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COMMENTARY

THE SIGNAL TRANSDUCTION MODEL OF CARCINOGENESIS

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Cancer is among the most common diseases of mankind. Regardless of cancer type, cancer-causing agent, or organ affected, the disease is generally characterized by unlimited proliferation of cancerous cells which fail to respond to physiological control mechanisms, thus destroying surrounding normal tissues, spreading to distant organs, and ultimately killing the host.

Because all cancers share these characteristics, it was soon suspected that there was a mechanism of carcinogenesis applicable to and responsible for the initiation and development of all cancer types. Many decades of research have generated a mountain of data on morphological, biochemical, and molecular aspects of all stages of the disease. A number of theories have evolved from these data, all of which have in common that they explain some of the experimental, clinical, and epidemiological evidence of the disease while never being applicable to each type of cancer and model system.

Currently the most popular view is that the cancer-causing agents and/or their metabolites interact with cellular macromolecules to form altered DNA/gene products [1, 2]. The alterations in gene expression then cause a variety of changes in the products of such genes which in some cases lead to continuous cell proliferation and the development of cancer.

However, such mechanisms fail to explain the well-documented carcinogenicity of peroxisome proliferators, of the antihistamine and mitochondrial proliferator, methapyrilene, or chronic irritation, all of which have, thus far, failed to yield detectable changes in DNA and/or gene expression. Moreover, a satisfactory explanation based on such views has yet to be provided for the well-documented organ and cell-type specificity of *N*-nitrosamines which often fails to correlate with the localization of DNA-adducts and altered genes in cells and tissues.

Research conducted in recent years has generated data which suggest an important role of the signal transducing system in the cascade of molecular events which ultimately lead to uncontrolled cell proliferation and cancer. This review highlights such findings which, upon integration with more established interpretations, have generated a new concept: the "Signal Transduction Model of Carcinogenesis".

The signal transducing system

All organs, tissues, and cells of the mammalian organism have the ability to function as individual entities and in concert, thus providing the basis for organized life. To achieve this, the organism is subdivided into a hierarchy of compartments, each of which although separated from the other compartments nevertheless is able to communicate with them and with the extracompartmental environment.

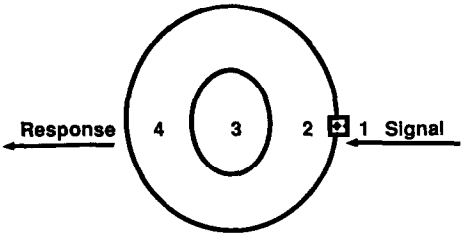


Fig. 1. The signal transducing system of the mammalian cell. Receptors (1) receive and bind extracellular signals, second messengers (2) "translate" this event into chemical reactions which initiate or inhibit the formation of products (4) by genes (3), thus resulting in a cellular response.

At the cellular level, each cell represents an individual compartment (Fig. 1) which is separated from adjacent cells by the cell membrane. Each cell demonstrates *receptors* which recognize and take up extracellular signals. This event then initiates a cascade of intracellular reactions known as *second messenger* pathways which ultimately activate one or several pertinent *genes* which encode the product/activity necessary for the appropriate response. Signals which activate the signal transducing system may be derived from adjacent cells, distant organs, or from the external environment of the organism. This elaborate mechanism enables the cell to adapt to changes in environment, organ function, and external challenges.

Receptors have been identified on the cell membrane, the nuclear membrane, in cytoplasmic organelles, on chromosomes, and in the cytosol [3], with the vast majority involved in responses to extracellular signals located on the cell membrane. Some receptors are fairly ubiquitous and are found in almost every cell, while others are highly specific and are found only in a few cell types where they usually regulate a cell-type specific function. Examples of signals for which specific receptors have been identified are: temperature, light, sound, pressure/tension, vitamins, hormones, neurotransmitters, growth factors, immunoglobulins, oxygen, choleratoxin, viruses (e.g. retroviruses, influenza

viruses), dioxins and related halogenated compounds. In keeping with the diversity of signals for which specific receptors have been identified, receptors regulate such vastly different functions as exocrine and endocrine secretion, hypertrophy and contraction of muscles, contraction/dilation of blood vessels and bronchi, heart rate, cell proliferation, and mood changes.

Physiological receptors can be classified into several families which share homologous structures and common mechanisms of action. Receptors for steroid hormones, thyroid hormone, retinoids, and vitamin D are soluble DNA-binding proteins believed to regulate the transcription of specific genes [4]. Another well-characterized family of receptors includes the receptors for peptides such as insulin, epidermal growth factor (EGF*), platelet-derived growth factor (PGF), gastrin-releasing peptide (GRP)/bombesin, and some lymphokines. These cell membrane receptors carry the tyrosine kinase complex on their intracellular domain which is activated via phosphorylation by binding of these ligands to the extracellular receptor domain [5]. Many neurotransmitters utilize cell membrane receptors which operate via ion channels that convey signals by altering the membrane potential. Among these receptors is the nicotinic cholinergic receptor and the type A receptor for γ-aminobutyrate. Many membrane receptors are mediated via adenylyl cyclase which catalyzes the formation of cyclic adenosine monophosphate (cAMP). Among the best studied receptors in this family are the β-adrenergic receptor and the muscarinic cholinergic receptor; serotonin, bradykinin and several hormones also operate via this receptor type. Guanine-nucleotide binding proteins (G-proteins) [6] activate several types of second messenger pathways including the adenylyl cyclase pathway and the inositol phosphate pathway [7] which, in turn, interacts with receptor-operated calcium channels [7].

Although passive diffusion is certainly one way that chemicals and drugs enter mammalian cells, especially if they are at a molecular weight of less than 100–200, growth factors, hormones, and neurotransmitters which regulate cell proliferation and differentiation under physiological conditions typically enter the cell via receptor-mediated uptake mechanisms. In view of the fact that drug/cell interactions generally do not create new cellular pathways but rather modulate preexisting normal ones, the investigation of the potential interaction of carcinogenic agents with such receptor-mediated signal transduction pathways is warranted. In this regard, the ability of membrane receptors to take up their physiological signals at minute quantities and against an enormous gradient via high-affinity ligand binding is of particular interest. As is generally accepted, the vast majority of human cancers are caused by chemicals. However, even in an environment which would be considered to have extremely high levels of pollution, the actual levels of cancer-causing agents are usually in the parts per million range. Likewise, even in a heavy smoker, the levels of tobacco-associated carcinogens which actually reach individual cells and tissues are in the picomolar and lower range. Receptors which operate via high-affinity ligand binding would therefore seem of particular interest for such research.

In light of the close functional interrelations between receptors, second messengers, and genes, disturbances of all three of these components of the signal transducing system could, in theory, lead to uncontrolled cell proliferation and ultimately cancer. This would imply that malfunctions at the levels of receptors and/or second messengers may, but would not have to in all cases, cause alterations in gene expression. This, in turn, may explain why alterations in gene expression are generally detected in a certain percentage of patients with a given type of cancer while higher incidences of genetic changes are usually less common.

In many cases, cell proliferation in response to external signals represents a reaction to the chronic stimulation of cellular production/activity by such agents. Cells and tissues can basically respond in two ways to such chronic stimuli. To satisfy the demand for enhanced production/activity, each preexisting individual cell either becomes larger (a phenomenon called "hypertrophy") or the cells undergo reversible proliferation (a phenomenon called "hyperplasia"). A typical example for hypertrophy is the enlargement of cardiac muscle mass in response to chronic hard exercise ("athlete's heart"). Examples for hyperplasia are the reversible proliferation of mucous cells of the airways caused by chronic exposure to irritants which necessitate increased mucous production as a response, or the reversible hyperplasia of hormone-producing cells in response to chronically elevated levels of their respective regulatory hormone. A logical consequence of this phenomenon is that the signal transducing pathway which regulates cell production/activity in response to extracellular signals has to be either closely associated with or even identical to the one which regulates cell proliferation in a given cell type. This, in turn, would imply that in addition to the family of so-called "growth factors", other biologically active agents which stimulate cell production/activity (e.g. some neurotransmitters) would also have to be considered as potential mitogens. This hypothesis will be dealt with in more detail later in this review (for details see "Genotoxic Chemicals and Cellular Receptors").

Neuroendocrine lung cancer and signal transduction

Neuroendocrine lung cancer, and its most malignant subtype, small cell cancer, in particular, are among the most common types of cancer found in humans [8]. Although small cell cancer demonstrates an overwhelming epidemiological link with cigarette smoking [8], efforts to reproduce this or either of the two other subtypes (carcinoid, atypical carcinoid) of

* Abbreviations: EGF, epidermal growth factor; PGF, platelet-derived growth factor; PNE, pulmonary neuroendocrine; DEN, *N*-nitrosodiethylamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; MB, mammalian bombesin; GRP, gastrin-releasing peptide; PAH, polycyclic aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and cAMP, cyclic adenosine monophosphate.

this cancer category in animal experiments remained unsuccessful for many decades [9].

Pulmonary neuroendocrine (PNE) cells, the putative origin of lung tumors with neuroendocrine differentiation [10], are sparse in healthy adult mammals but increase rapidly in number in response to a significant elevation of preexisting normal pulmonary oxygen levels (at birth [11], experimental hyperoxia [12]). On the other hand, PNE cells also demonstrate an increase in cell number upon chronic exposure to abnormally low lung oxygenation such as that found in patients with chronic respiratory disease [13], in residents at high altitude [14], or in laboratory animals exposed to hypoxia [15]. The proliferative reaction of PNE cells in response to deviations from normal pulmonary oxygen levels is believed to be mediated by receptors with sensitivity for oxygen [16]. PNE cells synthesize and secrete a host of neurohumoral products such as the peptides mammalian bombesin and calcitonin [17] as well as serotonin [18] and/or other agents many of which may constrict or dilate pulmonary blood vessels and bronchi. It has been postulated, therefore, that PNE cells play an important role in helping the lungs adapt to changes in oxygenation [11]. It is important to note that the increase in PNE cell secretion in response to altered lung oxygenation is always accompanied by proliferation of hyperplasia of PNE cells, thus supporting the hypothesis of a close association of regulatory pathways for cellular production and proliferation (see above).

Apart from abnormal pulmonary oxygen levels, a number of chemicals have been reported to cause PNE cell proliferation along with a stimulation of PNE cell secretion. Under physiological conditions, the neurotransmitter acetylcholine stimulates the synthesis and secretion of calcitonin and mammalian bombesin by PNE cells [19]. The biological effects of acetylcholine are mediated via high-affinity ligand binding of the neurotransmitter to cholinergic membrane receptors [20].

Investigations in human volunteers [21] and Syrian golden hamsters [22] have shown that nicotine causes hyperplasia of PNE cells along with an elevation of calcitonin production, thus mimicking the biological effects of acetylcholine. These findings are in accord with the well-documented high affinity of nicotine to a subtype of cholinergic receptors which have therefore been designated "nicotinic cholinergic" receptors [20]. Similarly, the carcinogenic *N*-nitrosamines *N*-nitrosodiethylamine (DEN) and 4-(methylnitrosamino) - 1 - (3 - pyridyl) - 1 - butanone (NNK) cause hyperplasia of PNE cells [23, 24] accompanied by an increase in neuropeptide secretion [25] in hamsters. Upon reassessment of these data, it seems likely that many smokers are exposed simultaneously to two different types of mitogenic agents. On the one hand, the development of chronic respiratory disease impairs the pulmonary ventilation, thus resulting in a relatively hypoxic condition. This, in turn, stimulates PNE cell secretion and mitosis. On the other hand, cigarette smoke also contains numerous noxious chemicals among which nicotine and nitrosamines have been shown to selectively stimulate PNE cells. It should be possible, therefore, to exploit such combined exposure in an animal experiment aimed at the induction of neuroendocrine lung tumors. Since the tolerance of animals to hypoxia is rather narrow, we decided to utilize hyperoxia (which is the physiological stimulus of PNE cells) for these attempts. Syrian golden hamsters were exposed to hyperoxia (60–70%) while receiving repeated subcutaneous injections with DEN or NNK. In both experiments, a significant number of the animals developed neuroendocrine lung tumors which secreted calcitonin and mammalian bombesin and were classified as atypical carcinoids by histopathology, immunocytochemistry, and electron microscopy [12, 24].

These data suggest that abnormal pulmonary oxygen levels have a promoter-like effect selective for PNE cells, thus leading to the development of lung tumors with neuroendocrine differentiation upon simultaneous exposure to carcinogenic nitrosamines. Future studies will have to clarify if this effect of altered oxygen levels on PNE cells is, in fact, mediated via stimulation of a separate oxygen receptor or results from an effect of oxygen on other receptor types such as the cholinergic receptor (e.g. up- or down-regulation of the receptor) and/or their associated second messenger pathways. Our view that abnormal pulmonary oxygen levels have a selective promoting effect in this system is supported by the pronounced prevalence of neuroendocrine lung cancer in smokers with chronic respiratory disease [26, 27] in conjunction with the virtual absence of this tumor type in nonsmokers [8], and failure to induce such tumors by tobacco-related chemicals in laboratory animals maintained under ambient air conditions [9].

The hamster experiments on the induction of neuroendocrine lung tumors suggested to us that cellular receptors which regulate secretion and cell proliferation of PNE cells may also play an important role in the mechanisms of PNE cell neoplasia. In this aspect, it is of particular interest that ligand binding to nicotinic cholinergic receptors stimulates the secretion of calcitonin and bombesin [19]. It is well established that mammalian bombesin (MB) acts as an autocrine growth factor in neuroendocrine cells [28], whereas calcitonin does not have a mitogenic effect [17]. MB is therefore believed to be one important mediator of neoplasia involving this cell type [28]. About 60% of all small cell cancers reportedly synthesize and secrete MB [29]. Specific bombesin receptors which mediate the selective uptake of MB have been identified in a variety of normal and neoplastic cell types and in many though not all neuroendocrine tumors [30]. It has been shown that experimental asbestosis in rats which causes a fibrosis-like response is accompanied by secretion of high levels of bombesin [31]. Likewise, hamsters exposed to hyperoxia demonstrate elevated levels of this neuropeptide [32]. On the other hand, treatment of hamsters with nicotine [19] or nitrosamines [25] alone without additional exposure to abnormal oxygen levels reportedly only increased the levels of calcitonin. Hence it appears that abnormal pulmonary oxygen levels are particularly important for the stimulation of bombesin secretion. The molecular mechanisms of this phenomenon still need to be elucidated. Much of our current knowledge on

the molecular biology of bombesin and other growth factors is derived from studies utilizing Swiss 3T3 cells. These cells are cultured fibroblasts which cease to proliferate once they have depleted the medium of growth factors and which reinitiate DNA synthesis and mitosis upon addition of new growth factors. 3T3 cells do not synthesize bombesin but express bombesin receptors and hence are a valuable system for investigations into molecular pathways activated by this receptor type independent of receptors which stimulate the synthesis/secretion of this neuropeptide. Such studies have identified a rapid flux of Na^+, K^+, H^+, and Ca^{2+} as some of the earliest intracellular events detectable after the binding of bombesin to its receptor [33, 34]. Moreover, they have also established that the addition of bombesin to the cell cultures results in an enhanced expression of c-myc and c-fos oncogenes [35]. This effect is believed to be mediated by Ca^{2+} mobilization and activation of protein kinase C [36] since inhibition of either one of these second messenger components reduces the over-expression of these oncogenes. These findings are important in that they reveal a functional association between the signal transduction pathway of bombesin and the levels of expression of protooncogenes commonly associated with neuroendocrine lung cancer. In response to these and similar findings, bombesin antagonists are currently being developed as potential therapeutics for neuroendocrine lung cancer. The majority of these agents are synthetic analogues of the substance P antagonist, peptide A [36, 37]. Likewise, efforts have been made to inhibit the mitogenic effect of bombesin by monoclonal antibodies to bombesin [38]. In view of the fact that the nicotinic receptor (mediates bombesin secretion/synthesis) and the bombesin receptor (mediates bombesin uptake) are both Ca^{2+} dependent, agents which inhibit Ca^{2+}-dependent cellular pathways should be able to block the mitogenic effect of this growth factor.

The exploration of a potential therapeutic effect of Ca^{2+}-channel blockers and calmodulin antagonists on lung tumors with neuroendocrine differentiation has been suggested previously because the second messenger pathways of most growth factors commonly expressed in this tumor category are Ca^{2+}/calmodulin dependent [39]. However, cancer chemotherapy with currently available Ca^{2+}/calmodulin antagonists is severely limited by the pronounced cardiovascular effects of such drugs [40]. In an effort to avoid such undesirable side-effects, the dihydropyridine analogue B859-35 is currently being developed as a potential therapeutic agent [41]. B859-35 is the (−)-isomer of the antihypertensive agent niguldipine [42] and, unlike the latter, demonstrates only minimal effects on blood pressure and cardiac function while retaining its inhibitory effect on Ca^{2+}/calmodulin [42]. Intragastric administration of B859-35 for 20 weeks to hamsters with hyperoxia/nitrosamine-induced neuroendocrine lung tumors resulted in a complete response (complete disappearance of lung tumors in all animals [41]). On the other hand, pulmonary adenomas of Clara cell origin which were induced in the same species by nitrosamines administered under ambient air conditions did not respond to B859-35 [41]. In keeping with these data, *in vitro* studies using human lung cancer cell lines have shown that B859-35 has a significant antiproliferative effect on the carcinoid-derived neuroendocrine line NCI-H727 at very low concentration levels (down to the picomolar range), whereas substantially higher levels are required to produce a similar effect in cell lines from different lung tumor types [43]. These findings are consistent with the view that B859-35 may prove to be an effective chemotherapeutic agent in some cases of neuroendocrine lung cancers, and clinical trials with this promising new agent have now started.

Genotoxic chemical carcinogens and cellular receptors

The term genotoxic carcinogens relates to chemicals which react with cellular DNA/genes and are believed to exert their carcinogenic effects via such mechanisms. Among these, *N*-nitrosamines are formed from amines and nitrite under acidic conditions in the mammalian stomach [44] as well as via physiological microorganisms in the saliva and in the gut [45]. The precursors of nitrosamines are found in a large variety of foods, beverages, and over-the-counter drugs. One of the most prominent routes of human exposure to this class of carcinogens is via tobacco products [46].

Nitrosamines are believed to require metabolic conversion into carcinogenic intermediates which react with cellular macromolecules, most notably DNA [47]. Nitrosamines demonstrate a striking organ and cell type specificity although the target of their carcinogenic action may vary among the species. Among the explanations offered for this phenomenon are: differences in isozymes [48], differences in the levels of formation of alkylated DNA molecules (DNA-adducts [49]), differences in the levels of DNA repair enzymes [1], and differences in the levels of expression of protooncogenes [50]. However, although cells which lack the oxidative enzyme systems believed to metabolize nitrosamines are mostly nontargets of nitrosamine carcinogenesis, there are many cell types and tissues which contain high levels of these enzymes but nevertheless do not respond with neoplasia to nitrosamine exposure. Likewise, the distribution pattern of DNA-adducts and of overexpressed and/or mutated protooncogenes after exposure to nitrosamines does not, in all cases, match the distribution pattern of the neoplastic response among organs and cell types [49, 50]. Moreover, it has been shown that at least in some cases the alkylated DNA-adducts disappear from the target tissues and cells many months before the onset of neoplasia [51].

The discovery that some nitrosamines have a selective proliferative effect on neuroendocrine cells [23] and Clara cells [52] in the hamster lung suggested to us that their selective biological effects are intrinsicaly linked with cell-type specific functions. However, only many years later, thanks to the progress in receptor pharmacology and neuroendocrinology, did it become possible to explore this hypothesis.

DEN causes selective proliferation of PNE cells in hamsters [23], rats [53], and rabbits [54] and causes

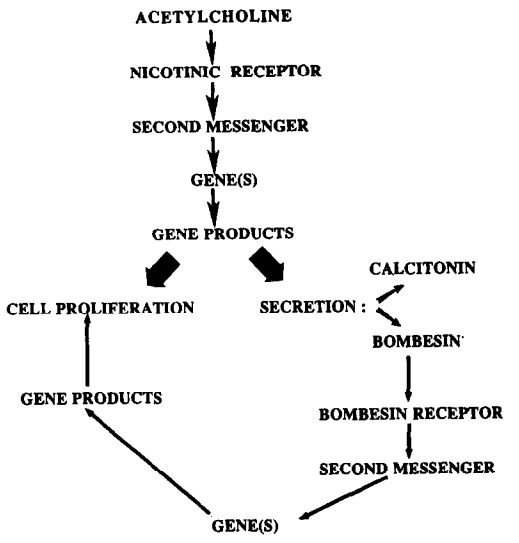
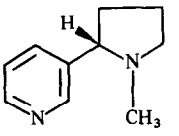


Fig. 2. Regulation of secretion and proliferation of pulmonary neuroendocrine cells under physiological conditions by the neurotransmitter acetylcholine.

Nicotine:



4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK):

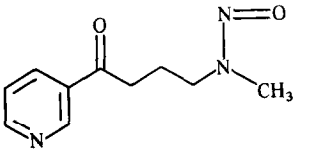
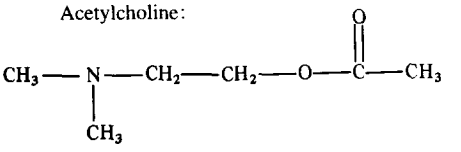


Fig. 3. Chemical structures of nicotine and the nicotine-derived 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

Acetylcholine:



N-Nitrosodiethylamine (DEN):

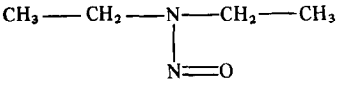


Fig. 4. Chemical structures of acetylcholine and *N*-nitrosodiethylamine (DEN).

neuroendocrine lung tumors in hamsters upon simultaneous exposure of the animals to hyperoxia [12]. The tobacco-specific nitrosamine NNK has a similar effect in hamsters [24]. It has been shown that the observed mitogenic effect in hamsters is accompanied by a pronounced elevation in the lung and serum levels of calcitonin [25], a secretory product of PNE cells [17]. Accordingly, DEN is not only a mitogen but also a secretagogue for this cell type, thus supporting our view that agents which stimulate cell secretion are potential mitogens (see above). Under physiological conditions, the secretion of the neuropeptides calcitonin and bombesin by PNE cells is stimulated by the neurotransmitter acetylcholine via high-affinity ligand binding to nicotinic cholinergic receptors (Fig. 2) [19]. Recently, we have also shown that acetylcholine stimulates the proliferation of well-differentiated neuroendocrine lung tumor cells *in vitro* at a rate comparable with that of the autocrine growth factor bombesin [55]. Accordingly, even the neurotransmitter which stimulates secretion by PNE cells under physiological conditions can act as a growth factor on this cell type. Studies in hamsters and in human volunteers have demonstrated that nicotine exposure results in a pronounced stimulation of PNE cell secretion and proliferation [21, 22]. This effect was inhibitable by specific antagonists of nicotinic cholinergic receptors, thus identifying this receptor type as a regulatory element for the observed biological effects on normal PNE cells. A similar growth factor-like effect of nicotine was also observed on well-differentiated neuroendocrine lung tumor cells *in vitro* [55]. In keeping with these data, the tobacco-specific nitrosamine NNK which has structural similarities with nicotine (Fig. 3) and the aliphatic nitrosamine DEN which has structural similarities with acetylcholine (Fig. 4) are strong mitogens in the same *in vitro* systems [56]. Their proliferative effect is inhibitable by specific antagonists of nicotinic cholinergic receptors but not by antagonists of muscarinic cholinergic receptors, thus suggesting interaction of the nitrosamines with nicotine receptors as part of the underlying molecular mechanisms. This hypothesis was supported by radioreceptor assays utilizing cell membrane fractions of hamster lung enriched with normal PNE cells [57]. In these experiments, saturable high-affinity ligand binding of radiolabeled nicotine which could be displaced by either one of the two nitrosamines was demonstrated. These data reveal, for the first time, uptake of nitrosamines by a cell-type specific high-affinity membrane receptor physiologically involved in the regulation of secretion and cell proliferation, thus offering a logical explanation for the documented selectivity of DEN and NNK for PNE cells. However, it is important to consider that the experiments with neuroendocrine cell lines required higher CO_2 levels (8%) than non-neuroendocrine cell lines in order to grow and respond with cell proliferation to nicotine or the nitrosamines. This may well be the *in vitro* correlate of the well-known mitogenic effects of hypoxia on PNE cells *in vivo*. In keeping with this interpretation, significant radioreceptor binding of nicotine in hamster lung

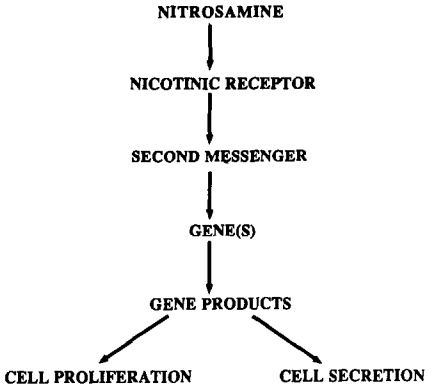


Fig. 5. Putative molecular mechanisms of nitrosamine-induced secretion and cell proliferation via direct interaction with the nicotinic cholinergic receptor in pulmonary neuroendocrine cells.

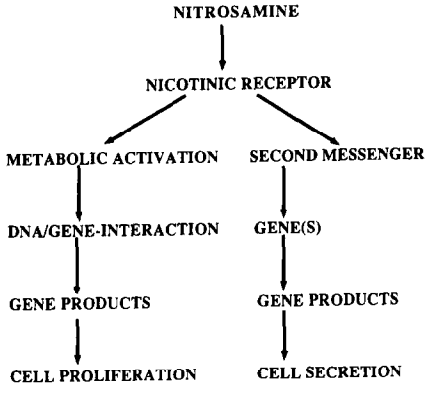


Fig. 6. Alternative molecular mechanisms of nitrosamine-induced cell proliferation mediated by DNA/gene interactions of active metabolites after uptake of the parent nitrosamine via nicotinic cholinergic receptors in pulmonary neuroendocrine cells.

was detectable only in animals that had been pre-exposed for several weeks to hyperoxia; such binding in normal hamster lung was negligible [57]. Although the molecular mechanisms of this effect on nicotinic receptors remain to be elucidated, it is possible that oxygen concentrations can alter the sensitivity of this receptor type to its ligands (e.g. up- or down-regulation). A mechanism such as this may explain why experiments with human small cell lung cancer cell lines conducted in another laboratory only showed a proliferative response to nicotine when exposed simultaneously to opioids [58]. Based on current knowledge, two alternative molecular mechanisms can be envisioned as a cause of nitrosamine-induced PNE cell neoplasia. On the one hand, it is possible that the nitrosamines initiate cell proliferation via direct interaction with the nicotinic receptor (Fig. 5). On the other hand, metabolic activation and subsequent changes in DNA/genes may occur once the nitrosamine has entered the cell via receptor uptake (Fig. 6). In both cases, interaction of the nitrosamine with the nicotinic receptor is the event responsible for the selectivity of the biological effect, thus identifying this most peripheral component of the cell's signal transducing system as a crucial trigger of neoplasia involving this cell type.

Laboratory rodents exposed to DEN or NNK while being maintained under ambient air conditions develop peripheral pulmonary adenomas [24, 52]. Pathogenesis studies in hamsters have established an origin of these neoplasms from Clara cells [24, 52]. In keeping with these observations, autoradiographic studies after *in vivo* administration of radiolabeled DEN found the most bound radioactivity in Clara cells under these conditions [59]. Contrary to the widely held belief that this is a reflection of high levels of oxidative enzyme systems in this cell type, alveolar type II cells which contain significant levels of the same enzyme systems did not bind significant levels of radioactivity in these experiments. In analogy to our recent experiences with mechanisms of PNE cell neoplasia (see above), we then explored the hypothesis that the signal transduction pathways which regulate Clara cell secretion may be involved in mediating the selective action of nitrosamines on this cell type. Clara cells synthesize and secrete a proteinous product which constitutes one of the components of lung surfactant [60]. Under physiological conditions, the secretion of this product by Clara cells is stimulated by the catecholamines epinephrine and norepinephrine via high-affinity binding to β-adrenergic cell membrane receptors [60]. Agonists with specificity for these receptors mimic this effect whereas agonists with specificity for α-adrenergic receptors do not [60]. In keeping with the hypothesis that the signaling pathways which regulate cell secretion also regulate cell proliferation, we have demonstrated a pronounced mitogenic effect of the β-adrenergic agonist isoproterenol on a well-differentiated human lung cancer cell line with features of Clara cells (NCI-H322) [61]. This effect was inhibited by a specific antagonist of β-adrenergic receptors, propranolol, but not by antagonists of other receptor types including α-adrenergic receptors [61]. These experiments thus establish a critical role for β-adrenergic receptors in the signaling pathways which regulate Clara cell proliferation. We then explored the possibility that nitrosamines may interact with the β-adrenergic receptors characteristic of this cell type. Utilizing again the cell line NCI-H322 (see above), we found that both DEN and NNK were strong mitogens in this system, an effect which was inhibitable by an antagonist of β-adrenergic receptors but not by antagonists of cholinergic or α-adrenergic receptors [61]. Radioreceptor assays with membrane receptor preparations of the same cell line subsequently revealed high-affinity ligand binding of the β-adrenergic agonist [^{125}I]Iodocyanopindolol and binding of this ligand was displaced by nonradioactive DEN and NNK (Schüller, unpublished results). Radioreceptor assays utilizing membrane receptor preparations of normal hamster lung periphery yielded similar data [62], thus identifying high-affinity ligand binding of the nitrosamines to β-adrenergic receptors as one of the molecular mechanisms mediating the observed selectivity of

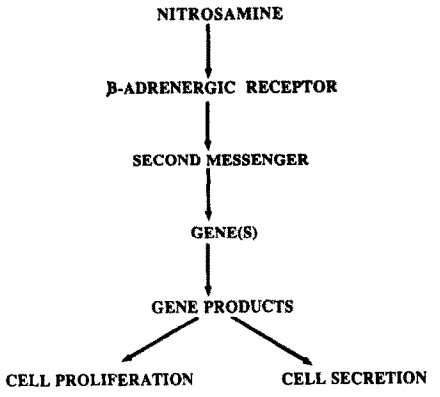


Fig. 7. Putative molecular mechanims of nitrosamine-induced cell proliferation and secretion mediated via direct interaction with β-adrenergic receptors in pulmonary Clara cells.

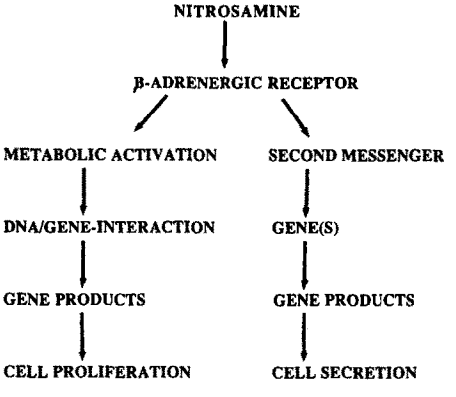


Fig. 8. Alternative molecular mechanisms of nitrosamine-induced cell proliferation by DNA/gene interactions of active metabolites after uptake of the parent nitrosamine via β-adrenergic receptors in pulmonary Clara cells.

these compounds for Clara cells. Similar to the case of neuroendocrine lung neoplasia (see above), our current knowledge therefore suggests two different mechanisms for nitrosamine-induced Clara cell carcinogenesis. On the one hand, chronic interaction of the nitrosamines with β-adrenergic receptors of Clara cells could result in a direct continuous stimulation of Clara cell proliferation via receptor-activated mitogenesis (Fig. 7). This in turn could lead, at least in a proportion of the cells, to reactive changes in the expression of second messengers and/or genes functionally linked with this receptor type. On the other hand, the nitrosamines could enter Clara selectively via uptake by β-adrenergic receptors and become subsequently metabolized into active intermediates which react with DNA/genes (Fig. 8). Again, both alternative molecular mechanisms attribute a key role to a membrane receptor which regulates secretion and proliferation in this cell type under physiological conditions.

The cited examples summarize the only currently available information on nitrosamine/receptor interaction. However, these findings may be just the tip of the iceberg. The autonomous nervous system with its two branches, the vagus and the sympathicus, regulate endocrine, exocrine, and neuroendocrine secretion in many different cell types and organs. These functions are mediated by receptor uptake of acetylcholine for the vagus [20] and by receptor uptake of the catecholamines for the sympathicus [63]. With respect to the many subtypes of cholinergic and adrenergic receptors which have been identified to date, the widely publicized organ and cell-type specificity of nitrosamines in general may well be the reflection of differences in affinity of the nitrosamine under study to these receptor subtypes. Moreover, it could be envisioned that receptors for simple amines like serotonin or histamine, both of which can stimulate cell secretion, may in some cases also bind nitrosamines which are structurally similar to their physiological ligands.

Polycyclic aromatic hydrocarbons (PAHs) are another common environmental contaminant, resulting from incomplete combustion of fossil fuels and organic materials. Unlike the nitrosamines, PAHs are not very selective in their effects and cause tumors in many tissues and cell types. Similar to many xenobiotics they are believed to be converted by oxidative cytoplasmic enzyme systems into active metabolites which react with the DNA molecule [64]. In some cases, it has been shown that the site of such DNA damage correlates with the locus of mutations induced by these chemicals in the H-*ras* gene [65]. Amplification and/or mutation of *ras* family protooncogenes have been demonstrated in many different tumor types [66, 67], are inducible by structurally different chemical carcinogens [67], and are believed to be an important factor in the initiation of neoplasia in such cases. On the other hand, it has been suggested that 3-methylcholanthrene and structurally similar PAHs cause estrogen-responsive mammary tumors in rats because they have structural similarities with steroidal estrogens. Estrogens which cause similar tumors experimentally are taken up by the mammary gland via high-affinity estrogen receptors [68]. These receptors are DNA-binding proteins which activate the transcription of several genes adjacent to the DNA sequence of their binding site [68]. The affinity of steroidal and nonsteroidal estrogens to this receptor type is believed to depend on the presence of a phenolic A ring in the molecule. Many PAHs have similar phenolic ring structures and many of the established nonsteroidal estrogens are, in fact, polycyclic aromatic compounds [68]. It is therefore intriguing to speculate that interaction of PAHs with the estrogen receptor may contribute to the carcinogenicity of this class of chemical carcinogens. However, ligand binding of PAHs to this receptor type has yet to be demonstrated. Another receptor type that has been implicated in the mechanisms of carcinogenesis by aromatic compounds including PAHs is the aromatic hydrocarbon receptor [Ah receptor; synonym 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) receptor]. Ligand binding to this receptor which is a cytosolic protein causes the induction of several drug-metabolizing enzymes including members of the cytochrome P450 family

[69, 70] and has been implicated in the regulation of epidermal cell proliferation and differentiation [71]. The Ah receptor is found in a great variety of tissues and cell types. It has been shown that several carcinogenic aromatic amines, the PAH 3-methylcholanthrene, benzo[*a*]pyrene, and benz[*a*]anthracene as well as TCDD bind to this receptor [69, 71, 72]. Studies using 3-methylcholanthrene or TCDD as ligands in conjunction with experiments in mice with polymorphism for enzyme induction by such ligands have aided greatly in an understanding of the molecular mechanisms of this receptor-mediated enzyme induction. These experiments have shown that ligand binding to the Ah receptor causes induction of the Ah locus, a gene which controls the activity of arylhydrocarbon hydroxylase and associate P450 isozymes as well as several other cytoplasmic enzyme systems [69, 71]. The tumor-promoting action of TCDD on PAH-induced carcinogenesis is believed to be mediated by ligand binding to this receptor [71]. If and how binding of PAHs and structurally related chemical carcinogens to the Ah receptor relates to other molecular mechanisms of PAH-induced carcinogenesis (changes of DNA/genes) still remain to be established. However, the Ah receptor is yet another example of a regulatory element which controls a type of cellular production (synthesis of cytoplasmic enzymes). Similar to the receptors which regulate secretion (see above), it is therefore likely that chronic stimulation of the Ah receptor by repeated exposure to ligands will also act as a mitogenic signal, thus providing a molecular mechanism for cell proliferation.

Non-genotoxic chemical carcinogens

The term non-genotoxic carcinogens relates to carcinogenic agents for which interaction with DNA/genes has not been shown despite many efforts. Among the most extensively studied representatives of this family of chemicals are the hypolipidemic drugs (synonym: peroxisome proliferators) such as clofibrate and the phthalate type plasticizers. These chemicals induce hepatomegaly and peroxisome proliferation in liver cells and cause hepatocellular carcinomas upon long-term administration while demonstrating no initiating activity when given as a single dose [73]. Peroxisome proliferators are non-mutagenic and do not interact with DNA [73, 74]. Clofibrate and many other members of this family induce several cytoplasmic enzymes including the peroxisomal enzymes enoyl CoA:hydratase-3-hydroxyacyl-CoA dehydrogenase, fatty acyl-CoA oxidase, as well as the microsomal P450IVA1, thus resulting in an increase in fatty acid β-oxidation [71]. Long-term exposure to clofibrate results in the accumulation of 8-hydroxydeoxyguanosine, and such oxidative DNA damage has been suggested as a possible factor responsible for the carcinogenicity of peroxisome proliferators [75]. On the other hand, a cellular receptor for clofibrate has been identified and purified recently [72]. Hence, it appears that in analogy to PAH and TCDD (see above), binding of peroxisome proliferators to a specific cellular receptor stimulates the genes which activate the induction of specific enzyme systems. The morphological correlate of induced peroxisomal enzymes is an increase in peroxisomes in which they are stored. Again chronic stimulation of such a receptor which controls the synthesis of a cellular product is likely to act as a mitogenic signal, an assumption supported by the observation that only long-term administration of peroxisome proliferators causes tumors.

Another interesting, though less well investigated, example of a non-genotoxic carcinogen is the antihistamine methapyrilene. Methapyrilene was widely used as a component of over-the-counter sleep aids until its withdrawal from the market upon the finding that it causes a high incidence of liver tumors in rats [76]. Electron microscopic examination of such tumors revealed that they were hepatocellular oncocytomas, a rare tumor type characterized by an overabundance of mitochondria [77] which to this day has not been induced experimentally by any other chemical. Subsequent pathogenesis studies revealed mitochondrial proliferation in portal areas of the liver after 2–4 weeks of methapyrilene treatment [78], and electron microsopic autoradiography showed that binding of radiolabeled methapyrilene was primarily to mitochondria in such areas [79]. Structurally similar analogues which share the antihistaminic property of methapyrilene did not cause mitochondrial proliferation and were not carcinogenic [80]. Subsequently, it was shown that methapyrilene induces lipid peroxidation in rat liver [81] and results in the formation of lamellar bodies resembling phospholipids ultrastructurally in rat liver cells *in vitro* [82]. Methapyrilene was then tested in a number of established mutagenicity assays, and studies on the formation of DNA-adducts were also conducted. Although the resulting data were somewhat conflicting, the initial general consensus was that the compound was nonmutagenic and did not react directly with DNA [83]. More recently, a significant increase in DNA methylation, as monitored by the formation of 5-methyldeoxycytidine, has been reported in methapyrilene-treated rats compared to animals receiving structurally related noncarcinogenic analogues which failed to cause such DNA changes [83]. These findings are in sharp contrast with studies using hepatocarcinogens other than methapyrilene which generally cause a pronounced hypomethylation in preneoplastic and neoplastic rat liver [84]. With respect to the role of DNA methylation on gene expression, DNA hypomethylation has been suggested as an essential step in hepatocarcinogenesis by genotoxic carcinogens [84]. Hence, it appears that although the reported hypermethylation of DNA by methapyrilene does, in fact, represent a change of the DNA molecule, such change is usually not associated with increased DNA synthesis or cell proliferation. In fact, the only experiments in the literature on chemically induced DNA hypermethylation achieved this effect with inhibitors of DNA synthesis [85].

In view of the initial use of methypyrilene as an antihistamine, it seems surprising that no efforts have been made to link the function of the compound as a potent antagonist of H_1 histaminic receptors with its carcinogenic properties. The H_1 histaminic receptor is a cell membrane receptor which operates via the phospholipase C/inositol triphosphate second

messenger pathway which, in turn, leads to the activation of a Ca^{2+}/calmodulin-dependent protein kinase C [86]. Ligand binding to this receptor affects several other receptor-mediated pathways including those of muscarinic cholinergic receptors and adrenergic receptors [86]. The observed proliferation of mitochondria which is unique to methapyrilene and does not occur with any of the structurally similar H_1 antagonists is possibly the morphological correlate of increased mitochondrial products (e.g. mitochondrial enzymes), a hypothesis which deserves further study.

Modulators of second messengers and carcinogenesis

Tumor promoters of the phorbol ester family have been widely used to investigate mechanisms of chemical carcinogenesis, and such studies have contributed substantially to the concept of "two-stage carcinogenesis" [87]. Like all other classes of tumor promoters, the phorbol esters are not carcinogenic *per se* but greatly enhance the carcinogenic response when administered repeatedly after the tumor initiator [87]. For many years, the underlying molecular mechanisms of this phenomenon were poorly understood. However, it was soon suspected that the phorbol esters may act by usurping the action of endogenous hormones and growth factors [87]. Chemical analysis by three-dimensional computer graphics of phorbol esters, teleocidins, and the marine toxin aplysiatoxin, all of which demonstrate similar tumor-promoting effects, has revealed similarities in the spatial arrangements of these molecules consistent with a receptor cavity model [88]. This, in turn, suggests interaction with a cellular receptor as the molecular mechanism responsible for the biological activity of these compounds. In keeping with this assumption, high-affinity saturable binding of phorbol esters to membrane-associated receptor sites has been demonstrated in a large variety of different cell types [87], thus suggesting that the receptor type in question is common to many tissues and cell types. The receptor binding of phorbol esters is inhibitable by teleocidins and aplysiatoxin [87], giving further support to the hypothesis that all of these compounds compete for the same receptor site. More recently, it has been demonstrated that this membrane associated binding site is protein kinase C [87] and that the phorbol esters can mimic the action of diacylglycerol which activates these kinases under physiological conditions [6]. In this context, it is important to note that the diacylglycerol/protein kinase C pathway is an intrinsic component of the signal transduction cascade of many physiological growth factors, neurotransmitters, and hormones [6] where it acts as second messenger activated by ligand binding to cell membrane receptors. It is conceivable therefore that repeated activation of protein kinase C by this class of tumor promoters subsequent to activation of a functionally linked receptor by a tumor initiator can amplify the mitogenic response caused by interaction of this external signal with the receptor. This, in turn, would imply that the chemical carcinogens commonly used as initiators in initiation/promotion experiments exert their carcinogenic effects to a significant extent via interaction with such receptors and/or other components of their associated signaling pathways. In keeping with this interpretation, studies on the regulation of cell proliferation in Swiss T3T fibroblasts have revealed that phorbol esters in fact compete with diacylglycerol for binding sites on protein kinase C and act synergistically with such receptor-mediated endogenous growth factors as insulin [33]. Moreover, addition of phorbol esters to quiescent Swiss 3T3 cells induced a pronounced increase in the levels of expression of c-fos and c-myc protooncogene products [35]. A similar effect on the expression of these protooncogene products was observed when the cells were treated with the physiological activators of protein kinase C, phospholipase C/diacylglycerol [83]. It hence appears that in many cell types activation of protein kinase C represents one of the steps in the signaling pathways which link receptor/ligand binding with alterations in the expression of protooncogenes associated with cell proliferation and that this step can be selectively potentiated by tumor promoters which share biological activities with the phorbol ester family.

In a seemingly unrelated line of studies, cAMP-dependent protein kinase has been identified as an important regulatory element for cell differentiation and proliferation [89]. Unlike other protein kinases, including protein kinase C (see above), cAMP-dependent protein kinases occur as a mixture of two different isozymes designated type I and type II [89]. The molecular structure of these isozymes has been studied extensively. Synthetic site-selective analogues of cAMP which bind to one or the other isozyme along with antisense oligonucleotides for their respective regulatory subunits (R I, R II) have been used to investigate their function [89]. It has thus been shown in several tumor systems that cAMP-dependent protein kinase can cause a strong inhibition of cell proliferation and that this growth-inhibiting property rests mainly with its type II isozyme [89]. Moreover, increases in the levels of type II isozyme are usually accompanied by decreases in type I isozyme [89]. On the other hand, an increased expression of type I protein kinase generally goes along with active cell proliferation [89]. In several tumor systems, administration of either site-selective cAMP analogues with high affinity to the type II isozyme or antisense oligonucleotides for the regulatory unit R I of type I isozyme has reversed malignancy as expressed by inhibition of cell proliferation along with a return to normal cell morphology [89]. Based on these findings, modulators of cAMP have recently become one major focus in the development of novel anticancer drugs [89].

When assessing these data, it is important to consider that cAMP and its associated kinases contribute to the second messenger pathway of G-protein coupled cell membrane receptors which act via the adenylyl cyclase pathway further downstream. This receptor family typically employs stimulatory (R_s) and inhibitory (R_i) receptors along with stimulatory (G_s) and inhibitory (G_i) G-proteins [7]. Binding of a ligand to stimulatory receptor causes conversion of adenosine triphosphate (ATP) to cAMP by adenylyl cyclase, whereas ligand binding to an inhibitory receptor results in inhibition of this enzyme [7].

Hence it is conceivable that the above-cited prevalence of the type I isozyme of cAMP-dependent protein kinase in actively proliferating normal and neoplastic tissues is, in many cases, the result of ligand binding to cAMP-associated stimulatory membrane receptors. As mentioned earlier in this review, the catecholamines, acetylcholine (muscarinic cholinergic receptor), serotonin, bradykinin and several hormones exert their biological effects via binding to this family of receptors, and many of these agents have been identified as mitogens. As has been exemplified by the two nitrosamines, DEN and NNK (see above), some chemical carcinogens can act as mitogens by mimicking the action of such physiological receptor ligands. Since mammalian cells generally possess multiple different receptor types, it is quite common to find membrane receptors of the adenylyl cyclase family in the same cell as receptors which utilize protein kinase C further downstream in their signaling pathway. Accordingly, cAMP-dependent protein kinase and protein kinase C associated with the diacylglycerol pathway may both be present in the same cell and participate in the regulation of proliferation.

Conclusions

The purpose of this review was to highlight data which suggest signal transduction pathways as mediators of cancer initiation and development. Although there are many excellent commentaries available on carcinogen metabolism, chemical/DNA interactions and molecular biology of protooncogenes, these area of study are not discussed in depth. However, the signal transducing system is more than just a receptor/ligand interaction and is also not limited to just some chemical reactions within second messenger pathways. Rather, this system is the complex functional entity of molecular events from the first contact of the cell with a signal, which in many cases involves receptor interaction, through subsequent intracellular second messenger-mediated reactions, down to activation or inhibition of gene-mediated responses. As was stated in a recent commentary in *Cancer Research*, "there are many things that control cell proliferation and an increase in mRNA levels of a given protooncogene upon entry of a previously quiescent cell into a proliferative state does not identify that protooncogene as growth regulatory" [90]. In fact, genes do not generally start a cellular activity unless they get a signal to do so. In many ways, cells can be compared with a computer in which the receptors are the keyboard to receive incoming signals while second messenger pathways are the software which converts the signals into a language that the computer program represented by the genes can understand and respond to appropriately. When looking at published data in *Cancer Research* from this perspective, it becomes obvious that the long sought after commonality of all cancer-inducing agents is that they all interact with one or several components of the signal transducing system. Because all components of this system mutually affect each other, it is logical than an identical response of a given cell (e.g. cell proliferation) can be caused by a molecular event at the level of receptors, second messengers, or genes. With respect to the crucial role of receptors for the initiation of cell proliferation in response to physiological stimuli and knowing that most pharmacologically active drugs do not create new cellular pathways but rather modulate preexisting normal ones, it would seem that the stage of cancer initiation in particular is likely to involve molecular events at the receptor level. A mechanism such as this would imply that many chemical carcinogens may act as "false signals" via receptor interaction, thus exerting the effects of pharmacological receptor agonists or antagonists. As is well established in receptor pharmacology, such ligand/receptor interaction can either result in a reversible receptor-mediated response which would be temporary or can cause irreversible changes in receptor configuration in which case the induced change in cell function would be permanent. Accordingly, some carcinogens operative via such a mechanism would require chronic exposure, whereas in other cases a single exposure would trigger an identical response. This, in turn, would result in changes of expression of second messengers and gene(s) which are functionally linked with this receptor. Moreover, a chemical which interacts with a cellular receptor while at the same time also interacting directly with other components of the signal transducing pathway further downstream (e.g. DNA/genes) or with several different receptor-mediated pathways would be more likely to cause cancer than an agent which only interacts with one component alone. It appears that some of the most potent chemical carcinogens among the polycyclic aromatic hydrocarbon class may fall into the latter category. Similarly, agents like the phorbol esters which interact with molecules involved in the second messenger pathways of several different receptors can modulate the carcinogenic response of several different initiating carcinogens. On the other hand, agents like the nongenotoxic peroxisome proliferators may illustrate that interaction with a receptor without detectable effects on DNA/gene expression can suffice for the induction of neoplasia although only in chronic exposure protocols.

This "Signal Transduction Model of Carcinogenesis" does not deny the possibility that occasionally one or several of the cancer-causing factors may be found within the cell as inheritable genetic alteration. However, such preexisting genetic alterations will always result in an abnormal response of the activity encoded by such a gene to the external signals which it receives via its respective receptors and second messengers. Accordingly, examples like the predisposition for the development of epidermal squamous cell carcinoma observed in patients with xeroderma pigmentosum represent an abnormal response to the external signal, UV light, because of a defect in the gene associated with this particular signal transduction pathway. Similarly, oncogenic viruses like the retroviruses enter the mammalian cell via cellular receptors, thus using preexisting normal signal transduction pathways to reach the genetic material where they cause changes resulting in an abnormal response of the affected genes to physiological and/or external stimulants for cell proliferation. Moreover, the "Signal Transduction Model of Carcinogenesis" provides a logical explanation for

the reported association of exposure to electromagnetic fields such as are created by electrical power cables and an increased cancer risk [91]. Chronic exposure to electromagnetic fields of this nature is likely to affect the function of cell membrane receptors which operate via ligand-initiated changes in membrane potential, a mechanism utilized by many receptors of growth factors and neurotransmitters. Even the somewhat controversial association between certain psychiatric disorders and a lower cancer risk could be explained on grounds that such disorders are characterized by defects in neurotransmitter signaling pathways which, as exemplified by the interaction of nitrosamines with acetylcholine receptors (see above), may also affect their response to chemical carcinogens.

Last but not least, the "Signal Transduction Model of Carcinogenesis" has far-reaching implications on the design of cancer therapy. If we assume that the majority of cancer-causing agents react with components of the signal transducing system thus "fooling" cells into a continuous proliferative response, this implies that, except for getting the wrong messages, these cells may, especially at an early stage of neoplasia, be perfectly normal and only continue to proliferate as an adaptive response. Consequently, attempts to cure this disease by killing the cancer cells would only result in renewed cell proliferation because the signal which has caused this reaction in the first place continues to be there. The numerous pitfalls in attempts to cure solid tumors by cytotoxic radio- and chemotherapy seem to support this view. On the other hand, the well-documented decrease in cancer risk after cessation of smoking [46] exemplifies quite well that once the signals which prompt the proliferative response are removed, the cells are able to return to normal performance. It therefore appears to be most promising to attempt to interrupt the vicious cycle of false signaling which causes cells to remain in a continuous proliferative state. In this regard, novel noncytotoxic chemotherapeutic agents like B859-35 [41–43], which interferes with Ca^{2+}/calmodulin or modulators of cAMP which interfere with adenylyl cyclase [89], are most promising. As outlined earlier in this review, the vast majority of signaling pathways including those of growth factors, hormones, and neurotransmitters utilize either Ca^{2+}/calmodulin-dependent or adenylyl cyclase-dependent second messengers. It could therefore be envisioned that in the future tumors will be classified and treated according to the spectrum of receptors and second messengers involved in their proliferative response.

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