al.* 1994, Barnes 1995, Al –Ali *et al.* 1998). NO also acts as a neurotransmitter of the inhibitory non-adrenergic non-cholinergic (iNANC) neural system in the airways (reviewed by Ricciardolo 2003). Low levels of NO, produced probably by cNOS, protect against bronchoconstrictive stimuli, but this effect is not as marked as that elicited by β2-agonists (Gaston *et al.* 1994, Al-Ali *et al.* 1998, Mulrennan *et al.* 2004). NO modulates the pulmonary vascular tone, counteracts hypoxic vasoconstriction and ventilation/perfusion matching, and acts as an airway neurotransmitter of bronchodilator nerves (Gaston *et al.* 1994, Ricciardolo 2003, Ricciardolo *et al.* 2004). NO has diverse activities in respiratory defense mechanisms. It is produced by activated macrophages to destroy disease causative agents; it inhibits neutrophils adhesion in vessel walls, modulates chemotaxis and increases ciliary motility (Al-Ali *et al.* 1998, Riccardolo *et al.* 2004). However, high NO levels produced by iNOS, for example in asthma, might evoke various deleterious effects, such as damage to airway epithelium, increase in vascular permeability and infiltration of inflammatory cells (Mulrennan *et al.* 2004).

Indirect effects are often due to large amounts of NO, and consequent modification of proteins, lipids, and nucleic acids by reactive nitrogen species formed in the reaction of NO with $O_2^{\bullet-}$ and O_2 (Davis *et al.* 2001). NO reacts rapidly with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^{\bullet}$)(Fig. 1), a highly toxic metabolite and a potent oxidant. Controlled generation of peroxynitrite may play a role in host defense, but excessive generation can lead to lung damage (Gaston *et al.* 1994, Al-Ali *et al.* 1998). Furthermore, peroxynitrite causes nitration of tyrosine, leading to the formation of nitrotyrosine, a compound which is used as a marker for peroxynitrite formation (Davis *et al.* 2001). However, there is also peroxynitrite-independent mechanisms by which nitrotyrosine can be formed (Davis *et al.* 2001). The complexity of NO chemistry in part may help explain the sometimes opposing results obtained in the research of the role NO in pathogenesis of the diseases.

In conclusion, expression of iNOS and consequent high levels of NO apparently protect cells against bacterial cytotoxins (Moncada and Palmer 1991, Nathan & Xie

1994), but they have also been connected to the pathobiology of respiratory tract disorders, such as progression of inflammatory diseases of the airways, lung parenchyma (Kharitonov *et al.* 1994, Lakari *et al.* 2002) There are several reports on NOSs in various types of cancers, focused mainly on iNOS and eNOS, while only few reports exists on nNOS (Asano *et al.* 1994, Thomsen *et al.* 1994, Cobbs *et al.* 1995, Rosbe *et al.* 1995, Thomsen *et al.* 1995, Bentz *et al.* 1997, Rajnakova *et al.* 1997, Takahashi *et al.* 1997, Ambs *et al.* 1998a and 1998b, Gallo *et al.* 1998, Hajri *et al.* 1998, Klotz *et al.* 1998, Nussler *et al.* 1998, Wilson *et al.* 1998, Zhao *et al.* 1998, Swana *et al.* 1999, Marrogi *et al.* 2000a and 2000b, Vakkala *et al.* 2000, Lewko *et al.* 2001). Both suppressive (Dong *et al.* 1994, Juang *et al.* 1998) and promotive (Jenkins *et al.* 1995, Edwards *et al.* 1996, Jadeski *et al.* 1999, Jadeski *et al.* 2000) effect on tumor growth and metastasis by the NOSs have been suggested.

2.3 Cigarette smoke and asbestos fibers as generators of free radicals

Cigarette smoke contains over 4700 chemical compounds of which free radicals and other oxidants are present in high concentrations (Pryor & Stone 1993). There are more than 10^{15} radicals per puff in gas-phase cigarette smoke, and fresh smoke contains NO levels up to 300 ppm (Pryor & Stone 1993). Two different populations of free radicals in cigarette smoke have been identified, one in the tar and one in the gas phase. The main radical in the tar phase is a quinine/hydroquinone complex, which is capable of reducing molecular oxygen to superoxide radicals. The gas phase of cigarette smoke contains small oxygen– and carbon-centered radicals that are much more reactive than the tar-phase radicals (Chow 1993). The release of ROS from phagocytes enhances further the oxidative burden of lung, as smoking increases the sequestration of neutrophils in the lung and superoxide release from these cells (MacNee *et al.* 1989, Rahman & MacNee 1996). Smoking also increases the level of nitric oxide metabolites in exhaled breath condensate (Balint *et al.* 2001). This oxidative burden either inhaled or generated by inflammatory leucocytes, has potent injurious effect on cells such as airway epithelium, promoting increased epithelial permeability and damaging lung matrix and proteins taking part in lung defense. Increased airspace epithelial permeability and oxidative alteration of protease/antiprotease balance are considered to have an important role in the pathogenesis of smoking-induced emphysema and COPD (Rahman & MacNee 1996)

The association between deleterious effect of cigarette smoke and genetic alterations leading to carcinogenesis have also been studied. Mutation of the p53 gene, leading to production of a mutant protein which has lost its inhibitory effect on the neoplastic process, has been suggested to be commonest genetic alteration occurring in human malignancies. A significant relationship has been found between exposure to cigarette smoke and overexpression of p53 protein in malignant bronchial tumors (Gosney *et al.* 1993).

Asbestos is a generic term for a group of naturally occurring hydrated silicate fibers whose tensile strength and resilient structural and chemical properties are ideally suited for various construction and insulating purposes (Kamp *et al.* 1999). Asbestos fibers

cause progressive pulmonary fibrosis (asbestosis), pleural disease and malignancies such as bronchogenic carcinoma and malignant mesothelioma.

The toxic effects of asbestos fibers are mediated by multiple mechanisms; one of them is ROS -induced pulmonary toxicity (Halliwell & Gutteridge 1989, Goodglick *et al.* 1990). Asbestos fibers produce ROS by at least two principle mechanisms. The first involves the iron content of the fiber, augmenting $OH^{\bullet}$ formation through iron -catalyzed reactions, i.e. the Fenton reaction. The second mechanism is based on the release of ROS upon activation of inflammatory cells such as pulmonary alveolar macrophages and neutrophils. Asbestos fibers also generate RNS such as NO, and peroxynitrite ($ONOO^{\bullet}$). Reactive oxygen species, especially $OH^{\bullet}$, RNS and $ONOO^{\bullet}$, can alter biological macromolecules including proteins, cell membrane lipids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) resulting in cellular dysfunction, cytotoxicity, and possibly malignant transformation. Asbestos fibers activate transcription factors such as nuclear factor kappa B, which in turn leads to upregulation of antioxidant enzymes, most importantly manganese superoxide dismutase (Kahlos *et al.* 1998, Kinnula 1999). Figure 2 shows pathways for the formation of reactive oxygen and nitrogen species by asbestos fibers.

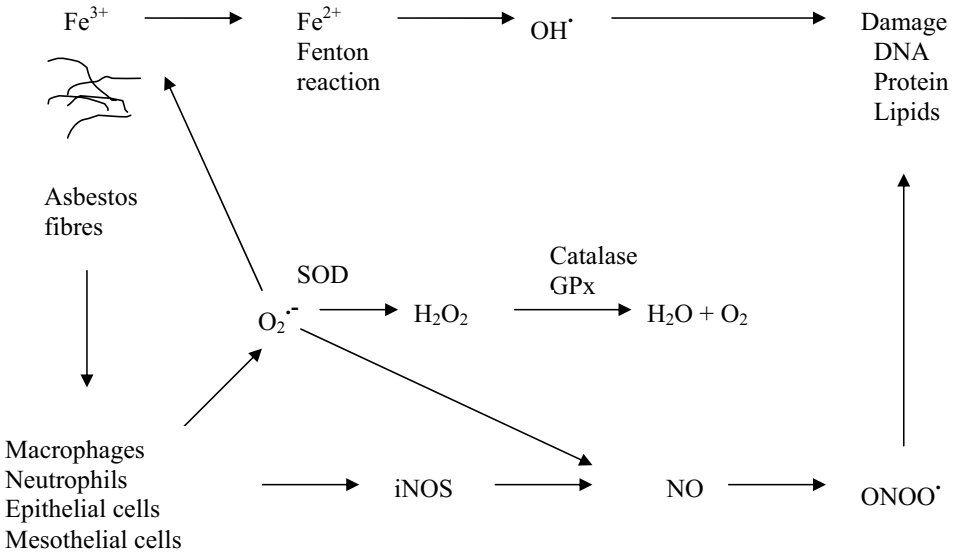

Fig. 2. Formation of reactive oxygen and nitrogen species by asbestos fibers. Modified from Kamp & Weitzman 1999

2.4 Vascular endothelial growth factor (VEGF) and its association with RNS/ROS

VEGF is a highly specific mitogen for vascular endothelial cells (Neufeld *et al.* 1999). It is a central regulator of vasculogenesis in normal organ development and in growing tumors; it contributes to wound healing, and increases the permeabilization of blood vessels. Five VEGF isoforms are generated by alternative splicing from a single VEGF gene. There are two tyrosine-kinase receptors of VEGF, i.e. FLT1 and KDR/FLK-1, expressed in endothelial cells. The third VEGF receptor, FLT-4, is expressed in lymph vessels (Neufeld *et al.* 1999).

In normal lung VEGF is expressed in capillary endothelial cells (Favre *et al.* 2003), activated macrophages (Monacci *et al.* 1993, Matsuyama *et al.* 2000) bronchial and alveolar epithelium (Berse *et al.* 1992, Tsokos *et al.* 2003) and bronchial glandular cells (Tsokos *et al.* 2003). Hypoxia, hypoglycemia, activated oncogenes and tumor suppressor genes such as K-ras (Konishi *et al.* 2000) and p53 (Ambs *et al.* 1998c, Ravi *et al.* 2000) and a variety of cytokines and growth factors potentiate the expression of VEGF (reviewed in Neufeld *et al.* 1999). Factors that increase VEGF production (reviewed in Neufeld *et al.* 1999) include hypoxia inducible factor (HIF), fibroblast growth factor 4 (FGF-4), platelet derived growth factor (PDGF), tumor necrosis factor-α (TNF- α) , transforming growth factor-β (TGF-β), keratinocyte growth factor (KGF), insulin-like growth factor 1 (IGF-1), interleukin-1β (IL-1β) and interleukin-6 (IL -6). Other cytokines such as interleukin-10 (IL-10) and interleukin-13 (IL-13) can inhibit the release of VEGF.

NO can up-regulate VEGF expression, and contribute to the blood vessel-permeabilizing effect of VEGF and to VEGF-stimulated vasodilatation. In turn, VEGF up-regulates the production of NO suggestingthat there may be a positive feedback loop between these agents. Increased permeabilization of blood vessels by VEGF leads to extravasation of proteins and the development of extravascular fibrin gel, which provides a matrix for the growth of endothelial and tumor cells and allows invasion of stromal cells into the developing tumor (reviewed by Neufeld *et al.* 1999).

Tumor growth depends on angiogenesis. It has been estimated that the largest size that a tumor can grow to without angiogenesis is only a couple of millimeters in diameter (in Cotran *et al.* 1994). Several growth factors influence tumor angiogenesis. These include VEGF, fibroblast growth factor (FGF), platelet – derived growth factor (PDGF), transforming growth factor – beta (TGF-β) and angiopoietins (reviewed by Neufeld *et al.* 1999).

2.5 RNS/ROS in lung diseases

2.5.1 RNS/ROS in normal lung

It is known, that the basal levels of each of the three NOS mRNAs in various tissues are different both in their sizes and in their contents, suggesting that regulation for differential expression of the three NOS genes in various human tissues may occur by alternative splicing of the NOS mRNAs in tissue –specific patterns (Park *et al.* 2000).

In the normal human lung, constitutive NOS (cNOS) immunoreactivity, indicating nNOS and eNOS, was detected in submucosal nerves and endothelium, but there was substantial variability among the 10 parenchymal specimens in the degree of circumferential staining of individual vessel lumen and the number of vessels showing immunolabeling of endothelium (Kobzig *et al.* 1993). No labeling of epithelium, smooth muscle, macrophages or other elements was seen for cNOS (Kobzig *et al.* 1993). Another study found a major site of nNOS expression in capillary endothelial cells in the alveolar septa (Luhrs *et al.* 2002). Anti-iNOS labeling (Kobzig *et al.* 1993) showed marked immunostaining of epithelial cells in histologically normal large, cartilaginous airways. Parenchymal samples showed patchy immunolabeling of bronchiolar respiratory epithelium, with many negative and some positive airways within the same specimen. Endothelium was variably positive. Alvelolar macrophages (AM) were occasionally positive. Focal linear staining within alveolar walls was seen in areas of macrophage and larger vessel iNOS labeling, and this was interpreted to represent patchy capillary staining (Kobzig *et al.* 1993).

2.5.2 RNS/ROS in lung diseases

Asthma is probably the most widely studied of obstructive lung diseases thought to involve the NOSs. In asthma, iNOS is induced in the bronchial epithelial cells (Hamid *et al.* 1993) leading to increased level of exhaled NO compared with healthy subjects (Kharitonov *et al.* 1994, Kharitonov *et al.* 2001, reviewed by Andreatis *et al.* 2003). The increased levels of exhaled NO in asthma originate predominantly from the lower airway and are related to airway eosinophilic inflammation, which is a key feature of allergic diseases (reviewed by Ricciardolo 2003). Exhaled NO measurement is a valuable diagnostic tool and can be used also in the evaluation of treatment efficacy and disease severity in asthma (Kharitonov *et al.* 2001, Ricciardolo 2003). Inhaled corticosteroid treatment reduces exhaled NO (Kharitonov *et al.* 1996) probably by direct inhibition of iNOS induction or by suppressing the proinflammatory cytokines that induce iNOS (Guo *et al.* 2000).

NOSs are thought to be involved in the development of vascular lung diseases, such as primary pulmonary hypertension. In this disease, the expression of eNOS is downregulated in the endothelium of pulmonary vessels (Giaid *et al.* 1995), and this has

been suggested to cause the characteristic features of this disease, i.e. pulmonary vasoconstriction and increased smooth muscle layer in the pulmonary vessels (reviewed by Ricciardolo 2003). As discussed above, NO signaling is mainly mediated by the guanylate cyclase/cyclic guanylate monophosphate pathway. The effects of this second messenger system are limited by enzymatic degradation through phosphodiesterases (PDEs), and beneficial effects of the oral PDE-5 inhibitor i.e. sildenafil have been reported in treatment of primary pulmonary hypertension (Ghofrani *et al.* 2004).

The presence and levels of NOSs have been studied in a variety of parenchymal lung diseases. In usual interstitial pneumonia, the end-stage fibrotic lesions were found to be negative for iNOS, but positive immunostaining was seen in myofibroblasts, alveolar macrophages (AM), regenerating type II pneumocytes and bronchial epithelium (Lakari *et al.* 2002). Some eNOS positivity could be detected in the ciliated columnar epithelial cells, AM, bronchial epithelial cells endothelial cells and regenerating type II pneumocytes (Lakari *et al.* 2002). In contrast, myofibroblasts revealed distinct eNOS but no iNOS immunoreactivity (Lakari *et al.* 2002). Another study of early stage fibrosing alveolitis found strong expression of nitrotyrosine and iNOS in macrophages, neutrophils, and alveolar epithelium in lungs (Saleh *et al.* 1997). This was correlated with the disease activity in a study of exhaled NO in fibrosing alveolitis, where exhaled NO levels were increased in active disease (Paredi *et al.* 1999).

In sarcoidosis and extrinsic allergic alveolitis biopsy specimens, iNOS immunoreactivity was positive in granulomas, especially in the giant cells, AM, regenerating type II pneumocytes and bronchial epithelium (Lakari *et al.* 2002). Also eNOS was positive in granulomas, being localized in epithelioid cells, AM and bronchiolar epithelium (Lakari *et al.* 2002). In tuberculotic lung tissue levels of iNOS, eNOS and nitrotyrosine, but not nNOS were significantly elevated in the inflammatory zone of the tuberculous granulomas, and in the nongranulomatous pneumonitis zone compared with control subjects (Choi *et al.* 2002).

In cystic fibrosis patients, the exhaled NO level was significantly reduced compared to normal subjects (reviewed by Kharitonov *et al.* 2001), despite strong neutrophilic inflammation in the airways. This might be due to a deficiency of iNOS observed in cystic fibrosis (Downey *et al.* 2000). Also in primary ciliary dyskinesia, the exhaled NO levels was lower than in normal subjects, but in contrast, in bronchiectasis both exhaled NO levels and iNOS expression in tissue were increased (reviewed by Kharitonov *et al.* 2001).

2.5.3 RNS/ROS in mesothelioma

The development of mesothelioma is tightly connected with previous asbestos exposure. Toxicity of asbestos fibers is suggested to be based on multiple factors, including the structure of the fibers and the ability of the fibers to stimulate the generation of RNS and ROS (reviewed by Kamp & Weitzman 1999, reviewed by Kinnula 1999). Asbestos fibers contain iron, which can generate hydroxyl radicals in the Fenton reaction (Figure 1) (Weitzman & Craeffa 1984, Halliwell & Gutteridge 1989, Kamp & Weitzman 1999). Iron

chelators, such as desferrioxamine, can prevent this reaction (Weitzman *et al.* 1988) and also inhibit lipid peroxidation accelerated by asbestos fibers (Halliwell & Gutteridge 1989). RNS and ROS can alter biological macromolecules including DNA, RNA and cell membrane lipids and this might lead to cytotoxicity, cellular dysfunction and malignant transformation (reviewed by Kamp & Weitzman 1999, reviewed by Kinnula 1999). For unknown reasons the sensitivity to asbestos fibers toxicity depends on the pulmonary cell type, and it has been suggested that mesothelial cells would be more sensitive compared with epithelial cells (reviewed by Kinnula 1999).

Rabbit pleural mesothelial cells (Liu *et al.* 2000) exposed to crocidolite asbestos fibers in primary culture showed elevated oxidant stress as assessed by the fluorometric microplate assay i.e. the 2'7'-dichlorodihydrofluorescein-diacetate (DCDHF-DA) method. This assay detects H_2O_2 and various peroxides and can be used in the assessment of oxidant-related reactions in the cells. DCDHF-DA, a fluorescent probe, diffuses into the cell and is oxidized to the highly fluorescent compound 2', 7'-dichlorofluorescein in the presence of intracellular H_2O_2 and hydroperoxides (Rosenkranz *et al.* 1992). The same method indicated that depletion of glutathione by buthionine sulfoximine (BSO) enhanced oxidant stress in human MeT-5A mesothelial cells exposed to crocidolite asbestos fibers (Ollikainen *et al.* 2000).

Asbestos fibers can induce the generation of nitrite in rat alveolar macrophages, as these cells obtained from animals inhaling asbestos fibers showed elevations in their nitrite/nitrate levels (Quinlan *et al.* 1998). Pulmonary epithelial cells exposed to crocidolite asbestos fibers exhibited upregulation of iNOS mRNA and a decrease in the GSH levels, and these effects being regulated by the iron content of asbestos fibers (Park & Aust 1998b). Inhalation of asbestos fibers leads to accumulation of nitrotyrosine in rat pleural mesothelium, at alveolar duct bifurcations and within bronchiolar epithelium and alveolar macrophages *in vivo* (Tanaka 1998). Asbestos fibers caused the formation of 8-hydroxyguanosine (8-OHgua) (a marker of oxidative DNA damage), and this was attenuated by N^G-nitro-L-arginine-methyl-ester (L-NAME), an L-arginine analogue and a competitive inhibitor of NOS (Chao *et al.* 1996, Chen *et al.* 1996). This suggested a role for NO in generation of DNA damage. NO enhanced the mutagenicity of crocidolite asbestos *in vitro* (Park & Aust 1998a). Interleukin-2 mediated cytotoxicity in malignant mesothelioma has been suggested to be mediated by NO (Porta *et al.* 2002).

On the other hand, NO has been suggested to protect mesothelial cells from malignant transformation. Simian virus 40 infection down-regulated the expression of nitric oxide synthase in human mesothelial cells, thus potentially favoring the survival of transformed, potentially neoplastic, cells by inhibiting the synthesis of NO (Aldieri *et al.* 2004).

In human mesothelioma samples iNOS was overexpressed, as was the case also with reactive mesothelial cells, and in this respect those cells are different from the normal mesothelial cells (Marrogi *et al.* 2000a). The expressions of eNOS or nNOS in mesothelioma have not been characterized. NO and VEGF are thought to interact in tumor associated angiogenesis (Neufeldt *et al.* 1999). VEGF and VEGF type C and their receptors were noted to be expressed in the majority of cases in human malignant mesothelioma tumors (Ohta *et al.* 1999). VEGF, FAF-1 and -2 and TGF-b immunoreactivity was present in 81, 67, 92 and 96% of mesothelioma samples, and

expression levels correlated significantly with intra-tumoral microvascular density and prognosis (Kumar-Singh *et al.* 1999)

Anti-VEGF -antibodies or inhibitors of VEGF-receptors provide a new target option in cancer treatment. Recently published results of a randomized clinical trial have indicated that adding bevacizumab, an anti-VEGF antibody, to routine treatment, prolonged survival in metastatic colorectal cancer (Kabbinavar *et al.* 2003, Hurwitz *et al.* 2004). According to phase I-II trials, no single anti-angiogenic treatment for mesothelioma will be effective by itself, but might be useful in combination with chemotherapy (Catalano *et al.* 2004). In murine mesothelioma model, inhibiting multiple mechanisms of angiogenesis using two angiogenesis inhibiting agents (human pigment epithelium-derived factor and human vascular endothelial growth factor receptor-1) inhibited tumor growth, prolonged survival and reduced microvessel density (Merritt *et al.* 2004). *In vitro* studies indicate that novel agents such as antisense oligonucleotide that inhibit VEGF and VEGF-C expression or VEGF and VEGF-C receptor antibodies can all reduce the levels of VEGF and VEGF-C and these agents have inhibited mesothelioma cell growth (Masood *et al.* 2003).

2.5.4 RNS/ROS in dysplasia and lung cancer

Studies on NOSs expression in dysplasias have mainly focused on iNOS. Increased expression of iNOS compared with normal tissue has been found in dysplasias at various sites. In normal prostate, there was no evidence of iNOS expression, but it was found in benign prostatic hyperplasia (Gradini *et al.* 1999). cNOS was expressed in both normal and hyperplastic prostate (Gradini *et al.* 1999). Barrett's esophagus showed elevated iNOS compared with paired gastric control tissues (Wilson *et al.* 1998). iNOS expression was increased in human oral premalignant epithelial lesions, but no iNOS was found in samples of normal buccal mucosa (Chen *et al.* 2002). The level of iNOS expression was correlated with the severity of the dysplasia (Brennan *et al.* 2000). The expressions of the NOS subtypes in bronchial dysplastic lesions have not been characterized.

Some studies have assessed the role of NOSs in human lung cancer by indirect methods (Arias-Diaz *et al.* 1994, Fujimoto *et al.* 1997, Ambs *et al.* 1998a, Liu *et al.* 1998). An increased level of nitrite/nitrate was found in the bronchoalveolar lavage (BAL) fluid of squamous cell lung carcinoma patients, suggesting increased tumor-associated NO production (Arias-Diaz *et al.* 1994). Also Liu *et al.* (1998) found that the level of nitrite was significantly higher in BAL fluid of cancer patients compared with control subjects. The level of exhaled NO from cancer patients was significantly higher than that in the control group, and the nitrite generation from cultured AM obtained from patients with lung cancer was enhanced (Liu *et al.* 1998).

The NOS activity was higher in lung adenocarcinomas than in other types of lung cancers or normal lung samples (Fujimoto *et al.* 1997).The NOS activity did not correlate with grades or T stage, but cancer tissues from patients with N2 disease tended to have lower activity than those from patients with N0 or N1 disease. Fujimoto *et al.* (1997) analyzed the correlation between intensity of immunohistochemical NOS

immunoreactivity and NOS activity. They observed that the presence of NOS was detected both in tissue samples with high and low NOS activity. Immunoreactivity for nNOS was detected in all cases, iNOS in half of the cases, but eNOS immunostainings were mainly negative (Fujimoto *et al.* 1997).

A significant correlation between NOS activity and p53 gene mutation frequency was found in early-stage lung adenocarcinoma (Fujimoto *et al.* 1998). A p53 DNA sequence analysis revealed that 5 of the 8 p53 mutation-positive samples in the high NOS activity category had a G: C –to T: A transversion, which was reported to be a major change induced by NO (Fujimoto *et al.* 1998).

Ambs *et al.* (1998a) assessed the correlation of NOS activity and VEGF expression. According to their results, Ca^{2+}-dependent NOS activity indicative of the presence of the nNOS and/or eNOS, was reduced in tumor tissue compared to normal tissue. Ca^{2+}-independent NOS activity, denoting iNOS expression, was mainly low or undetectable in lung tissues and did not differ significantly between carcinomas and normal tissue. However, high activities were found in three squamous cell carcinomas (SCC). The immunohistochemical expression of iNOS was found in these samples in tumor – infiltrating monocytes, and endothelial cells lining larger vessels; only one SCC had a small focus of tumor cells that stained for iNOS. Immunohistochemistry revealed VEGF positivity in 50% of the SCC and carcinoids, but not in any adenocarcnomas. The regions of iNOS positivity did not overlap with areas of VEGF positivity, which suggests that iNOS and VEGF were induced by different stimuli.

The association between iNOS and VEGF in NSCLC has been evaluated also in one study, which found iNOS, COX2 and VEGF immunoreactivity in 48%, 48% and 58% of the tumors of the study subjects, respectively (Marrogi *et al.* 2000b), and their levels correlated with microvascular density at the tumor-stromal interphase. These workers found more iNOS immunoreactivity in adenocarcinomas and large cell carcinomas. iNOS levels correlated positively with VEGF status, but no correlation was found between patient survival and iNOS levels. Angiogenesis inhibitors for VEGF-regulated pathways have been proposed to be useful for radiosensitization in the treatment of NSCLC (Raben *et al.* 2004). Nevertheless, more detailed information of the relative importance of VEGF in NSCLC is needed, as also nonangiogenic phenotypes of some NSCLC exist, and in this group antiangiogenic treatment might not be useful (Kerbel 2004).

Liu *et al.* (1998) examined lung cancer patients and detected expression of cNOS in nerves, endothelium and alveolar macrophages but not in cancer cells. In control subjects they found cNOS expression in endothelium and nerve elements. In cancer patients iNOS expression was present in epithelium, gland cells, endothelium, alveolar macrophages, tumor-associated macrophages, chondrocytes, and fibroblasts (Liu *et al.* 1998). In that study, two of nine adenocarcinomas and one alveolar cell carcinoma showed iNOS staining, this was negative in all squamous, large cell and small-cell carcinomas (Liu *et al.* 1998). AM and tissue-associated macrophages from patients with primary lung cancer, but not from control subjects, were strongly iNOS positive.

Lewko *et al.* (2001) found nNOS expression in 21 out of 32 of NSCLC cases, the strongest granular cytoplasmic reaction being in the basal cell layer of the neoplastic foci. No association with nNOS, p53 and degree of tumor differentiation was detected (Lewko *et al.* 2001). Thus, nNOS was not thus considered as a marker of highly malignant tumors in the NSCLC. In contrast, intense NOS activity has been associated with low tumor

grade in some other malignancies, such as gynecological (Thomsen *et al.* 1994) and breast cancers (Thomsen *et al.* 1995, Reveneau *et al.* 1999, Tschugguel *et al.* 1999)

2.5.5 RNS/ROS in COPD

The pathogenesis of COPD is suggested to be mediated in part by RNS and ROS (Rahman & MacNee 1996). The major risk factor for COPD is tobacco smoke, which not only contains large amounts of free radicals but also stimulates ROS generation by inflammatory cells with consequent ROS/RNS mediated epithelial cell injury (Pryor & Stone 1993).

The increased oxidative stress in COPD can have direct and indirect consequences which lead to the pathognomonic changes observed in lung tissue (MacNee 2000). The inflammation cascade is probably initiated by cigarette smoke and other irritants, which cause activation of macrophages in the airways. These cells secrete neutrophil chemotactic factors, such as interleukin-8 (IL-8) and leukotriene-B_4 (LTB_4) (Barnes 2000). Neutrophils release proteases which destroy connective tissue in lung parenchyma and cause emphysema (Barnes 2003). In normal lung proteases are inhibited by protease inhibitors like α_1-antitrypsin and tissue inhibitor of matrix metalloproteinases. Superoxide, which is one component involved in cigarette smoke (Pryor *et al.* 1993) related inflammation, can also react with NO and enhance the formation of peroxynitrite (MacNee 2000)**,** a potent oxidant, causing multiple injurious effects on the cells. These consequences include activation of matrix metalloproteinases and inactivation of the α_1-antitrypsin in the lung (Pryor *et al.* 1993). A hereditary deficiency of α_1-antitrypsin causes alveolar wall disruption and the development of emphysematous lesions (Sandford *et al.* 1997). In turn eNOS has been proposed to be a potential susceptibility gene in the pathogenesis of emphysema in α_1-antitrypsin deficiency (Novoradovsky *et al.* 1999). *In vitro* models suggest that cigarette smoke extract can decrease iNOS (Hoyt *et al.* 2003) and eNOS (Su *et al.* 1998) mRNA transcription and NO production from cultured murine and human epithelial cell lines. VEGF inhibition has in an experimental setting caused endothelial cell apoptosis associated with lung emphysema (Kasahara *et al.* 2001). It is yet to be seen, whether the VEGF –inhibitors, now being studied in the treatment of cancer (Kabbinavar *et al.* 2003, Hurwitz *et al.* 2004), will have side effects that cause emphysematic lesions of the lung.

With respect to iNOS expression and distribution in COPD patients somewhat conflicting results have been reported (van Straaten *et al.* 1998, Maestrelli *et al.* 2003). Van Straaten (1998) reported iNOS immunoreactivity in macrophages and occasionally in endothelial cells. eNOS immunoreactivity was present in endothelial cells in all of the large vessels studied, but only a small proportion of the capillaries in the alveolar interstitial tissue showed eNOS immunoreactivity. eNOS immunoreactivity was found also in endothelial cells and macrophages and at the brush border of bronchiolar epithelial cells. Immunoreactivity for iNOS or eNOS in the alveolar walls was not reported (van Straaten *et al.* 1998). Patients with severe emphysema showed lower percentages of iNOS and eNOS –positive macrophages compared to patients with only

mild emphysema, and this was suggested to reflect a relatively inactive defense system in severe emphysema (van Straaten *et al.* 1998).

Maestrelli *et al.* (2003) came to opposite conclusions about the association of NOS expression with the disease severity. iNOS staining was found in the alveolar wall and alveolar macrophages, in smooth muscle, in adventitia of pulmonary arteries and bronchial wall (Maestrelli *et al.* 2003). The number of iNOS-positive cells in the alveolar wall was significantly increased in severe COPD with respect of control smokers. Also the extent of nitrotyrosine staining in alveolar wall tended to be greater in severe COPD patients compared to control smokers (Maestrelli *et al.* 2003).

Studies on NO levels in exhaled air in COPD have been variable. Some studies show an elevated exhaled NO level which is not reduced by inhaled corticosteroids as is the case in asthma, whereas most studies have reported low exhaled NO levels in stable and high NO levels in unstable COPD (Ansarin *et al.* 2001, Kharitonov *et al.* 2001). Nevertheless, also elevated exhaled NO levels in stable COPD have been demonstrated (Corradi *et al.* 1999). The low levels of exhaled NO in COPD have been suggested to be related to the high reactivity of NO with superoxide and NO consumption in the formation of peroxynitrite (Eiserich *et al.* 1998). Other potential reasons could be weak or transient iNOS expression in COPD which consequently leads to a reduction in the amounts of NO exhaled in this disease. This alternative would then suggest that tissue injury in COPD is not related to persistent or direct iNOS induction in the airways but more likely to other oxidant enzymes (Ichinose *et al.* 2003) or to other ROS generated by neutrophils and alveolar macrophages (Repine *et al.* 1997). The airway distribution and immunoreactivity of all NOSs have not been systematically investigated in COPD, and it is not known if their immunoreactivity levels differ from those of non-smokers and smokers without COPD.

In conclusion, though many studies have examined this topic, there remain many open questions on the role of NO and the expression of all NOSs in the development of lung cancer, mesothelioma and COPD. Also the association of NOSs expression with lung tumor grade and prognosis remains unclear.

2.6 Mesothelioma

Mesothelioma is a tumor arising from mesothelial cells, usually in pleura, or rarely in peritoneum or pericardium. Three main histological subtypes of mesothelioma are distinguished; epithelioid, sarcomatoid and biphasic (Travis *et al.* 1999). The mesothelioma cells are highly radio- and chemoresistant owing to various intracellular mechanisms including overexpression of antioxidant enzymes (Sinha *et al.* 1990, Kahlos *et al.* 1998, Kinnula & Crapo 2004). Mesothelioma carries a dismal prognosis, with a median survival of less than a year after the diagnosis (Walz *et al.* 1990).

In most patients, the development of mesothelioma is associated with an occupational exposure to asbestos fibers (Mossman *et al.* 1996). The possible molecular mechanisms of asbestos induced lung cell injury have been discussed in chapters 1.4.3 and 1.5.

After asbestos exposure, the lag time to the development of the mesothelioma is 25-50 years. Asbestos is a fibrous material, which was widely used after World War II in the construction industry as an insulation material and as a fire retardant. In Finland, the peak for asbestos use as construction material was in the 1970's, and it was only prohibited in the year 1994. At the moment, mesothelioma is still a relatively rare disease, as in Finland the frequency of new mesothelioma cases is approximately 70 per year (Mattson 2000). Due to the lag time between exposure and the development of the disease, mesothelioma rates are still increasing. For instance in Britain there is now about 1800 deaths per year (Treasure *et al.* 2004). The peak of the epidemic is expected to occur in 2015-2020, when the death rate is likely to be 2000 per year in the United Kingdom (Treasure *et al.* 2004).

There is no curative treatment for mesothelioma. At the time of diagnosis, the disease has usually spread so widely that the surgical treatment is no longer an option. Nevertheless, in local disease, surgery can be considered. The operation, extrapleural pneumonectomy, entails removal of all the parietal pleura, the pericardium, and the diaphragm in addition to the whole lung on that side. Experimental chemotherapy has been given, but most of the patients still die within two years after diagnosis. Palliative treatments against pain include radiotherapy, pleurectomy, pleurodesis and medication (Mattson 2000). To clarify the benefit of surgical treatment, a trial is needed, and a pilot feasibility study (the mesothelioma and radical surgery "MARS" trial, funded by Cancer Research UK) is now in progress (Treasure *et al.* 2004).

2.7 Bronchial preneoplastic lesions and lung cancer

The formation of tumors has been hypothesized to occur in several stages: initiation, promotion and progression. Genetic changes accumulate in a cell and eventually lead to the development of an invasive cellular clone. Major molecular alterations in lung cancer include dysfunction of tumor suppression genes, such as p53 and p16, and putative autocrine loops and growth stimulation by proto-oncogene products (Fong & Minna 2002). Morphologically distinct preneoplastic changes (hyperplasia, metaplasia, dysplasia, and carcinoma in situ) can be observed in bronchial epithelium before an invasive cancer develops (Fong & Minna 2002). In metaplasia a differentiated tissue is transformed, for example when normal bronchial ciliated pseudostratified columnar epithelium is changed to stratified squamous epithelium (MacSween *et al.* 1992). Metaplasia can further develop into dysplasia, which is characterized by a disorganization of epithelium and anaplasia of cells without overt invasion.

Lung cancer is the second commonest cancer type in men and the fourth commonest in women in Finland (Finnish Cancer Registry 2003). The incidence in men is 32.9/100 000 and in women 9, 4 /100 000, and the predicted number of cases for the year 2004 is 1367 men and 568 women (Finnish Cancer Registry 2003). The most common reason for lung cancer is tobacco smoking, which is responsible for 90% of lung cancer cases. Other causative agents include asbestos, quartz, chrome, nickel, arsenic and polycyclic aromatic hydrocarbons. Exposure to asbestos doubles the risk of lung cancer, if combined with

smoking, asbestos increases the risk to fifty-fold. Environmental radon inhaled in high concentrations can cause lung cancer especially in smokers (Lubin *et al.* 1994).

Lung cancer arises mostly from the epithelial or neuroendocrinal cells of the bronchial tree. According to the WHO classification of lung tumors, the four commonest cancer types cover 95% of lung tumors, i.e. squamous cell carcinoma, small cell carcinoma, adenocarcinoma and large cell anaplastic carcinomas (Travis *et al.* 1999). The two main histological subgroups are separated for treatment options; 80% of tumors are non-small cell lung cancers (NSCLC) and the remain 20 % of the tumors are small cell lung cancers (SCLC). The grouping has been made because of substantially different prognosis and treatment modalities of these two lung cancer categories. SCLC has usually metastasized at the time of diagnosis and is treated by chemotherapy. Median survival of SCLC patients with complete response to medication is 14 months (Mattson 2000). The five years survival of SCLC is 3% in men and 4% in women (Finnish Cancer Registry 2003).

The prognosis of NSCLC depends on the age of the patient and the histological subtype and stage of the disease. The anatomic extent of the disease is evaluated by the TNM staging; where T describes the size of the primary tumor, N the extent of the nodal metastases and M the distant metastases (Mountain 1997). The prognosis of younger patients is better. With respect to the histological subtypes, adenocarcinoma carries the best prognosis with 5 years survival rates of 16% in men and 24 % in women; the respective figures for squamous cell carcinoma are 16% in both sexes (Teppo 2001).

The primary treatment for NSCLC is resection of the tumor by anatomical lung resection. It is also the only curative treatment available. A surgical treatment can be considered in those patients with stage I-IIIA NSCLC. The outcome of surgery is depended at which stage the disease has progressed to before the patient is operated. The results are best in stage I carcinomas, where the five year survival is 76-84%. The outcome declines clearly in stage III tumors, where the five years prognosis is at best 26% (Salo 2001). Other treatment modalities include chemotherapy and radiotherapy alone or combined with surgery.

The overall outcome of lung cancer is poor. Predicted 5-year relative survival rates for patients with lung cancer diagnosed in 1999 to 2001 are 9% for men and 13% for women (Finnish Cancer Registry 2003).

2.8 Chronic obstructive pulmonary disease

COPD is defined as a disease state which is characterized by the presence of airflow limitation due to chronic bronchitis or emphysema, and the airflow obstruction is generally progressive and mostly irreversible (American Thoracic Society 1995). COPD is the fourth leading cause of death in the world (Pauwels *et al.* 2001). The prevalence of COPD has been estimated in Finland in population over 64 years to be 12.5% in men and 3% in women, and 35% of smoking men suffered from this disease (Isoaho *et al.* 1994).

Cigarette smoking is the major known risk factor for COPD (Siafakis *et al.* 1995, Pauwels *et al.* 2001). Another, but rare, known cause of emphysema is the hereditary deficiency α_1-antitrypsin enzyme (Sandford *et al.* 1997). Previous studies have reported

that only 10-20% of smokers develop COPD (Fletcher *et al.* 1976), but recent studies suggest that up to 50% smokers might develop the disease (Lundbäck *et al.* 2003). The factors which predispose an individual to the development of the COPD are poorly characterized (Bascom 1991).

There are three major components to the disease: chronic bronchitis, airway obstruction and destruction of alveolar septae leading to development of lung emphysema (Kinnula 2000). COPD is characterized by chronic airway inflammation and obstruction (American Thoracic Society 1995). Mucus secretion, caused by metaplasia of submucosal glands and goblet cells, is a major feature in COPD (Barnes 2000).

Studies of bronchoalveolar lavage (BAL) and induced sputum reveal that the inflammatory cell profile in large airways in COPD consists of activated neutrophils (Thompson *et al.* 1989, Keatings *et al.* 1996). Bronchial biopsies show also infiltrates of mononuclear $CD4^+$-positive, and especially $CD8^+$-positive T-lymphocytes, the latter also known as Tc or cytotoxic T-cells (Saetta *et al.* 1993). There multiple inflammatory mediators thought to be involved in COPD such as lipids, chemokines, cytokines and growth factors include leukotriene-B_4 (LTB_4), TNF-α, Interleukin-8 (IL-8), growth related oncogene alpha (GRO-α) and TGF-β (Saetta *et al.* 2003, Barnes *et al.* 2003). COPD affects typically lung parenchyma, where at the site of destruction there is an accumulation of $CD8^+$ T-lymphocytes and macrophages (Saetta *et al.* 1998).

Fibrosis of the bronchioli leads to irreversible obstruction of the small airways. The emphysematic changes are in part explained by imbalance of elastase/antielastase-enzymes. An increase in tissue degradating protease enzymes, such as matrix metalloproteinases has been detected in BAL fluid of COPD patients (Finlay *et al.* 1997). The protease-antiprotease imbalance- theory does not fully explain the changes leading to emphysema. There are smokers who have elevated neutrophil levels, i.e. potent elastase releasers, in their lungs due to cigarette smoking, but yet they do not develop emphysema.

It has been suggested that the development of emphysema might be connected with vascular changes (Liebow 1959) and endothelial cell apoptosis of alveolar walls (Kasahara *et al.* 2001). In lung tissue, a VEGF receptor inhibitor SU5416 has in an experimental setting caused airspace enlargement (Kasahara *et al.* 2001). Significantly lower levels of VEGF have been reported to be associated to the mutation at position 936 of the 3' untranslated region of the VEGF gene, but this polymorphism was not associated with the development of smoking –related COPD (Sakao *et al.* 2003). Emphysematous changes were induced by alveolar wall apoptosis in mice, which were given active caspase -3 and protein transfection reagent Chariot (Aoshiba *et al.* 2003). However, in patients with COPD has been reported also enhanced bronchial expression of VEGF and its receptors, suggesting that these factors might have a role in airway and vascular remodeling (Kranenburg *et al.* 2005). Thus, the role of VEGF in the development of COPD is not unambiguous.

There are no current therapies that reduce the progression of this disease. Smoking cessation is the only known intervention that prevents the accelerated decline of the lung function in COPD. Long term use of corticosteroids does not have any effect on the clinical course of COPD nor do these drugs inhibit the progression of the disease, in contrast to asthma, another obstructive airway disease. The steroid resistance in COPD might be linked to increased oxidative stress, as this can impair the binding of

glucocorticoid receptors to DNA and the translocation of these receptors from the cytoplasm to the nucleus (Barnes *et al.* 2003). The use of inhaled combination of long acting β2-agonists and steroids has been claimed to reduce the number of COPD exacerbations (Calverley *et al.* 2003). Increased knowledge of cellular and molecular changes is needed to provide new targets for the development of treatment modalities in COPD. These include antagonists of inflammatory mediators such as leukotriene B4 inhibitors, chemokine inhibitors, TNF- α inhibitors, antioxidants and iNOS inhibitors (Barnes 2003, Saetta *et al.* 2003).

Prognosis of patients with severe COPD is generally poor, as described in British Medical Research Council –study (1981). Long term domiciliary oxygen therapy was able to prolong their five year survival from 30% to 50% (Medical Research Council Working Party 1981).

3 Aims of the study

This study was designed to analyze the presence of nitric oxide synthases in tissue samples of smoking and asbestos exposure related malignant and non-malignant lung diseases, and to evaluate the role of glutathione and iNOS in asbestos fibers-induced pulmonary cell and DNA toxicity. The specific aims were:

1. To assess the effects of asbestos fibers-exposure on pulmonary cell viability, on tail extent moments as markers of DNA damage, and on intracytoplasmic iNOS induction detected by flow-cytometry. A further aim was to analyze the glutathione content of bronchial and mesothelial cells, and also the immunoreactivity of heavy and light subunits of γ-GCS in bronchial epithelium and in non-neoplastic and non-inflamed pleura.
2. To analyze the immunohistochemical reactivity of iNOS in normal pleural mesothelial tissues for control, malignant mesotheliomas, malignant mesothelioma cell lines and metastatic pleural adenocarcinomas. An additional purpose was to evaluate the association of iNOS immunoreactivity with apoptosis, vascular density and survival; and also the expression of iNOS mRNA in four malignant mesothelioma cell lines.
3. To analyze the immunohistochemical reactivity of eNOS in malignant mesothelioma and its association with expression of VEGF, its receptors FLK1 and FLT1, vascular density and survival. Furthermore, the aim was to assess the expression of eNOS mRNA in two malignant mesothelioma cell lines.
4. To analyze the immunohistochemical reactivity of iNOS, eNOS and nNOS and nitrotyrosine in the metaplastic and dysplastic bronchial lesions, and in the lung tissue samples from non-smokers, smokers and COPD patients
5. To analyze the immunohistochemical reactivity of iNOS, eNOS and nNOS in non-small cell lung cancer. Additionally, the aim was to evaluate the association of all three NOS enzyme immunohistochemical reactivity with the type, grade, apoptotic index, and proliferation of tumors and the survival of patients.

4 Materials and methods

4.1 Materials

4.1.1 Cell culture (I, II, III, V)

Transformed human mesothelial cells (MeT-5A) (I, II, III) and human A549 alveolar epithelial cells (I, V) were obtained from American Type Culture Collections (Rockville, MD, USA). MeT-5A cells were maintained in RPMI 1640 (Gibco, Paisley, UK) growth medium with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Eggenstein, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) and A549 cells in Nutrient Mixture F-12 Ham (Gibco) growth medium with L-glutamine supplemented with 15% heat-inactivated fetal bovine serum (Gibco). Immortalized human bronchial epithelial cells, BEAS-2B (V), were obtained from the National Cancer Institute, Laboratory of Human Carcinogenesis (Dr C Harris, Bethesda, MD). The BEAS-2B cells were grown on uncoated plastic flasks (Nalge Nunc International, Roskilde, Denmark) in Bronchial Epithelial cell Growth Medium (BEGM, Clonetics, USA).

Mesothelioma cell lines M14K (II, III), M25K (II), M28K (II) and M38K (II, III) were originally established from the tumor tissue of untreated patients with malignant mesothelioma (Pelin-Enlund *et al.* 1990). These mesothelioma cell lines were grown in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.03% L-glutamine (all from LTI, Life Technologies, Paisley, UK). Human lung carcinoma cells, i.e. CALU-6, Sk-MES-1, A427 (V) (American Type Culture Collection), were grown in Alpha Minimal Essential Medium (α-MEM, Gibco) supplemented with 10% FBS, 100 U/ml penicillin, and 10 µg/ml streptomycin. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2.

4.1.2 Tissue samples (I, II, III, IV, V)

The tissue specimens of non-neoplastic and non-inflamed pleura (n=6 (I), n=5 (II), n=6(III)), non-neoplastic bronchial epithelium and healthy lung ((n=5 (I)), n=4 (III), n=13 (IV)), lung samples of smokers' with normal lung function (n=22 (IV)) and COPD patients (n=20 (IV)), inflamed mesothelium (n=4 (II)), malignant mesotheliomas (n=38 (II), n=36 (III)), pleural metastatic adenocarcinomas (n=25 (II)), and NSCLC lung tumors (n=89 (V)) were retrieved from the files of the Department of Pathology, Oulu University Hospital. The samples were obtained from patients going through a diagnostic or therapeutic pleural or lung tumor surgery. Additionally, bronchial biopsy specimens of metaplastic and dysplastic bronchial lesions (n=24 (IV)) were included, these samples were obtained from patients examined due to suspicion of a lung tumor.

In article IV, lung tissue samples (to represent control group, smoker group or COPD group) were obtained from the lung tissue distant from the lung tumor. Two tissue blocks were chosen from each patient, one from the level of cartilage-containing bronchus and the other from peripheral lung (IV).

Malignant mesotheliomas were subclassified into epithelial, sarcomatoid and biphasic subtypes according to the criteria given by AFIP (Battifora & McCaughet 1994) (II) or WHO (Travis *et al.* WHO 1999) (III). Metastatic adenocarcinomas consisted of tumors originating from lung, breast, kidney and liver (III). The NSCLC tumors (V) were subclassified into squamous cell carcinoma, adenocarcinoma, including four bronchioloalveolar carcinomas, and large cell carcinoma (WHO 1999).

Clinical data (I, II, III, IV, V), such as sex, age, smoking history, asbestos exposure, clinical diagnosis and survival, were obtained from hospital records. The stydy protocol was accepted by the ethical committee of the University of Oulu and Oulu University Hospital.

4.2 Methods

4.2.1 Cell exposures (I)

Semiconfluent cell cultures were exposed to 1-10 μg/cm^2 crocidolite (International Union Against Cancer, Johannesburg, South Africa) or to 0.05-5 mM H_2O_2 (Riedel-deHaën, Seelze, Germany) at 37° C as indicated. The concentrations of crocidolite and H_2O_2 were lower in the exposures of MeT-5A cells than A549 cells, since preliminary experiments indicated that A549 cells were more resistant than MeT-5A cells to both of these agents. In additional experiments, the cells were preincubated with 0.2 mM or 1 mM buthionine sulfoximine (BSO) for MeT-5A and A549 cells, respectively, for 16-18 hours to cause inhibition of γGCS, or with 2 mM L-arginine-methyl ester (L-NAME) to inhibit NO generation. The concentrations of these compounds were selected on the basis of previously published investigations where the concentrations had been tested (Buckley

1991, Kinnula 1992, Watkins 1997, Kinnula 1998, Park 1998b, Quinlan 1998, Belloq 1999, Järvinen 2000)

4.2.2 Cell viability (I)

The microculture tetrazolium dye colorimetric assay (XTT) (Boehringer, Mannheim, Germany) was used to assess cell viability according to the manufacturer's instructions. The results were analyzed using the Victor multilabel counter (1420 Wallac Inc., Turku, Finland).

4.2.3 Comet assay (I)

The comet assay (single-cell gel electrophoresis) was performed under alkaline conditions (pH 13) according to the method of Singh *et al.* (1991) with slight modifications. After exposure, the cells were detached by trypsinization, centrifuged and resuspended in preheated (37º C) 0.5% low melting point agarose (LMPA, Bio-Rad, Hercules, CA, USA). The cell suspension was then applied to dry slides (Assistant, Germany), precoated with 1% normal melting agarose (International Biotechnologies Inc, New Haven, CT, USA), and allowed to harden for 10 min at 4º C. The slides were then immersed in a cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100) for at least 1 h at 4º C. The hydroxylation of guanine in the 8-position (8-OHgua) is the probably most frequent and most mutagenic oxidative modification in DNA. In selected experiments, the slides were treated with formamidopyrimidine glycosylase (FPG) for 30 min (modified comet assay). This enzyme has apurinic/apyrimic (AP) endonuclease activity that recognizes and cleaves 8-OHgua lesions i.e. the oxidized bases formed during oxidative stress (Collins *et al*. 1996). The microscope slides were placed in an electrophoresis tank, to allow DNA to unwind for 20 min in freshly made alkaline electrophoresis buffer (1 mM EDTA, 300 mM NaOH). Electrophoresis was conducted at room temperature for 15 min at 24 V and 300 mA. The slides were then neutralized three times with Tris buffer (0.4 M, pH 7.5) and stained with ethidium bromide. All the steps were performed under yellow light in order to prevent additional DNA damage. The slides were scored using a fluorescent microscope attached to a video camera connected to a personal computer-based image analysis system (Komet 4.0; Kinetic Imaging Ltd, Liverpool, UK). A total of 600 cells from three separate experiments were scored (200 cells from each culture). H_2O_2 was used as a positive control for oxidant mediated cell damage throughout the comet study. Single cells were analyzed under a fluorescence microscope as described. Tail extent moments, as markers of DNA damage, were calculated as tail length multiplied by the percentage of DNA in the tail. The 6 h and 48 h exposures were separate experiments.

4.2.4 Glutathione content (I)

Total glutathione content was determined spectrophotometrically following the reduction of 5, 5'-dithiobis (2-nitrobenzoic) acid by NADPH in the presence of glutathione reductase (Halliwell *et al*. 1990). The glutathione content is expressed as nmol/mg protein. Protein was measured by the method of Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA).

4.2.5 Flow cytometry (I)

The detection of intracytoplasmic iNOS was done flow-cytometrically by using a fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse anti-iNOS antibody (Transduction Laboratories, Lexington, KY) as the primary antibody. The MeT-5A and A549 cells were trypsinized, washed once with phosphate-buffered saline (PBS), incubated for 15 min in cold 70% ethanol, and washed twice with PBS. The cells were incubated for 30 min with the primary antibody or with the isotype control, and after two washings with PBS, 10 000 cells were analyzed from each sample with a flow cytometer (FACSort flow cytometer, Becton Dickinson).

4.2.6 RT-PCR for eNOS (III, V) and iNOS (II, V)

Expression of eNOS and iNOS was investigated by using reverse transcription polymerase chain reaction (RT-PCR) in the cultured human non-malignant transformed mesothelial cells MeT-5A, mesothelioma cell lines M14K, M25K M28K and M38K (II, III), immortalized human bronchial epithelial cells i.e. BEAS-2B (V) and in the human carcinoma cell lines A549, CALU-6, Sk-MES-1, A427 (V).

The oligonucleotide primers were designed according to cDNA sequence data published earlier (Marsden 1992, Geller 1993). Total cellular RNA was extracted from the cells using a kit for RNA isolation (RNEasy®, Qiagen, Hilden, Germany). One μg of RNA was treated with DNase I (Pharmacia Biotech, Milwaukee, WI, USA) at 37^{o}C for 10 min and at 75 ^{o}C to eliminate possible DNA contamination of the samples, and reverse-transcribed with 100 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Paisley, UK) and the respective antisense primer (eNOS 5'- AGC TCG CTC TCC CTA AGC TGG TAG G -3', 10 or 7 pmol for mesothelioma cell lines or human lung carcinoma cell lines, respectively; iNOS 5'-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3', 5 or 10 pmol for mesothelioma cell lines or human lung carcinoma cell lines, respectively) at 42°C for 45 min in a 20 μl reaction mixture containing 1 U RNase inhibitor (5 Prime→3 Prime, Boulder, CO, USA). The cDNA was PCR-amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) using 1 U of DNA polymerase (Dynazyme, Finnzymes, Espoo, Finland) and the respective sense primer

(eNOS 5'- GCA TCA CCT ATG ACA CCC TCA GCG -3', 10 or 7 pmol for mesothelioma cell lines or human lung carcinoma cell lines, respectively; iNOS 5'- TGC CTG GCA AGC CCA AGG TCT ATG TTC AGG AC-3', 5 or 10 pmol for mesothelioma cell lines or for human lung carcinoma cells, respectively) in a 100 µl reaction volume containing 1.5 mM $MgCl_2$. The thermal profile involved 43 or 35 cycles (eNOS and iNOS, respectively) of denaturation at 94°C for 50 sec, primer annealing at 64 or 62°C for 50 sec, and extension at 72°C for 50 sec or 1 min 30 sec (eNOS and iNOS, respectively). PCR products were electrophoresed in an ethidium bromide-stained 2 % agarose (SeaKem, Rockland, ME) gel and visualized under UV-light. The amplification products of the eNOS and iNOS RT-PCRs were 277 base pairs (bp) and 500 bp in length, respectively. Negative controls were established in each experiment by substituting the RNA sample with water and by leaving the reverse transcriptase enzyme out of the RT-reaction for each of the samples.

4.2.7 Immunohistochemistry (I, II, III, IV, V)

4.2.7.1 Handling of the tissue samples (I, II, III, IV, V) and the cell pellets (II)

All the tissue material had been fixed in 10% buffered formalin and embedded in paraffin. Four micron thick sections were cut from a representative paraffin block, and the sections were deparaffinized in xylene and rehydrated in a descending ethanol series. Endogenous peroxidase was consumed by incubating the sections in 0.1% hydrogen peroxide in absolute methanol for 10 minutes. To enhance immunoreactivity, the sections were incubated in 10 mM citrate buffer (pH 6.0) and boiled in a microwave oven for 10 min. In article I, the paraffin blocks were then incubated with 2% milk powder to diminish background staining. The sections were then incubated with the primary antibodies (see Table I). The immunostaining was performed using the Histostain-Plus Bulk Kit (Zymed laboratories Inc, South San Francisco, CA, USA) and the chromogen used was aminoethyl carbazole (AEC) (Zymed Laboratories Inc), except for detection of FVIII related antigen and PCNA where the immunostaining was performed with the avidin-biotin-peroxidase-complex technique using diaminobenzidine-hydrogen peroxide as a chromogen. Negative control stainings were carried out by substituting PBS (I,II,IV) non-immune goat (III), mouse (II,III) or rabbit (I, II, III, V) serum or rabbit primary antibody isotype control (I,IV) for the primary antibodies.

For immunocytochemistry, the cell pellets (II; III data not shown) derived from mesothelioma cell lines M14K (II, III), M25K (II), M28K (II) and M38K (II, III) were fixed in 10% neutral formalin overnight, after which the formalin was removed, and melted 2% agar was laid over the pellets. The agar blocks were further embedded in paraffin. Four µm thick sections were cut from the cell blocks and stained for the polyclonal anti-iNOS (II) or anti-eNOS (data not shown) antibody.

4.2.7.2 Immunohistochemistry antibodies

The primary antibodies used in immunohistochemistry are listed in the Table 1.

Table 1. The primary antibodies used in immunohistochemistry.

Antibody	Dilution	Source
γGCSh and γGCSl		
Polyclonal rabbit	1:1000(I)	A gift from Dr. Terrance Kavanagh, University of Washington, Seattle, USA
iNOS		
Polyclonal rabbit	1:100(IV)*,1:200(II,IV,V)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Monoclonal mouse	1:60(II)	Transduction Laboratories, Lexington, KY,USA
eNOS		
Polyclonal rabbit	1:50(III,V)1:100(IV)**	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
nNOS		
Polyclonal rabbit	1:200(IV,V)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Nitrotyrosine		
Rabbit polyclonal	1:100(IV)	Upstate, Lake Placid, NY, USA
FVIII related antigen		
Polyclonal rabbit	1:50(II)	Dako, Dakopatts, Denmark
VEGF		
Monoclonal mouse	1:250(III)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
FLT1		
Polyclonal gout	1:100(III)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
KDR/FLK1		
Monoclonal mouse	1:250(III)	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
PCNA		
Monoclonal mouse	1:50(V)	Dako, Glostrup, Denmark

* To ascertain the results for iNOS in COPD (IV), the immunohistochemical stainings were done twice with two different lots of the antibody, at the first time with dilution 1:200 and at the second time with dilution 1:100, with internal positive control sample of sarcoid granuloma. ** The pilot stainings with eNOS antibody dilution 1:50 used in malignant lung tissue samples (III, V) exhibited intense background in COPD tissue samples. Therefore the dilution 1:100 was used in study IV.

4.2.8 Evaluation of the intensity of the immunohistochemical stainings (I, II, III, IV, V)

The results of immunostainings were assessed under a light microscope by two (I, II, III, V) or three (IV) investigators independently and blindly, and the association of the evaluations or the Kappa value was counted ($p<0.001$, Fisher's exact test (II); Kappa value 0.38 (IV)), or by two investigators together and consensus was formed on cases where the opinions differed (V). The results of immunostainings were assessed by the

intensity of the stainings (negative, weak, moderate, strong or very strong) and quantity of the stainings was assessed by interpreting the percentage of positively stained cells. Additionally a combined score for the immunostaining was counted for statistical analysis, based on both qualitative and quantitative immunostaining (II, III, V).

The vascular density (II) was estimated as the number of positively stained blood vessels/high power field (HPF). In each tumor section, a minimum of 10 HPFs were analyzed. The proportion of PCNA-positive cells (V) was counted in at least six HPFs. PCNA positivity was classified as follows: 1≤1%, 2=1-10%, 3=11-50%, 4=51-75%, 5≥75% of cells positive.

4.2.9 3'-end labeling of DNA in apoptotic cells (II, V)

In order to detect apoptotic cells (II, V); in situ labeling of the 3´-ends of the DNA fragments generated by apoptosis-associated endonucleases was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA). The sections, after being dewaxed in xylene and rehydrated in ethanol, were incubated with 20 mg/ml Proteinase K (Boehringer Mannheim GmgH, Mannheim, Germany) at room temperature for 15 minutes. The endogenous peroxidase activity was blocked by incubating the slides in 2% hydrogen peroxide in PBS, pH 7.2. The slides were then treated with terminal transferase enzyme and digoxigenin-labelled nucleotides after which anti-digoxigenin-peroxidase solution was applied on the slides. The color was developed with diaminobenzidine after which the slides were lightly counterstained with haematoxylin.

Cells were defined as apoptotic if the whole nuclear area of the individual cell labeled positively. Apoptotic bodies were defined as small positively-labeled globular bodies in the cytoplasm of the tumor cells which could be found either singly or in groups. To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies in tumor cells were counted in 10 HPFs and this figure was divided by the number of tumor cells in the same HPFs.

4.2.10 Statistical analysis

SPSS for Windows (Chicago, IL, USA) was used for statistical analysis (I, II, III, IV, V). The data are expressed as means ±SD or ±SEM. The significance of the associations was determined using Fisher's exact probability test (II, III, IV, V), correlation analysis (II, V) and two-tailed Student's *t*-test (I, II, III, IV, V). The survival analysis was performed by the Kaplan-Meier curve (II, III, V) and significance of associations was tested by the log-rank (II, V), Breslow (III, V) and Tarone-Ware (III, V) tests and Cox's regression analysis (V). Probability values $P<0.05$ were considered statistically significant.

5 Results and discussion

5.1 Evaluation of methodology

The main aim of this study was to characterize the presence of the NOS enzymes in lung tissue and neoplasia. Also the ROS related pathways in asbestos fiber-induced lung cell injury were evaluated.

Patient materials were collected from the archives of the Pathology Department, Oulu University Hospital. Consecutively operated patients for mesothelioma (II, III) or NSCLC (V) were included to the study. Clinical characteristics were screened for patients who had gone through lung resection in a certain time period, and those who fulfilled the inclusion criteria for smoking history and lung function tests were included (V). Thus the relatively limited size of samples in the studies was dependent on the amount of operatively treated lung tumor patients. The control groups were small, but it is difficult to obtain control tissue samples from healthy patients. In this case, control tissue samples were obtained from patients who had gone through a diagnostic or therapeutic lung resection. The differences in the studied parameters in the statistical analysis might have been clearer if it would have been possible to obtain a larger patient series.

The diagnostic criteria for the malignant lung diseases were unambiguous, since they were based on histological definitions in routine clinical use (Battifora *et al*. 1994 II, III), (WHO 1999 IV). COPD was defined by irreversible bronchus obstruction (FEV1 < 80% and FEV% <70) (V). The international guidelines for definition of COPD are somewhat variable, and often there is greater focus on the obstruction of the large airways and poorer definition of the peripheral tissue damage. Furthermore, the assessment of the peripheral lung tissue damage by lung function tests by MEF_{50}, MEF_{25} or diffusion capacity is not straightforward. There is a correlation between alveolar damage seen at the tissue level, reduced diffusion capacity and bronchus obstruction but the correlation is not particularly strong.

Immunohistochemistry was used to assess NOSs stainings in lung tissues. This method is a widely used diagnostic tool in clinical practice, and thus readily available. It provides direct, cell specific information about the expression of the studied protein in a tissue *in vivo* but there are some problems associated with this method. Since there is

heterogeneity especially in cancer tissues, the sample might not have been taken from the most representative site. Also in COPD, the amount of lung tissue damage can be patchy and not necessarily similar in all parts of the lung. The sensitivity of immunohistochemistry can be diminished by the small amount of a studied antigen or the antigens can be destroyed during handling of the samples. Also the concentration and affinity of specific antibodies can influence the sensitivity. The specificity of immunohistochemistry depends on the quality and concentration of antibodies (Naukkarinen 1999). The specificity can be tested by using an internal positive control, or staining a control tissue known to be positive, as was done in this study. Negative controls can be either tissues which do not contain the studied antigen or immunologic controls. In this study, negative control staining was carried out by substituting antibodies with PBS, non-immune mouse or rabbit serum, or isotype control. It is also possible that positive immunohistochemical staining occurs even though the protein is in an inactive form. The evaluation of immunohistochemical staining under the light microscope is subjective, and at best semiquantitative. This problem is diminished if two investigators achieve best agreement on results, or they assess the samples separately and the interobserver variability is counted by a correlation coefficient, such as a Kappa value (Stenkvist *et al*. 1983).

Apoptotic cell detection in tissue sections of tumors was conducted in this study by in situ 3'-end labeling of DNA fragments generated by apoptosis-associated endonucleases. Problems concerning this method have been discussed in a review article (Soini *et al*. 1998). The extent of apoptosis may vary in different areas of tumors, and apoptotic cells may appear in clusters. To avoid erroneous results, a sufficient number of fields should be included in the analysis, and a high –power lens should be used for the identification of apoptotic cells. An erroneous count of total number of tumor cells in the field can readily change the apoptotic index. In addition to problems with the detection of apoptotic cells, the sensitivity and specificity of the used method can be a source of misinterpretation. In this study, apoptotic cells were identified by evaluating the morphological changes present in the cell. This excluded false positive results due to staining artifacts. Additionally, such staining artifacts usually result in a high amount of nuclear staining in a given field in contrast to apoptosis, which usually involves individual cells. Areas near necrosis were avoided due to increased amount of fragmented cells in such areas. DNA fragmentation can be present in cells damaged in ways other than apoptosis, for instance cells undergoing necrosis. In addition, positive and negative controls were used in the TUNEL procedure.

Cultured cells were used in this study to evaluate iNOS and eNOS expression at the cellular level in malignant lung cells, and for assessment of the extent of cell injury induced by asbestos fibers. The cell culture environment differs from the *in vivo* situation in various ways. Since cell culture is carried usually with one cell type in a single experiment, it is unable to assess the interaction of different cell types and cytokines. Furthermore also the environmental oxygen pressure may be different from that *in vivo*. Thus, results obtained in cultured cells can not directly be extrapolated to the situation *in vivo*. Nonetheless cultured cells do provide a practical and useful tool to obtain information about the generation of cell and DNA damage with different exposure conditions.

The capability of mesothelioma and lung cancer cell lines to express iNOS and eNOS mRNA *in vitro* was assessed by RT-PCR. This method is extremely sensitive, and the required sample size is small. The problem with this method is that it readily produces false positive results due to contamination. To exclude that possibility, negative control samples containing water instead of RNA were included in each assay. Another problem with this method is that sometimes mRNA is produced but it is not translated into active protein in a cell. Therefore the expression of iNOS and eNOS at the protein level was confirmed by immunohistochemistry of mesothelioma cell pellets. iNOS protein was positively detected by immunocytochemistry (II) and also eNOS was positive in mesothelioma cell pellets (data not shown).

The cell viability after oxidant and fiber exposure was assessed by the microculture tetrazolium dye colorimetric assay (XTT assay; Boehringer, Mannheim, Germany). This provides a more practical way to measure cell viability than the traditional trypan blue method. Nevertheless, XTT is a rather insensitive method to assess cell damage, as the association of cell viability with the amount of DNA damage in the Comet-method after oxidant and fiber exposure was poor. The single cell gel electrophoresis, i.e. Comet –method is a sensitive method to detect DNA single –strand breaks in individual cells, these being caused by potential mutagens or carcinogens. It is also easy to perform and required sample sizes are small. However, the sample size used in this study i.e. 200 cells per experiment, is much larger than the 50 cells often described in the literature.

Flow cytometry was used to evaluate the effect of fiber exposure on iNOS protein in mesothelioma and lung carcinoma cell lines (I). Flow cytometry measures scattered light and fluorescence from single cells as they pass through the focus of a high-intensity of light source at very rapid rates. Thus flow cytometry provides a quick and sensitive way to obtain quantitative information about a particular constituent in a cell population (Watson 1991). One disadvantage of this method is the absence of a direct visual record and furthermore the results may be affected by other factors e.g. by specificity of antibodies, handling of samples, standardizing and calibration of the equipment.

In conclusion, several methods were chosen in order to obtain a reliable and comprehensive characterization of the NOS enzymes in lung, as well as the pathways involved in this phenomenon and also in ROS related lung cell damage.

5.2 Glutathione and iNOS in fiber-induced cell and DNA toxicity in mesothelial and bronchial cells (I)

5.2.1 The effect of asbestos fibers and oxidant exposures on mesothelial and bronchial cell viability

To compare fiber-induced toxicity with the oxidant effects, MeT-5A mesothelial and A549 lung epithelial cells were exposed to asbestos fibers and H_2O_2 (I). Exposure of MeT-5A and A549 cells to asbestos fibers at concentrations of 1, 2 and 10 $\mu g/cm^2$ caused

a marginal cell injury only in the MeT-5A cells as measured by the absorbance decrease in XTT (Figure 1A in I). Statistical test used was paired samples t-test. The results were checked subsequently by one-way ANOVA, which revealed a statistically significant difference between groups in MeT5A- cells ($p<0.001$). The results obtained with both statistical methods were consistent with each other. In post hoc analysis (Dunnett) the groups exposed to the two doses of the crocidolite asbestos fibers i.e. the 2 μg/cm^2 ($p=0.035$) and 10 μg/cm^2 ($p=0.001$) differed statistically significantly from the control group, but there was no statistical significance between these two exposure groups ($p=0.10$). The mean absolute absorbance values in MeT5A cells were 2.44±0.04 (SD), 2.08±0.21 (SD) and 1.81±0.22 (SD) in control, 2 μg/cm^2 and 10 μg/cm^2 exposures, respectively. There was no statistically significant difference in A549 cells between the crocidolite asbestos exposure groups ($p=0.524$). The mean absolute absorbance values in A549 cells were 2.89±0.14 (SD), 2.78±0.11 (SD), 2.73±0.28 (SD) in control, 2 μg/cm^2 and 10 μg/cm^2 exposure groups, respectively.

Also exogenous H_2O_2 exposure caused a statistically significant loss of cell viability in MeT5A cells measured by the decrease in absorbance in XTT ($p<0.001$) (Figure 1B in I). In post hoc analysis (Dunnett) both the 0.1 mM H_2O_2 ($p=0.002$) and 0.5mM H_2O_2 ($p<0.001$) exposure groups differed statistically significantly from the control group, and between these two exposure groups there was also significant difference ($p<0.001$). There was no difference in the A549 cells between the H_2O_2 exposure groups ($p=0.60$). The mean absolute absorbance values in MeT5A cells were 2.25±0.19(SD), 1.69±0.22 (SD) and 0.32±0.0.02 (SD) in control, 0.1 mM H_2O_2 and 0.5 mM H_2O_2 exposures, respectively. The mean absolute absorbance values in A549 cells were 2.70±0.24 (SD), 2.64±0.16 (SD), 2.52±0.30 (SD) in control, 0.1 mM H_2O_2 and 0.5 mM H_2O_2 exposures, respectively.

In previous studies, asbestos fibers have evoked apoptosis in various lung cell types, including mesothelial cells (Broaddus *et al*. 1997, BéruBé *et al*. 1996, Liu *et al*. 2000) and alveolar macrophages (Hamilton *et al* 1996). The present study (I) results are in line with studies describing an increase in DNA damage evoked by asbestos fibers in cultured lung cells (Ollikainen *et al.* 1999, Liu *et al.* 2000). These genotoxic changes do not necessarily lead to marked loss of cell viability (Kinnula *et al.* 1994, Jaurand *et al.* 1997). Asbestos fibers have also been shown to have proliferative effects on mesothelial cells, at least *in vitro* (Driscoll 1996). The present study showed that asbestos fibers exposure caused less prominent loss of mesothelial cell viability compared with oxidant exposure (I). On the other hand, increased DNA breaks were observed in asbestos fibers -exposed mesothelial cells in COMET assay (I). The survival of cells containing DNA modifications may contribute to asbestos-related malignant transformation.

5.2.2 Asbestos fibers -induced DNA damage related toγ-GCS and glutathione content

DNA breaks induced by asbestos fibers were measured by Comet –assay. A statistically significant increase in the mean tail extent moment, an indicator of DNA strand breaks, was observed in MeT-5A cells after exposure to asbestos fibers (Figure 2 in I). The

unmodified Comet assay detects DNA single-strand breaks, but it is not specific for oxidatively damaged DNA. The most frequent and most mutagenic lesion of oxidative modification of DNA is probably the hydroxylation of guanine in the 8-position (8-OHgua). Formamidopyrimidine glycosylase (FPG) is an enzyme which introduces breaks at sites of oxidative damage in DNA. This enzyme removes 8-OHgua and leaves apurinic/apyrimidinic (AP) sites that have been converted to strand breaks by associated AP endonuclease activity (Collins *et al.* 1996). To analyze specifically oxidatively damaged DNA, the Comet assay was modified by addition of FPG-enzyme (see methods). Inclusion of FPG-enzyme further elevated the mean tail extent moments when compared to the unmodified assay in both cell lines (Figure 2 in I). Thus, modification of Comet assay by the FPG-enzyme was an additional tool in this investigation not only for oxidant related DNA damage, but also for asbestos fibers induced DNA damage.

Glutathione is one of the most important antioxidants in the detoxification of reactive oxygen and nitrogen species (Wink *et al.* 1998). The development of single strand breaks was significantly potentiated in both cell lines by pre-treatment of the cells with BSO, which is known to evoke glutathione depletion by inhibiting the rate limiting enzyme of γ-GCS (Figure 3 in I). The mesothelial MeT-5A cells were far more vulnerable compared with the bronchial A549 cells to exogenous H_2O_2 as measured by XTT (I). This difference is in line with the lower intracellular glutathione content of MeT-5A cells compared to A549 cells (23.5±4 nmol/mg protein and 90.1±10 nmol/mg protein, n=4) (I). Consistent with this, A549 cells were more resistant against oxidant and asbestos fibers -related DNA breaks than MeT-5A cells.

The earlier findings have emphasized the importance of intracellular glutathione content against DNA base modifications in mammalian cells (Lenton *et al.* 1999). This is in line with the results of the present study, where glutathione depletion enhanced asbestos fibers -induced DNA toxicity in both cell lines (I). *In vivo* glutathione may also have an important role in protecting genetic material against oxidative insults. The immunoreactivity of subunits of the rate limiting enzyme of glutathione synthesis i.e. γ-GCSh and γ-GCSl, is readily assessable by immunohistochemistry in tissue samples. Indeed, *in vivo* human pleural mesothelium was mostly negative in all of cases for both subunits of γ-GCS, while bronchial epithelium exhibited weak to moderate immunoreactivity in all the five cases investigated (Figure 5 in I). This low γ-GCS expression reflecting intracellular glutathione levels in human mesothelium may have a crucial impact on the development of oxidant- and asbestos fibers –induced DNA alterations. Thus, low glutathione content might in part contribute to the increased sensitivity of human pleural mesothelium to asbestos fibers toxicity compared with bronchial epithelium.

5.2.3 Asbestos fibers- induced DNA damage related to NO and iNOS

The previous studies have not been able to differentiate the effects of various oxidants in fiber-induced DNA damage, and on the other hand iNOS induction associates with the development of various lung diseases, such as inflammatory airway (Barnes 1995) and

interstitial lung diseases (Saleh *et al.* 1997). To investigate the possible role of nitric oxide synthase in asbestos fibers –induced DNA damage, both MeT-5A cells and A549 cells were treated with L-NAME, an unselective NOS inhibitor (I). These experiments were conducted without and with the FPG enzyme, but no significant effect on the development of the tail moments in the Comet assay could be seen in either case (I). In MeT-5A cells, which are the more sensitive cell type, mean extent tail moments were 19.2±0.7 in asbestos exposed and FPG treated cells (4 μg/cm^2, 48 h, n=3, for each experiment 200 cells were counted) and 23.1±0.7 (n=3, for each experiment 200 cells were counted) in asbestos exposed L-NAME treated cells (I). The statistical method used in Comet assay was independent samples two tailed t-test (I). The results suggest that at least *in vitro* NOS inhibition might not modify asbestos fibers –induced DNA damage.

The importance of NO-mediated mechanisms in asbestos fibers exposure, such as iNOS induction, has been reported (Tanaka *et al.* 1998). Inhalation of asbestos fibers has increased the expression of nitrotyrosine in rat pleural mesothelium *in vivo* (Tanaka *et al.* 1998). In this present *in vitro* study, L-NAME had no effect on the mean tail extent moment in asbestos-exposed cells (I), and therefore the effect of this exposure on iNOS induction was further explored by flow cytometry (I). Both the MeT-5A and A549 cells lines were positive for iNOS protein at the basal stage, A549 cells being more strongly positive (I). Exposure to asbestos fibers (crocidolite 2 or 10 μg/cm^2) did not increase iNOS protein expression in either cell line (Figure 4 in I). Results obtained with cultured cells cannot, however, be directly extrapolated to the situation *in vivo*. Since activated inflammatory cells potentiate the toxicity of asbestos fibers (Kinnula *et al.* 1995b), nitric oxide may cause DNA damage *in vivo*. This suggests that the induction of iNOS in lung cells *in vivo* might be linked to cytokines and not to the direct exposure of the target cell to the fibers.

In conclusion, in asbestos-exposed cells the Comet assay was a sensitive method for observing DNA strand breaks (I). Together with the oxidative effects evoked by prolonged asbestos fibers exposure, the low intracellular glutathione content may have a decisive role in the development of DNA strand breaks. Marginal γ-GCS immunoreactivity was seen in human mesothelium (I). Consequently the possibly low glutathione content may have important effects on the development of oxidant- and fiber-induced DNA damage in these cells. During exposure to asbestos fibers, the inflammatory cells are activated (Kinnula *et al.* 1995b), and this in combination with the low γ-GCS expression in human mesothelial cells (I), may further enhance the oxidant effects of asbestos fibers *in vivo*.

5.3 iNOS immunoreactivity in healthy and inflamed pleura, malignant mesotheliomas and pleural metastatic adenocarcinomas (II)

The results of immunoreactivity of iNOS (II) in pleural tissues are summarized in Table 2. In the present study, strong iNOS immunoreactivity was observed in the majority, i.e.

28/38 (74%) of malignant mesotheliomas (II). The immunoreactivity for iNOS was diffuse intracytoplasmic and finely granular. In contrast, iNOS immunoreactivity was negative in all but one of non-neoplastic, non-inflamed pleural tissue samples, while as expected, all inflamed non-neoplastic pleural tissue samples were iNOS positive (II). The results of the present study are in accordance with one previous study on the iNOS immunoreactivity in mesothelioma (Marrogi *et al.* 2000a): these workers found 86% of mesothelioma samples to be iNOS positive. Epithelial and biphasic subtypes of mesotheliomas seemed to be significantly ($p=0.001$ with the poly- and $p=0.023$ with the monoclonal antibody) more often stained positively for iNOS than the sarcomatoid subtype (II), suggesting that this might be a special character of the epithelial mesothelioma. Furthermore, malignant mesothelioma cell lines M14K, M25K, M28K and M38K and transformed mesothelial cells (Met5A) expressed iNOS assessed by RT-PCR (Figure 2 in II) and immunohistochemically (II, data not shown) suggesting that neoplastic mesothelioma cells are capable of synthesizing iNOS also *in vitro*.

With respect to the metastatic pleural adenocarcinomas, 24/25 (96%) were iNOS positive (II). iNOS was significantly more often positive in metastatic adenocarcinomas than in mesotheliomas ($p=0.021$ with the poly- and $p<0.001$ with the monoclonal antibody) (II). The development of mesothelioma is connected with asbestos fibers exposure. The toxicity of asbestos fibers to mesothelial cells has been suggested to be mediated in part by RNS, as discussed in chapter 2.4.3. The greater amount of iNOS positive pleural metastatic adenocarcinomas compared with the mesotheliomas (II) suggests that also factors other than asbestos can evoke induction of iNOS in mesothelioma. These include increased levels of cytokines and growth factors such as IL-6 (Monti *et al.* 1994) and IL-8 (Gaffy *et al.* 1999) produced by mesotheliomas. However, the abundant iNOS immunoreactivity in mesothelioma in the present study (II) is in line with the experimental results showing that asbestos fibers upregulate the formation of RNS in the lungs and pleura (Choe *et al.* 1998, Tanaka *et al.* 1998). There are conflicting results about the involvement of NO in the metastatic potential of tumor cells, as both inhibitory (Xie *et al.* 1998) and promotive (Edwards *et al.* 1996) effects on metastatic capacity have been reported. The results of the present study (II) support the hypothesis of a promotive effect of NO in tumor metastasis and also suggest that the production of iNOS inducing cytokines may be higher in pleural tissues compared with other metastatic sites.

Strongly iNOS positive non-cancerous cell types (II) in tumor tissue included lung alveolar macrophages and macrophages of the tumor tissue. Further, intense iNOS immunoreactivity was seen in neutrophils while lymphocytes were negative. Also endothelial cells and in fibroblasts of the tumor stroma were positive. The amount of iNOS positive stromal macrophages and neutrophils between mesotheliomas and carcinomas did not differ significantly. There was no association between the number of positive stromal macrophages and neutrophils and the iNOS –positive mesotheliomas and/or carcinoma cases (data not shown).

Two iNOS antibodies were used to test the reproducibility of the iNOS immunostaining in the material. Generally, the polyclonal iNOS antibody gave a stronger immunoreaction compared with the monoclonal iNOS, whereas the monoclonal iNOS antibody showed less background staining (II). There was a significant association between iNOS expression between the results obtained with the two iNOS antibodies

(p=0.002) in malignant mesotheliomas and in the whole material consisting also of the adenocarcinomas (p<0.001) (II). With both antibodies, there were significantly more often iNOS negative mesotheliomas than metastatic adenocarcinomas (p=0.021 with the poly- and p<0.001 with the monoclonal antibody) (II). Sarcomatoid mesotheliomas displayed significantly more often no immunostaining compared to epithelial or mixed mesotheliomas (p=0.001 with the poly- and p=0.023 with the monoclonal antibody) (II).

Table 2. Results of immunohistochemical expression of iNOS in non-neoplastic mesothelium, mesotheliomas and metastatic adenocarcinomas studied with the polyclonal iNOS antibody, n=number of cases.

iNOS	Non-neoplastic mesothelium	Reactive mesothelium	Epithelial mesothelioma	Sarcomatoid mesothelioma	Biphasic mesothelioma	Pleural metastatic adenocarcinoma
	n	n	n	n	n	n
-	4	0	2	6	2	1
+	1	3	17	4	2	18
++	0	1	5	0	0	6
Total	5	4	24	10	4	25

5.3.1 iNOS immunoreactivity in mesothelioma and metastatic adenocarcinoma and its association with apoptosis, vascular density and survival (II)

As iNOS was abundantly found in pleural malignancies in the present study (II), NO production might contribute to the progression of these tumors. One possible route is the modulation of apoptosis by NO, as it is one of the known apoptosis inducing factors (Soini *et al.* 1998). Apoptosis contributes to the tumor growth in a complex manner and apoptosis extent is known to vary depending on the tumor type (Soini *et al.* 1998). On the other hand, NO may either inhibit or favor apoptosis depending on NO concentration or cell type (Shen *et al.* 1998). In the present study (II) the average apoptotic index in mesotheliomas was 1.07% (range 0.0-4.8%). There was no statistically significant association between iNOS expression and apoptosis in mesotheliomas (p=0.07 for the monoclonal and p=0.95 for the polyclonal antibody) (II). iNOS immunoreactivity did not associate with patient survival in mesothelioma (p=0.251 and p=0.170 for the poly- and monoclonal antibody, log rank test) (II). In metastatic adenocarcinomas, the mean apoptotic index was 1.73% (range 0.07-7.01%) and it did not associate with iNOS immunoreactivity (p=0.73 for mono- and p=0.37 for polyclonal antibody) (II). These results may be a reflection of a dual role of NO in apoptosis. Simian Virus 40 (SV40) infection, which is linked with the development of mesothelioma and known to inhibit apoptosis, had a suppressive effect on asbestos fibers-induced iNOS mRNA expression and NOS activity in cultured mesothelial and mesothelioma cells (Aldieri *et al.* 2004). This was suggested to be one of the mechanisms leading to decreased apoptosis (Aldieri

et al. 2004). NO has been reported to inhibit APO-1/FAS mediated apoptosis (Mannick *et al.* 1997, Dimmeler *et al.* 1998). Bcl-2 protein family is a group of closely related proteins, which includes both apoptosis-promoting (for instance bax) and apoptosis-inhibiting (for instance bcl-2) members (Soini *et al.* 1998). These proteins operate at cell cycle check points in which it is determined whether a cell is ushered towrd survival or death (Soini *et al.* 1998). Apoptosis caused by NO is regulated also by the bcl-2 protein family, as bcl-2 can inhibit NO induced apoptosis (Kim *et al.* 1998). Bcl-2 is seldom found in mesotheliomas and mesothelial cell lines, contrary to the frequently found bax expression (Segers *et al.* 1994, Soini *et al.* 1999). On the other hand, NO causes accumulation of wild type p53 and bax and results in increased apoptosis (Ambs *et al.* 1998a). The p53 gene is called a guardian of the genome, as in the case of failured DNA repair, p53 triggers apoptosis and prevents malignant transformation (Soini *et al.* 1998). Although p53 mutations are common in human malignancies, they are absent in malignant mesotheliomas (Mor *et al.* 1997). There exists a negative feedback loop between NO and iNOS synthesis, as accumulation of wild type p53 leads to inhibition of iNOS synthesis (Ambs *et al.* 1998a). Thus the effects of NO in apoptosis are regulated in a complex manner.

Angiogenesis, supported by the production of angiogenic factors such as VEGF, is essential for the development of a solid tumor, as tumor cell survival requires blood-borne nutrients (Neufeld *et al.* 1999). Tumor vascularization may be influenced by expression of iNOS, as it upregulates the synthesis of VEGF (Ziche *et al.* 1997). iNOS expression might thus act as a promoting factor for tumor progression through stimulation of angiogenesis in mesothelial tumors. In mesotheliomas, the average number of vessels/HPF was 8.7 (range 1-58), and it did not have statistically significant association with iNOS expression ($p=0.29$ for the monoclonal and $p=0.25$ for the polyclonal iNOS antibody) (II). In metastatic adenocarcinomas, the average vascular density was 6.88/HPF (range 1.38-20.40) (II). There was no statistically significant difference in vascular density in metastatic adenocarcinomas as compared with mesotheliomas ($p=0.33$) (II). Furthermore there was no statistically significant association between iNOS expression and vascular density in metastatic adenocarcinomas either ($p=0.37$ for the monoclonal and $p=0.82$ for the polyclonal antibody) (II). As there was no significant association with iNOS expression and vascular density in malignant mesotheliomas (II), it could be proposed that iNOS synthesis by mesothelioma tumor cells may not have a crucial role in angiogenesis. This is in contrast with a *in vitro* study, where NO produced by iNOS provided a growth advantage in lung cancer cells with mutant p53 by inducing VEGF and neovascularization (Ambs *et al.* 1998c) Thus the effect of NO on angiogenesis may be variable also according to the progenitor cell type. However, microvessel density *per se* does not necessarily reflect the total angiogenic activity of a tumor, and all tumor microvessels are not functionally equal (Hlatky *et al.* 2002). Further, angiogenesis is regulated in a complex manner by various angiogenic factors (Folkman 2003). Also tumor stromal cells are capable of synthesizing iNOS and might be involved in the regulation of angiogenesis of the tumors.

In conclusion, patient survival did not associate with iNOS immunoreactivity, and thus it is not a major prognostic factor in mesothelioma. Further, strong iNOS immunoreactivity was not associated with apoptosis or vascular density. This might be due to complex regulation of apoptosis and neovascularization in mesothelioma by

various other factors in addition to NO, and also stromal cells may have an effect on these phenomena.

5.4 Immunoreactivity of eNOS, VEGF, FLK1 and FLT1 in mesothelioma and their association with vascular density and survival (III)

In the present study, most mesotheliomas were positive for eNOS 32/36 (89%) (III). eNOS immunoreactivity was cytoplasmic and diffuse, and often granular (Figure2a in III). There are no previous reports on eNOS immunoreactivity in mesothelioma. The results of immunohistochemistry for eNOS, VEGF, FLK1 and FLT1 are collated in Table 3. eNOS immunoreactivity did not differ significantly between sarcomatoid or other subtypes of mesotheliomas (p=0.16), assessed in these restricted number of cases (III). Non-neoplastic cells exhibiting eNOS immunoreactivity included endothelial cells of the blood vessels, pulmonary macrophages, bronchial epithelial cells, and fibroblasts in the reactive tumor stromal tissues (III). There was no eNOS immunoreactivity detectable in alveolar epithelial cells, lymphocytes, adipocytes and smooth muscle cells in the walls of the vessels (III). Infrequent positivity was seen in normal mesothelial cells but reactive mesothelial cells were strongly stained (III). Thus eNOS was localized both in malignant and non-malignant lung cells.

RT-PCR experiments of cultured mesothelioma cell lines M14K and M38K and non-neoplastic MeT-5A cells indicated that these cells are capable of synthesizing eNOS mRNA (III). Also the positive eNOS immunoreactivity was seen in the cell pellets from these cell lines (data not shown). These experiments suggest that neoplastic mesothelial cells are capable of expressing eNOS also *in vitro*.

The immunoreactivity for VEGF, FLK1 and FLT1 was cytoplasmic and diffuse; immunoreactivity was seen in 17(47%), 24 (67%) and 25 (69%) mesotheliomas, respectively (III). This positivity was seen also in metaplastic alveolar cells (III). Immunoreactivity of non-neoplastic cells represented endothelial cells of the blood vessels, where VEGF, FLK1 and FLT1 positivity was seen (III). Non-neoplastic mesothelial, bronchial, and alveolar epithelial cells were negative, as was the case also with alveolar macrophages (III). Thus immunoreactivity of VEGF and its receptors was not as widely dispersed as eNOS positivity. VEGF or eNOS immunoreactivity did not correlate with the VEGF receptors FLT1 (VEGF p=0.30; eNOS 0.36) and FLK1 (VEGF p=0.38; eNOS p=0.72), but there was a correlation between these two receptors (p=0.013) (III). In epithelioid or biphasic mesotheliomas, FLK1 and FLT1 immunoreactivity (p=0.007 and 0=0.011, respectively) was more often seen than in sarcomatoid mesotheliomas, but there was no association with VEGF (p=0.57) (III). However, there was a tendency to more frequent VEGF immunoreactivity if epithelioid mesotheliomas were compared with grouped biphasic and sarcomatoid mesotheliomas, but the difference was not statistically significant (p=0.06)(III). Similarly, in epithelioid mesotheliomas, there was more FLK1 positivity than found in the biphasic and

sarcomatoid counterparts ($p<0.001$) and also in FLT1, the immunoreactivity trends were similar, but not significant ($p=0.08$) (III). Between FLK1, FLT1, VEGF or eNOS immunoreactivity there was no significant association ($p=0.72$, $p=0.36$, $p=0.45$, respectively) (III).

The vascular density was 8.6±6.1, 10.9±/6.1, 8.7±2.1 vessels/HPF in epithelioid mesotheliomas, sarcomatoid and biphasic mesotheliomas, respectively (III). There was no significant association between eNOS, VEGF, FLK1 and FLT1 immunoreactivity and vascular density ($p=0.49$, $p=0.28$, $p=0.36$, $p=0.70$, respectively). Moreover, vascular density did not associate with tumor subtype ($p=0.18$).

Regardless of the strong immunoreactivity in mesothelioma cells eNOS positivity did not associate with patient survival ($p=0.22$, log rank). Likewise, no association was found with VEGF, FLK1 or FLT1 and survival ($p=0.11$, $p=0.18$, $p=0.19$, respectively). There was no difference was between sarcomatoid and epithelioid or biphasic mesotheliomas and survival ($p=0.28$).

There are few reports about the extent of VEGF positivity in human malignant mesothelioma. Ohta *et al.* (1999) found positive VEGF, KDR i.e. FLK1, and FLT1 mRNA in 75.9%, 90.7% and 74.1% of 54 malignant mesotheliomas, respectively. The mean VEGF, FLK1 and FLT1 densitometry index was higher than in normal pleural mesothelium in 31.5%, 42.6% and 20.4% cases, respectively (Ohta *et al.* 1999). These workers observed that immunohistochemically detected microvessel density was a negative prognostic factor (Ohta *et al.* 1999). In another study, VEGF positivity was found in 80.8% of mesotheliomas, and non-neoplastic pleura showed mostly weak to moderate VEGF reactivity (Kumar-Singh *et al.* 1999). There was no significant correlation between prognosis and VEGF immunoreactivity or microvascular density (Kumar-Singh *et al.* 1999). These workers did not find any association between the immunohistochemical expression levels of VEGF and intratumoral microvascular density or histological tumor type (Kumar-Singh *et al.* 1999). Although patients with high levels of VEGF immunoexpression had shorter survival, the association was not statistically significant (Kumar-Singh *et al.* 1999). The results of the present study (III) did not show as intense immunoreactivity for VEGF as this previous immunohistochemical study (Kumar-Singh *et al.* 1999), but the results otherwise were in line with that study concerning the association of VEGF with vascular density and prognosis (III).

VEGF has been associated strongly with tumor neovascularization (Neufeldt *et al.* 1999). At least in endothelial cells, VEGF seems to induce expression of eNOS (Kroll *et al.* 1997). Also FLK1 activation in endothelial cells leads to NO release (Kroll *et al.* 1997). NO in turn influences vascular angiogenesis by up-regulating the synthesis of VEGF (Kroll *et al.* 1997). eNOS might have an important role in tumor growth and metastasis by stimulating angiogenesis and cell migration (Jadeski *et al.* 2000). In mesothelioma there was no association between vascular density and eNOS immunoreactivity in the present study (III). Thus in malignant mesothelial cells, eNOS regulation by VEGF might be different from that occurring in endothelial cells and some other tumors. Some studies have suggested that eNOS levels would correlate with tumor progression (Lala *et al.* 1998, Thomsen *et al.* 1998). The present study (III) did not support this view in malignant mesothelioma, since the prominent eNOS immunoreactivity in mesotheliomas was not associated with prognosis (III).

In epithelioid and biphasic mesotheliomas, FLK1 and FLT1 immunostainings were more intense compared with sarcomatoid mesotheliomas (III). A similar trend was also seen with VEGF immunoreactivity, which was more pronounced when sarcomatoid and biphasic tumors were grouped together (III). In contrast to what might be expected, vascular density was not significantly lower in sarcomatoid tumors (III). Consequently, FLK1, FLT1 and VEGF immunoreactivities can be considered as characteristics of the epithelioid type of mesothelial cells. It is noticeable that the same kinds of differences between sarcomatoid and epithelioid mesotheliomas have been observed in the expression of different proteins such as manganese superoxide dismutase (Kahlos *et al.* 2000). Possibly because of its more constitutive expression, eNOS did not have such correlation with mesothelioma subtype (III). There was no difference in survival between sarcomatoid and epithelioid or biphasic mesotheliomas in the present study regardless of the immunoreactivity differences noted between different subtypes of mesotheliomas (III). This presumes that genomic expression which parallels phenotypic changes in malignant mesothelial cells might not influence their overall prognosis to any significant degree. Thus, it is not surprising that patient survival did not associate with VEGF, FLT1 or FLT1 expression (III).

In conclusion, in malignant mesothelioma eNOS immunoreactivity was strong (III). It was also shown that malignant mesotheliomas can synthesize VEGF and its receptors FLK1 and FLT1 (III), suggesting that these compounds have some effect on tumor angiogenesis. On the other hand they might also affect the growth of malignant mesothelioma cells in an autocrine manner. These factors were not significantly correlated with the vascular density (III), suggesting that their role is not critical. This might be explained by other confounding factors contributing to angiogenesis, such as the influence of inhibitors or promoters originating from the stromal and tumor cells.

Table 3. eNOS, VEGF, FLK1 and FLT1 immunoreactivity in mesothelioma, n= number of cases.

eNOS	Epithelioid mesothelioma	Sarcomatoid mesothelioma	Biphasic mesothelioma
	n	n	n
-	2	1	1
+	13	1	1
++	9	6	2
Total	24	8	4
VEGF			
-	10	5	4
+	7	2	0
++	7	1	0
Total	24	8	4
FLK1			
-	2	8	2
+	9	0	1
++	13	0	1
Total	24	8	4
FLT1			
-	5	6	0
+	7	2	1
++	12	0	3
Total	24	8	4

5.5 iNOS, eNOS, nNOS and nitrotyrosine in metaplasia-dysplasia-sequence of bronchial epithelium (IV)

Cellular proliferation and promotion of carcinogenesis might be enhanced by increased iNOS expression by oxidant mediated mechanisms. This proposal is in keeping with the importance of oxidants and cellular redox balance in carcinogenesis and cell proliferation (Oberley *et al.* 2002, Kinnula *et al.* 2004). In the present study iNOS, nNOS and nitrotyrosine immunoreactivities were was mostly moderate or strong in metaplastic and dysplastic bronchial biopsies (Table 7) (IV). Compared to normal-looking central bronchus epithelium of smokers' and patients with COPD, iNOS ($p<0.001$) and nNOS ($p<0.001$) was statistically significantly more often positive in metaplasias and dysplasias (IV). As central airways were not stained for nitrotyrosine, the comparison between normal bronchial epithelium and metaplastic/dysplastic epithelium was not available (IV). In contrast to iNOS, nNOS and nitrotyrosine, eNOS was mostly negative in metaplastic and dysplastic bronchial lesions (IV).

This clear immunoreactivity of iNOS and nNOS observed in the majority of cases in metaplasia-dysplasia-sequence of the bronchial epithelium represents a new and important finding (IV), as there are no previous published studies on NOSs in human

bronchial dysplasia. There are some other dysplasias where intense iNOS immunoreactivity has been observed, for example in oral (Chen *et al.* 2002) and esophageal (Wilson *et al.* 1998) precancerous lesions.

There are animal studies supporting the hypothesis of a promotive effect of iNOS on carcinogenesis. For example iNOS-deficient mice developed 80% less chemically induced lung tumors compared with the wild type mice (Kisley *et al.* 2002). Further, in bronchial dysplasia, the levels of antioxidant enzymes and related proteins (manganese superoxide dismutase and thioredoxin) are also elevated (Soini *et al.* 2003). These enzymes are known to be highly induced by oxidant stress (reviewed by Kinnula & Crapo 2004). Thus the positive nitrotyrosine immunoreactivity (a stable marker of RNS/peroxynitrite -mediated tissue injury) detected in the metaplasia-dysplasia-sequence of the bronchial epithelium (IV) is also in line with those observations. Clear iNOS, nNOS and nitrotyrosine positivity in the metaplasia-dysplasia-sequence of the bronchial epithelium compared to normal bronchial epithelium of smokers suggests that induction of NOSs in the bronchial epithelium might well be linked to the early stage of carcinogenesis.

5.6 Immunoreactivity of iNOS, eNOS and nNOS in NSCLC and their relation to tumor type, grade, proliferation, apoptosis and survival (V)

5.6.1 iNOS, eNOS and nNOS expression and immunoreactivity in NSCLC (V)

Preliminary studies i.e. RT-PCR for iNOS and eNOS of human lung carcinoma cell lines indicated that all of the investigated human lung carcinoma cell lines (A549, CALU-6, Sk-MES-1, A427) were capable of synthesizing iNOS mRNA *in vitro* (V). In contrast, eNOS mRNA was detectable only in non-malignant BEAS-2B cells and in malignant A427 cells (V). Compared with neoplastic mesothelial cell lines the lung carcinoma cell lines were different, because some of lung carcinoma cell lines (A549, CALU-6, Sk-MES-1) were not able to produce eNOS mRNA *in vitro* (V). In contrast, all neoplastic mesothelial cell lines produced both iNOS (II) and eNOS (III) mRNA. Non-malignant bronchial BEAS-2B cells were iNOS negative (V), as expected, but non-malignant mesothelial MeT-5A cells expressed iNOS mRNA (II). In line with its constitutive nature, eNOS was positive in both non-malignant bronchial BEAS-2B and mesothelial MeT-5A cell lines (III).

The outcome of immunohistochemical staining in the whole NSCLC patient material is presented in Tables 4-6 (V). iNOS positivity was seen in 36/89 (40 %) of lung cancer cases (V). In the iNOS negative tumor samples, nonetheless, strong positivity for iNOS was observed in intra-alveolar macrophages cells (Figure 2A in V). Also some iNOS

positive macrophages were found amongst the tumor cells were found in the tumor tissue (V). iNOS positivity was significantly less prominent in tumor cells compared with eNOS and nNOS positivity ($p<0.001$) (V). No association was found with iNOS and the histological type of the tumor ($p=0.40$). In contrast, grade I-II tumors exhibited significantly more cases showing iNOS immunoreactivity compared with grade III tumors ($p=0.024$).

The constitutive forms of NOS i.e. eNOS and nNOS showed immunoreactivity in 79/89 (89%) and 72/89 (81%) cases, respectively. According to the results, eNOS ($p<0.001$) and nNOS ($p<0.001$) positivities were found in tumor cells more often than iNOS positivity ($p<0.001$), while no significant difference was found between eNOS and nNOS ($p=0.10$). Adenocarcinomas exhibited significantly more often strong immunoreactivity for eNOS than squamous cell carcinomas ($p=0.016$) while no such association was found with nNOS immunoreactivity ($p=0.28$) (V). There was no association between grade of the tumor for eNOS ($p=0.90$) or nNOS ($p=0.76$) (V).

Reports of NOSs in lung cancer have evaluated the role of these enzymes mainly by indirect methods such as by measuring NOS activity in tissue homogenates (Fujimoto *et al.* 1997, Fujimoto *et al.* 1998), measuring nitrite/nitrate levels in bronchoalveolar lavage (BAL) fluid (Arias-Diaz *et al.* 1994, Liu *et al.* 1998) and by measuring the levels of exhaled NO from lung carcinoma patients (Liu *et al.* 1998). These studies have reported generally increased NOS activity. Nonetheless, also opposing results, such as reduced Ca^{2+} dependent NOS activity reflecting reduced eNOS and nNOS expression, have been observed in lung tumors compared with adjacent non-malignant tissue (Ambs *et al.* 1998a). One study attributed most of the NOS activity to the constitutive form of NOS in lung adenocarcinomas (Fujimoto *et al.* 1997).

There are a few studies which have examined the immunohistochemical characterization of NOSs in NSCLC: iNOS (Marrogi *et al.* 2000b) and nNOS (Lewko 2001) have been characterized, but the distribution of eNOS in NSCLC has not been described. Marrogi *et al.* (2000b) found iNOS positivity in 48% of NSCLC cases. Also Fujimoto and co-workers (1997), found slight to moderate iNOS immunoreactivity in approximately half of their cases. However, interpretation of this study results is limited by the fact that iNOS immunohistochemistry was performed in 16 of 72 studied cases (Fujimoto *et al.* 1997). The present study came to similar results, i.e. iNOS positivity was demonstrated in 40 % of the cases (V). In contrast to one published report associating iNOS immunoreactivity with adenocarcinoma subtype (Marrogi *et al.* 2000b); the present study did not find iNOS immunoreactivity to associate with adenocarcinomas or any other subtype of NSCLC (V). These conflicting results might in part be explained by the different proportions of various NSCLC subtypes in these two studies. In Marrogi's *et al.* study (2000b) the proportion of adenocarcinomas and squamous cell carcinomas were 52% and 27%, respectively, while in the present study (V) the corresponding proportions were 35% and 64%.

Contrary to results of Fujimoto *et al.* 1997 where most cases were eNOS negative, the present study found 89 % of the cases to be eNOS positive (V). The eNOS -antibody used (Fujimoto *et al.* 1997, Transduction Laboratories) was different from that used in the present study (V, Santa Cruz Biotechnology Inc). However, no significant difference was observed between these two antibodies in their abilities to detect eNOS (Vakkala *et al.* 2000b). Thus, the possible cause of difference between the present study results and

Fujimoto's (1997) findings perhaps is be due to the different fixation protocols or studied sample size rather than to the different antibodies used. In agreement with the results of the present study concerning nNOS immunoreactivity (V), also Fujimoto *et al.* 1997 and Lewko *et al.* (2001) found nNOS immunoreactivity in the majority of NSCLC tumors.

Table 4. Expression of iNOS, eNOS and nNOS in lung carcinoma according to a sum score formed of the intensity and quantity of NOSs staining; n= number of cases.

Sum score= intensity+quantity	iNOS (n)	eNOS (n)	nNOS (n)
Negative - Score 0	53	10	17
Low intensity + Score1-4	32	41	34
High intensity ++ Score 5-8	3	38	37

Owing to exhaustion of blocks, immunostaining could not be performed in all cases.

Table 5. Distribution of iNOS, eNOS and nNOS immunoreactivity according to the histological type of NSCLC, n=number of cases.

NSCLC subtype	Negative n (%)	Mildly positive, score ≤4, n (%)	Strongly positive, score >4, n (%)
Adenocarcinoma			
iNOS	17(65%)	7(27%)	2(8%)
eNOS	0(0%)	11(41%)	16(59%)
nNOS	4(15%)	9(33%)	14(52%)
Squamous cell carcinoma			
INOS	34(60%)	22(39%)	1(1%)
eNOS	10(18%)	27(47%)	20(35%)
nNOS	13(23%)	23(41%)	20(36%)
Bronchioloalveolar carcinoma			
iNOS	1(25%)	3(75%)	0(0%)
eNOS	0(0%)	2(50%)	2(50%)
nNOS	0(0%)	1(25%)	3(75%)
Large cell carcinoma			
iNOS	1(100%)	0(0%)	0(0%)
eNOS	0(0%)	1(100%)	0(0%)
nNOS	0(0%)	1(100%)	0(0%)

Table 6. Expression of iNOS, eNOS, and nNOS in different grades of lung carcinoma, n= number of cases.

Grade	Negative n (%)	Mildly positive, score ≤4, n (%)	Strongly positive, score >4, n (%)
Grade I			
iNOS	8(67%)	4(33%)	0(0%)
eNOS	0(0%)	9(69%)	4(31%)
nNOS	2(17%)	6(50%)	4(33%)
Grade II			
iNOS	18(46%)	18(46%)	3(8%)
eNOS	6(15%)	13(33%)	20(52%)
nNOS	7(21%)	14(42%)	12(37%)
Grade III			
iNOS	22(76%)	7(24%)	0(0%)
eNOS	4(14%)	14(48%)	11(38%)
nNOS	7(24%)	12(41%)	10(35%)

5.6.2 Immunoreactivities of iNOS, eNOS and nNOS and their association with cell proliferation, apoptosis and survival in NSCLC (V)

Cell proliferation was determined by assaying the levels of proliferating cell nuclear antigen (PCNA). The mean PCNA index was 27%±13% (range 50%, min.10%, max. 60%). PCNA did not associate with iNOS positivity (p=0.32). The mean apoptotic index in tumors was 1.23%±1.18% (range 6.06, min. 0.03, max. 6.09%). There was a tendency towards a higher apoptosis index in tumors with negative iNOS immunoreactivity compared with tumors with positive iNOS immunoreactivity (1.40±1.26 vs. 0.96±1.00 %, respectively), but the association did not reach the level of statistical significance (p=0.072). There was no association between PCNA or the apoptotic index for eNOS (p=0.41, p=0.46, respectively) or nNOS (p=0.17, p=0.32, respectively) (V).

In survival analysis iNOS immunoreactivity did not associate with survival (V). Similarly, eNOS immunoreactivity did not correlate with survival (p=0.11, log rank; p=0.19, Breslow; p=0.14, Tarone-Ware), and this was the case also with nNOS (p=0.06, log rank; p=0.04, Breslow; p=0.048, Tarone-Ware), although there was a tendency towards better survival in those cases where there was more pronounced nNOS immunoreactivity. In some malignancy types, such as gynecological cancers (Thomsen *et al.* 1994) and brain (Cobbs *et al.* 1995) tumors, intense nNOS expression has been associated with poorly differentiated tumors. This does not seem to be the case in NSCLC, according to the results of the present (V) and a previous report (Lewko *et al.* 2001).

In view of the fact that all NOSs contribute to the generation of NO, we also formulated a sum index (iNOS+eNOS+nNOS) (V) to see whether the total effect would have any impact on the patients' prognosis (1=low NOS expression, scores 0-4; 2=high NOS expression, scores 5-8). The high sum index in tumors associated with significantly better survival than the low sum index (Figure 3 in V, p=0.041, log rank; p=0.036, Breslow; p=0.038, Tarone-Ware). It has been reported that NOS activity from patients with N2 disease tended to be lower in those than with N0 or N1 disease while T stage did not correlate with NOS activity of the cancer tissues (Fujimoto *et al.* 1997). This is consistent with the results of the present study where a better prognosis was associated with the high NOSs sum index (V). Nevertheless, the sum index did not have an independent prognostic value according to Cox's multivariate analysis (p=0.12) (V). Only tumor size had an independent prognostic value (p=0.0079) when all the variables (tumor size, nodal metastasis, gradus, histological type, postoperative therapy, apoptotic index, proliferation index, NOSs sum index) were analyzed (V).

There are several pathways by which NO might take part in tumor development, for instance by inducing angiogenesis (Ziche *et al.* 1994) or by suppressing apoptosis (Mannick *et al.* 1997), both mechanisms would enhance tumor promotion. Nevertheless, NO has also been reported to retard tumor growth and induce apoptosis (Bonfoco *et al.* 1995). Stimulation of iNOS and NO by 17β-estradiol and progesterone leads to inhibition of proliferation in ovarian carcinoma cells (Keith *et al.* 2001). The present study did not find any correlation between apoptosis and proliferation as assessed by PCNA immunostaining (V) in NSCLC. This method, however, provides only an approximation of the evaluation of proliferating cells. Furthermore, there was not any significant association between apoptosis and the expression of the NOSs in NSCLC (V). This is in contrast with results from breast carcinoma samples, where apoptosis appeared to be associated with positive iNOS expression (Vakkala *et al.* 2000a). A similar kind of tendency has been found in ovarian cancer cell lines (Keith *et al.* 2001) and in pancreatic tumors (Hajri *et al.* 1998). Thus, the influence of NO in carcinogenesis may be complex as it seems to be cell-specific and depend on the NO concentration. Consequently, the growth of lung tumors might be modulated by expression of NOSs, even though no significant association with apoptosis or proliferation found in the present study (V).

Both immunoreactivity and activity of iNOS have been associated with disease progression in some malignancies, such as gynecological and breast cancers (Thomsen *et al.* 1994, Thomsen *et al.* 1995, Reveneau *et al.* 1999). However, not all studies have detected an association between tumor grade and eNOS or iNOS immunoreactivity, even including studies on breast cancer (Vakkala *et al.* 2000a & b). A positive estrogen and progesterone receptor status was correlated with eNOS immunoreactivity, suggesting that there is major NO synthesizing capability in malignant tumors with a higher degree of differentiation i.e. tumors which still harbor these receptors (Vakkala *et al.* 2000b). In NSCLC, an association between iNOS immunoreactivity and microvessel density was observed, but neither of them correlated with the prognosis of the patients (Marrogi *et al.* 2000b). The present study (V) exhibited increased iNOS immunoreactivity in 40 % of cancer samples and in grade I-II NSCLC (V) but this did not associate with survival (V); this is in line with previous reports in NSCLC (Marrogi *et al.* 2000b). Therefore the effect of iNOS in tumor progression might be dependent in part on the progenitor cell type.

In summary, intense eNOS and nNOS immunoreactivities were found in lung carcinoma cells (V). The immunoreactivity of iNOS was less abundant (V). Better survival of the patients was associated with a high sum index of NOSs immunoreactivity, but on the other hand only tumor size had an independent prognostic value in the multivariate analysis (V). These results imply that NOSs activity might have an effect on survival, possibly through regulation of tumor size. To evaluate this hypothetical connection, further studies are needed.

5.7 iNOS, eNOS, nNOS and nitrotyrosine in healthy lung compared with smokers' and COPD lung (IV)

Results of iNOS, eNOS, nNOS and nitrotyrosine immunostainings in COPD are presented in Table 7 (IV). Positive, patchy, mostly weak, iNOS immunoreactivity was found in central bronchial epithelium, bronchiolar epithelium and alveolar macrophages in healthy lung of non-smokers, smokers, and in the COPD groups (IV). Also iNOS positive neutrophils were detected (data not shown) (IV). Alveolar epithelium was negative, except for one case from a non-smoker (IV). There was no significant difference between the groups with respect to their iNOS immunoreactivity ($p=0.421$, $p=0.905$, $p=0.461$, $p=0.222$ for central bronchus epithelium, bronchial epithelium, alveolar macrophages and alveolar epithelium, respectively) (IV). Strong positive immunoreactivity for eNOS was found in alveolar macrophages, especially in the COPD group (IV) but there were no differences between the groups ($p=0.129$) (IV). Less prominent positivity for eNOS was found also in central bronchus epithelium ($p=0.446$), bronchiolar epithelium ($p=0.513$) and alveolar epithelium ($p=0.699$) and peripheral lung vessels ($p=1.0$) in all studied groups, once again there were no differences between groups with respect to eNOS immunoreactivity (IV). nNOS was negative of all cases in peripheral lung samples in all groups, except in bronchiolar epithelium of two non-smokers where positive signs were detected (IV).

Several studies have investigated iNOS in COPD, mostly in sputum samples (Rutgers *et al.* 1999, Ichinose *et al.* 2000, Ichinose *et al.* 2003). There are also two studies which have examined iNOS in lung specimens of COPD patients, but they have found variable NOS expression (van Straaten *et al.* 1998, Maestrelli *et al.* 2003). The earlier study found lower expression of iNOS and eNOS in the alveolar macrophages of severe COPD patients compared to subjects with mild emphysema, occasional iNOS and eNOS immunoreactivity in endothelial cells and the brush border of bronchiolar epithelium, and apparently negative immunoreactivity in alveolar epithelium (van Straaten *et al.* 1998). In contrast, a recent study found higher iNOS immunoreactivity in type II pneumocytes in patients with severe COPD compared to smokers without COPD (Maestrelli *et al.* 2003). On the other hand in a study on unselected patients, focal iNOS positive alveolar wall staining has been interpreted to represent patchy capillary staining and not hyperplastic type II cells (Kobzig *et al.* 1993). Additionally, in one sputum study macrophage iNOS expression in stable COPD and controls was very similar, while the levels of exhaled NO in COPD was associated with the numbers of eosinophils in the sputum (Rutgers *et al.*

1999). Based on previous and present (IV) findings it appears that iNOS is not significantly induced in the alveolar epithelium of the lungs of smokers or in patients with mild to moderate COPD or in the alveolar macrophages of stable COPD subjects. Small levels of iNOS might remain undetected by immunohistochemistry. In the present study (IV), high iNOS expression in these COPD samples was, however, excluded by using positive controls i.e. sarcoid granulomas where iNOS immunoreactivity is known to be strong (Lakari *et al.* 2002). In preliminary studies (not shown) the results were also observed to be similar with the two commercial iNOS antibodies used in the previous and present staining experiments. The results (IV) do not exclude the possibility of iNOS induction during COPD exacerbation.

There was a significant difference in the level of immunostaining for nitrotyrosine between non-smokers and smokers without COPD or with COPD in peripheral lung alveolar macrophages specimens (IV). In the non-smoker group**,** alveolar macrophages of all 13 cases were negative while 7/20 cases of smokers and 12/21 of COPD subjects were nitrotyrosine positive (p=0.025, p=0.001**,** respectively) (IV). The difference was even more marked when non-smokers were compared to the combined group of smokers and COPD patients (p=0.004) (IV). Nitrotyrosine positivity could be detected in only five cases in alveolar epithelium of non-smokers but it was positive in most samples from smokers and COPD patients (non-smoker vs. smoker groups, p=0.065; non-smoker vs. COPD groups, p=0.062) (IV). When a comparison was made between non-smokers and combined groups of smokers and COPD, the difference was statistically significant (p=0.049) (IV).

Consistent with previous studies in sputum samples (Ichinose *et al.* 2000, Kanazawa *et al.* 2003, Sugiura *et al.* 2003), in the present study alveolar macrophages of the COPD patients were more often nitrotyrosine positive than macrophages of the normal lung (IV). However, nitrotyrosine was also more often positive both in the alveolar macrophages and alveolar epithelium of smokers without COPD than in the corresponding cells of non-smokers (IV). This suggests that the destruction of airspaces occurring during emphysema progression in sensitive subjects might be modulated by oxidative/nitrosative stress possibly leading to cellular apoptosis (Aoshiba *et al.* 2003), but also other factors might be needed for development of emphysematic lesions. In addition nitrotyrosine can be considered as a very sensitive marker of oxidative/nitrosative phenomena in the lung.

Nitrotyrosine is considered to be a stable end product and biomarker of both ROS (produced by enzymes such as myeloperoxidase) and RNS related cell injury (van der Vlient *et al.* 1999, Reiter *et al.* 2000, Davis *et al.* 2001) and is closely linked association to apoptosis (Liversidge *et al.* 2002). Apoptosis is considered as one of the causes for emphysematous changes in the lung (Aoshiba *et al.* 2003, Tuder *et al.* 2003). The positive nitrotyrosine immunoreactivity in smokers (IV) without a clinical COPD diagnosis (i.e. no decline in their lung function parameters) suggests that nitrotyrosine can be considered as a sensitive marker for the impact of tobacco smoking on the lung. The nitrotyrosine positivity observed in this study (IV), in relation to the negative iNOS immunoreactivity in the alveolar epithelium in COPD, indicates that alveolar epithelial injury might be oxidant/NO mediated, but iNOS might not be the major source of NO. Other possible sources of NO include eNOS and nNOS. However, eNOS produces relatively small amounts of NO and nNOS immunoreactivity was mainly negative in the present study

(IV), thus these enzymes might not play a significant role in formation of nitrotyrosine. One must also remember that tobacco smoke itself contains large amounts of NO (Pryor & Stone 1993). The NO inhaled in cigarette smoke, in combination with ROS, might be a source for peroxynitrite formation in lungs. The numbers of ROS producing cells, such as alveolar macrophages and neutrophils become elevated in smoker's lung and these cells have also been shown to express iNOS especially during COPD exacerbation (Barnes 2000). Moreover, especially neutrophils are potent generators of ROS and can form these species by several other oxidant generating mechanisms such as NADPH oxidase and myeloperoxidase, the levels of which have also been shown to associate with tissue destruction and nitrotyrosine positivity (Fantone *et al.* 1985, van der Vlient *et al.* 1999, Davis *et al.* 2001, Gaut *et al.* 2002). This also suggests that the nitrotyrosine mediated injury is not necessarily related solely to iNOS induction in the epithelium, but more likely to the increased oxidative/nitrosative stress caused by inflammatory cells in COPD and maybe in part to NO inhaled in tobacco smoke. Oxidant injury in COPD is still further enhanced by the evident imbalance between the oxidants/antioxidant systems and the decline in the antioxidant defense in smokers' lung (Harju *et al.* 2002). Although nitrotyrosine immunoreactivity was increased in both smokers and COPD groups compared with non-smokers (IV) these two smoker groups did not differ significantly from each other. This suggests that in addition to RNS also other factors are needed in the development of COPD changes.

In an experimental setting VEGF inhibition has caused endothelial cell apoptosis associated lung emphysema in animals (Kasahara *et al.* 2001). It remains to be determined, whether the VEGF -inhibitors now being introduced into the treatment of cancer (Kabbinavar *et al.* 2003, Hurwitz *et al.* 2004) will possess side effects leading to emphysematic lesions of the lung.

In conclusion, nitrotyrosine is a sensitive early marker of ROS/RNS mediated effects in the lungs of smokers. Inducible NOS was not detected by immunohistochemistry in the alveolar epithelium in non-smokers, smokers and COPD, but was positive both in alveolar macrophages and neutrophils, cell types which are abundantly present in the lungs of smokers. Also eNOS was present in a variety of cell types in non-smokers, smokers and COPD, but nNOS was never detected. The precise role of NOSs in the development of COPD lung changes remains to be clarified

Table 7. Immunohistochemical expression of inducible NOS (iNOS), endothelial NOS (eNOS), neuronal NOS (nNOS) and nitrotyrosine in the major cell types of non-smokers, smokers without COPD, subjects with COPD and bronchial dysplastic lesions, n= number of cases.

Group	iNOS				eNOS				nNOS				Nitrotyrosine			
	Immunoreactivity, combined score				Immunoreactivity, combined score				Immunoreactivity, combined score				Immunoreactivity, combined score			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Control group, n=13																
central bronchus epithelium	7	6	-	-	3	9	1	-	13	-	-	-	NA	NA	NA	NA
bronchiolar epithelium	8	4	-	-	5	8	-	-	11	2	-	-	13	-	-	-
alveolar epithelium	11	1	-	-	10	2	1	-	13	-	-	-	8	4	1	-
alveolar macrophages	6	5	1	-	1	4	2	5	13	-	-	-	13	-	-	-
peripheral vessels	12	-	-	-	12	1	-	-	13	-	-	-	13	-	-	-
Smokers, non-COPD, n=20																
central bronchus epithelium	13	5	2	-	9	9	2	-	20	-	-	-	NA	NA	NA	NA
bronchiolar epithelium	12	7	1	-	5	10	3	2	20	-	-	-	17	1	-	-
alveolar epithelium	20	-	-	-	16	3	1	-	20	-	-	-	5	12	1	-
alveolar macrophages	5	13	2	-	5	7	2	6	20	-	-	-	11	7	-	-
peripheral vessels	20	-	-	-	19	1	-	-	20	-	-	-	18	-	-	-
COPD, n=22																
central bronchus epithelium	15	7	-	-	11	8	3	-	22	-	-	-	NA	NA	NA	NA
bronchiolar epithelium	16	5	1	-	8	10	3	-	22	-	-	-	19	2	-	-
alveolar epithelium	22	-	-	-	19	3	-	-	22	-	-	-	6	14	1	2
alveolar macrophages	6	11	5	-	2	3	2	15	22	-	-	-	9	10	2	-
peripheral vessels	22	-	-	-	20	2	-	-	22	-	-	-	21	-	-	-
Metaplastic /Dysplastic bronchial lesions, n=24	2	9	7	6	17	5	-	-	8	5	3	6	4	9	4	-

Owing to exhaustion of the blocks, immunostainings could not be performed in all cases. NA = not available.

6 Conclusions

The present study assessed the role of reactive oxygen species, especially nitric oxide mediated pathways in the development of asbestos fiber -induced cell and DNA damage, and the immunoreactivity of different NOS isoforms in mesothelioma, the bronchial metaplasia-dysplasia-sequence, NSCLC, and lung specimens from smokers and COPD patients, and the results were compared with those obtained from normal pleura and lung tissue.

It was shown *in vitro* that asbestos exposure caused a minor cell injury only in the mesothelial MeT-5A cell, but not in the bronchial A549 cells. The MeT-5A cells had a lower intracellular glutathione content, and they were also more sensitive to oxidant effects measured by H_2O_2 exposure. The mean tail extent moment, a marker of DNA strand breaks, increased significantly in both MeT-5A and A549 cell lines after exposure to asbestos fibers. The pre-treatment with BSO, an inhibitor of the synthesis of the anti-oxidant enzyme γ-GCS, potentiated the amount of DNA single-strand breaks. On the other hand, pre-treatment with L-NAME, an inhibitor of NO generation did not have any effect on the mean tail moment. Asbestos fiber exposure did not cause any induction of iNOS protein expression in either cell line measured by flow cytometry. The immunohistochemical expression of γ-GCS heavy – and light subunits was negative or very weakly positive in healthy pleural mesothelium, while bronchial epithelium showed weak to moderate immunoreactivity of these subunits.

In normal pleura, iNOS expression was negative, but inflamed reactive mesothelium exhibited iNOS positivity. Malignant pleural mesotheliomas and metastatic pleural adenocarcinomas were in most cases iNOS positive. Sarcomatoid mesotheliomas were more often negative for iNOS as compared with epithelial or mixed mesotheliomas. No association was found between iNOS immunoreactivity and apoptosis, vascular density or patient survival. Non-malignant mesothelial cells MeT-5A and all malignant mesothelioma cell lines were capable of synthesizing iNOS mRNA *in vitro*.

In normal pleural mesothelial cells, eNOS was occasionally positive, but reactive mesothelial cells were strongly stained. Immunoreactivity for eNOS was seen in most cases of malignant mesotheliomas. There was no significant difference in the immunostaining of eNOS between the different histological subtypes of mesothelioma.

Non-neoplastic mesothelial cells were negative for VEGF, FLK1 and FLT 1. Half of the mesothelioma cases were positive for VEGF, and most of the cases were positive for FLK1 and FLT1. There was no significant association with VEGF immunoreactivity and mesothelioma subtype, while FLK1 and FLT1 immunoreactivity was more often seen in epithelioid or biphasic subtypes compared with sarcomatoid mesotheliomas. FLK1 and FLT1 immunoreactivities were mutually associated, but not with VEGF expression. There was no association between FLK1, FLT1 or VEGF and eNOS immunoreactivity. Vascular density did not associate with tumor subtype, FLK1, FLT1, VEGF or eNOS positivity. Patient survival did not associate with mesothelioma subtype, FLK1, FLT1, VEGF or eNOS immunoreactivity. *In vitro* studies indicated that the non-malignant mesothelial cell line, MeT-5A, and the malignant mesothelioma M14K and M38K cell lines were all capable of synthesizing eNOS mRNA.

Both metaplastic and dysplastic bronchial lesions were clearly positive for iNOS, nNOS and nitrotyrosine. Nitrotyrosine was higher in alveolar macrophages of smokers and COPD than in the non-smokers and in the alveolar epithelium of smokers and COPD than in the non-smokers. Immunohistochemistry was used to detect the presence of iNOS; this enzyme was mainly non -detectable or weak in the bronchial epithelium and alveolar epithelium of non-smokers, smokers and stable COPD. In all groups eNOS was most prominently expressed in alveolar macrophages while nNOS was negative in all major cell types of the lung.

In NSCLC tissue samples, the expression of iNOS was detected in less than the half of the cases, while most cases showed weak to intense positivity for eNOS and nNOS. Strong eNOS staining was seen more often in adenocarcinomas than in squamous cell carcinomas, and iNOS immunoreactivity was seen more often in grade I-II tumors than in grade III tumors. There was no significant difference between the low and high apoptotic indexes or between the low and high proliferation rates of tumors in any instance of NOS staining. The patients with tumors showing high levels nNOS immunoreactivity tended to have better survival than the others. Similarly, the patients with tumors showing extensive expression of iNOS, eNOS and nNOS, as determined by a combined sum index, enjoyed a better survival than those with a low sum index for these enzymes.

Overall, NOS synthases immunoreactivity was prominent in mesotheliomas and bronchial dysplasias, and moderate in NSCLC. High levels of NOS isoenzymes immunoreactivity were associated with patient survival in NSCLC. Lung biopsies from smokers and COPD patients exhibited intense nitrotyrosine immunoreactivity, compared with control lung biopsies, and in all these three groups the immunoreactivities of the NOS isoenzymes were mostly weak.

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