Advances in Cancer Biomarkers as Applied to Chemical Exposures: The *ras* Oncogene and p21 Protein and Pulmonary Carcinogenesis

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Pulmonary carcinogenesis due to occupational and environmental exposures to chemical carcinogens such as polycyclic aromatic hydrocarbons presents an interesting model for study of possible oncogene-related cancer biomarkers. Polycyclic aromatic hydrocarbons are important respiratory carcinogens and have been shown to cause specific mutational lesions that can lead to the activation of the ras oncogene and expression of its p21 protein product; ras oncogene activation and p21 expression frequently are detected in human lung cancers. In addition, the p21 protein is detectable via immunoblotting techniques in the serum of lung cancer patients and in selected persons in exposed worker cohorts at risk for the development of lung cancer. Thus, the ras oncogene and p21 protein may be useful biomarkers for monitoring pulmonary carcinogenesis in exposed populations.

A significant challenge in occupational health is the adequate surveillance of workers with potential exposures to a wide variety of suspect carcinogens. Traditional industrial hygiene approaches to the monitoring of the ambient environment have been deemed insufficient because of the multiple steps required between external exposure to a carcinogen and the ultimate production of neoplastic changes.[1] Thus, biological monitoring has focused on markers of internal dose (the amount of carcinogen or its active metabolite present in biological fluids such as serum or urine) or biologically effective dose (the amount of activated carcinogen that has interacted with critical target molecules in the cell, eg, DNA-carcinogen adducts).[2] These approaches, however, remain compound-specific and thus necessitate multiple tests for a worker with many different chemical exposures over time or with exposures to complex mixtures of carcinogens. Furthermore, even the identification of a specific increase in biologically effective dose does not necessarily guarantee that a target cell has become committed to the cancer pathway and thus will cause clinical disease.[3]

Chemical carcinogens presumably cause cancer by producing genetic damage (eg, initiators that produce point mutations in genes or chromosomal breaks with translocations of genes) or by epigenetic phenomena (eg, promoters that influence signal transduction and could thus secondarily alter the levels of expression of genes). However, in these cases, it is apparently the effect on a specific subset of the genome that contributes to the cancer pathway.[3] This subset includes genes that normally exert stimulatory (oncogenes) or inhibitory (antioncogenes) effects on cell growth and division.[4] Anti-oncogenes or tumor suppressor genes likely play a major role in many instances of tumorigenesis,[5] but the potential role of chemical carcinogens in their inactivation remains to be defined, except for the recent suggestion that aflatoxin-induced carcinogenesis in the liver is produced by specific point mutations in the anti-oncogene protein p53.[6] On the other hand, considerable progress has been made in elucidating the potential role of chemical carcinogens in contributing to tumorigenesis via the activation of oncogenes.[7]

Although it is clear that no single gene can account for all the changes in all tissues in the multistage

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progression from normalcy to malignancy and that oncogenes interact with one another and with anti-oncogenes in the process,[8] certain oncogenes have emerged as particularly important in the development of certain human tumors and of special relevance to chemical carcinogenesis. The prototypical example of such an oncogene is *ras*.[9]

It is generally conceded that *ras* represents a nexus for the control of cellular proliferation and thus could be important to the development of many cancers.[4,9] Indeed, *ras* frequently is found to be activated in many human cancers, including those of special relevance to chemical carcinogenesis such as lung cancer;[9,10] for example, many occupational and environmental chemical carcinogen exposures are via the respiratory route and in many cases result in an increase in pulmonary malignancy.[11] Furthermore, in in vitro and animal models it has been shown that these same chemical exposures are capable of activating the *ras* gene.[9] Thus, the *ras* gene represents a likely candidate for a potential cancer biomarker in cohorts with these chemical exposures; the evidence for its possible application as a cancer biomarker will be considered here with particular emphasis on common respiratory carcinogens such as polycyclic aromatic hydrocarbons (PAHs) and pulmonary carcinogenesis.

ras Oncogene and Chemical Carcinogenesis

Three major classes of *ras* genes have been identified. H-*ras* and K-*ras* were first identified in the genome of acute transforming retroviruses, H-MuSV (Harvey virus) and K-MuSV (Kirsten virus), respectively. H-MuSV and K-MuSV were isolated from solid tumors in rats (rat sarcomas, hence the name of the gene) infected with the ecotropic chronic mouse retrovirus MuLV. H-*ras* and K-*ras* have been shown to have normal homolog (cellular proto-oncogenes) in the human genome on chromosomes 11 and 12, respectively. N-*ras* has not been found in acute transforming retroviruses but was isolated directly from the genome of human neuroblastoma cells and shown to have a normal homolog on chromosome 1.[9,10]

To produce transformation of cells in culture, *ras* genes can be activated either by the introduction of specific point mutations (eg, at codons 12, 13, 61, 63 or 119) or by overexpression of the normal proto-oncogene.[9,10] Evidence suggests that these *ras* gene activations can be accomplished by carcinogens of occupational concern. The best studied of these carcinogens have been the PAHs, including benzo(*a*)pyrene (BP) and dimethylbenzanthracene (DMBA).[7,9] PAHs are ubiquitous compounds usually formed during the combustion or other high-temperature processing of carbon-containing compounds. Besides being significant environmental contaminants and critical carcinogenic components of cigarette smoke, they are important occupational carcinogens in a wide variety of industrial settings (including coke production, iron and steel founding, coal gasification, aluminum production, rubber/tire manufacturing, and petroleum refining/petrochemical production).[12,13] It should be noted that in most of these situations, exposure is via the respiratory route, and, in many cases, epidemiologic evidence suggests an increase in lung cancer in the exposed cohorts.[12,13]

In vitro experiments have demonstrated that selected PAHs can activate *ras* genes via production of point mutations at the critical susceptible positions in the coding sequences.[14] This has been documented in vitro and in vivo for many different PAHs in many different systems.[7,9,15,16] For example, mouse skin tumors induced by BP or DMBA have been shown to contain G → T transversions in the 12th codon of the H-*ras* gene as an early event.[7] Likewise, G → T transversions at codon 12 of H-*ras* have been detected in human fibroblast cell lines transformed by BP.[17] The presumed ultimate carcinogenic metabolite of BP, the diol epoxide, is known to bind primarily to guanosine at the N^2 position in native DNA[18] and induces predominantly G → T transversions in several systems.[19,20] Thus, PAH-induced point mutation is one likely mechanism for *ras* gene activation. In addition, activation of *ras* genes by overexpression can be produced by carcinogenic chemicals such as PAHs that produce alterations in noncoding sequences of the gene. For example, the 5′ flanking region of the human H-*ras* gene contains a number of Sp1 transcription factor binding sites[21] as well as two sets of positive and negative elements that affect the efficiency of H-*ras* oncogene transformation.[22]

There are seven GC runs within this 5′ flanking region, several of which are within the Sp1 transcription factor binding sites. These GC runs have been shown to be preferred sites of alkylation in vitro by guanine alkylating agents, including PAHs such as BP.[23] Such alkylation once again could produce mutations that directly affect gene expression, for example, through the altered binding of transcription factors or through recombinational and translocational events during DNA repair. For instance, support for the latter is provided by evidence that recombinant *ras* genes without mutations in the coding region can transform cells in culture[24] and the fact that H-*ras* is translocated in an erythroblastic cell line derived from a PAH-induced leukemia.[25] Thus, it is possible that an important mechanism of PAH induction of cancer is via activation of *ras* genes by point mutation or overexpression.

As with all genes, the *ras* genes exert their effect in cells through the expression of their protein product. *Ras* genes encode a protein of 189 amino acids of molecular weight 21 kd, hence designated p21. Contained in all eukaryotic cells on the inner surface of the plasma cell membrane,[9,10] p21 binds GTP, has GTPase activity, and apparently functions as a G-protein in some unidentified signal transduction pathway.[9,10] Point mutations in the coding sequences of the *ras* gene produce amino acid substitutions in p21 that affect this function by causing conformational changes in the protein.[26,27] Protein conformational effects may likewise explain the activation of *ras* by overexpression because even for the normal proto-oncogene encoded protein, a small proportion of molecules in the cell will exist in the same conformation as that assumed by the mutant transform-

ing proteins; at normal levels of expression, the presence of this small number of p21 molecules in a transforming conformation apparently has no physiologic effect, but with overexpression, a sufficient number of p21 molecules in this minority conformation is generated to result in cell transformation.[27] These conformational effects may relate to differences in GTPase activity mediated by the binding of another protein, GTPase activating protein.[4,9] How this translates into signal transduction is unclear.

It is clear that p21 interacts with several other intracellular proteins that may be important to signal transduction.[8] These may be involved in a phospholipase C-phosphatidylinositol-protein kinase C pathway.[28] It is clear that p21 is vital for cell division.[4,9] Furthermore, it is apparent p21 is responsible for the transforming effect of *ras* genes. For example, when p21 is microinjected into cells in culture, transient cell transformation is produced, with the cells gradually reverting to a normal phenotype as the protein is degraded intracellularly,[29] and, conversely, when monoclonal antibody directed against p21 is introduced into *ras*-transformed cells, the cells temporarily revert to a normal phenotype as the antibody binds to the protein, cancelling its effect.[30] In summary, there is a growing body of evidence that certain common carcinogens of occupational and environmental concern (such as PAHs) can activate *ras* genes and that the resultant expression of the p21 protein product is capable of producing cell transformation, which is believed to represent an important step in the progression from normalcy to malignancy.

ras, p21, and Lung Cancer

As noted above, a common mode of exposure to PAHs is via the respiratory route. Thus, based on the above evidence, one might expect to find *ras* gene activation and p21 protein expression in a significant proportion of lung cancers because the majority of human lung cancers are related to exposure to cigarette smoke, which contains PAHs as an important component,[13] and lung tissue of smokers is known to contain PAH-DNA adducts.[31] There is animal evidence to support this expectation. For example, G → T transversions, which can be produced by PAHs, are the most frequently detected mutations in *ras* genes in BP-induced mouse lung tumors;[32] in addition, such changes were noted at a very early stage of tumorigenesis (ie, in lung adenomas less than 1 mm in diameter).[32]

Studies of DNA from human lung cancers or cancer cell lines provide similar evidence. Several studies have shown activated K-, H-, and N-*ras* genes (with mutations at codons 12 and 61 primarily) in these cells, particularly for adenocarcinomas (27% to 55% of adenocarcinomas), 17% to 50% of all lung cancers), and the mutation profile of the activated genes revealed that G → T transversions were the most frequently detected.[33–35] At least one of these studies provides evidence that these events are smoking related and that they may occur early in the carcinogenic process.[33,34] One of these studies also included an epidermoid carcinoma of the lung with a nonmutated H-*ras* gene probably activated by overexpression.[35] Another study has demonstrated *ras* gene amplification (which presumably would result in overexpression) in eight human lung cancers; several of these were found to contain codon 12 point mutations as well, but no point mutations were detected in the coding sequences of three others.[36]

The findings of these studies on DNA from human lung cancers imply that *ras* gene point mutations in the coding sequences may play an important role in PAH-induced pulmonary carcinogenesis but that *ras* gene overexpression may be of lesser importance. However, studies of *ras* mRNA and p21 protein expression in human lung cancers provide a somewhat different picture. For example, enhanced expression of H- or K-*ras* mRNA has been noted in 4 of 4 human lung cancers.[37] Immunocytochemical study with monoclonal antibody of p21 protein expression in 73 primary bronchial carcinomas revealed a high percentage (48%) with overexpression with some variability by histologic type (61% of squamous cell carcinomas, 32% of adenocarcinomas, and 30% of small cell carcinomas).[38] In addition, a study of immunoblotting with monoclonal antibody to p21 of tissue extracts from 23 human lung tumors demonstrated 4- to >10-fold overexpression in comparison with adjacent normal tissue of apparently normal p21 in 10 of the tumors (32%) and only one adenocarcinoma (4%) exhibited expression of an apparently structurally altered p21 (as evidenced by different electrophoretic mobility); p21 overexpression appeared to occur predominantly in squamous cell lesions (82%).[39] Thus, the results of these studies imply that *ras* gene overexpression may indeed be quite important in pulmonary carcinogenesis.

These discrepancies remain to be explained. Partially they may be due to different mechanisms of activation in different tumor types, eg, *ras* mutation in adenocarcinomas and *ras* overexpression in squamous cell carcinomas, or they may reflect the fact that what occurs at the gene level does not always necessarily translate to events at the protein level.

Because the protein product of the *ras* gene is responsible for the physiologic effect, further study of p21 protein expression in lung cancer would be particularly useful. In addition, it has been demonstrated that p21 reaches the extracellular environment of cells in culture and should thus be accessible to study in easily obtainable biological fluids in vivo rather than having to rely on tissue analysis.[40–42] This has been demonstrated in animal models where p21 can be detected by immunoblotting with monoclonal antibodies in the serum of animals with tumors expressing the *ras* gene.[41,42] Furthermore, p21 can be detected by similar techniques in the serum and urine of cancer patients.[41,43,44] This technique appears to be adequately sensitive, specific, and reproducible for study in human populations.[40]

We have applied this approach to the study of non-small cell lung cancer patients with histories of cigarette smoking. Initial results showed frequent elevation (>5-fold) of p21 in the serum of these patients compared with normal unexposed controls without evidence for the presence of mutated forms of p21 by electrophoretic

mobility.[44] More recent studies using p21 mutant-specific antibodies have tended to confirm this, ie, frequent (in approximately 34% of cases) elevation (>5-fold) of normal H-*ras* p21 and infrequent (in approximately 3%) expression of mutated K-*ras* p21 in the serum of lung cancer patients regardless of histologic subtype among non-small cell cases (P. W. Brandt-Rauf et al, work in progress). These results would be consistent with those for p21 protein expression in lung cancer tissue noted above. Furthermore, these results confirm that altered expression of p21 can be detected in the sera of patients with cancer associated with a known PAH-related carcinogenic exposure, cigarette smoke.

In addition, four patients with non-small cell lung cancer were observed with serial serum p21 determinations over the course of their therapy.[45] Two patients had no response to therapy, nor did their pattern of serum p21 expression evidence any improvement over time, suggesting they still had a significant residual burden of tumor, albeit not clinically apparent. With 2 years, both patients had suffered a relapse and succumbed to their disease.[45] These findings suggest that serum p21 levels may actually precede and thus predict clinical recurrence of lung cancer. This also raises the possibility of detecting altered p21 expression in the sera of clinically healthy persons at risk for the development of pulmonary neoplasms caused by occupational or environmental exposures at a point in the disease process before the clinical presentation, thus predicting those in an exposed cohort who will develop lung cancer.

p21 Expression in Occupational Cohorts with Respiratory Exposure to Chemical Carcinogens

p21 protein expression has been evaluated in the serum of several cohorts with respiratory exposure to carcinogens in the workplace. A cohort of 10 unexposed controls and 8 Finnish foundry workers with known workplace high exposures to PAHs (>0.05 μg of BP/m^3 8-hour time-weighted average) and historically at high risk for the development of lung cancer (due to their occupational exposure) were examined for PAH-DNA adducts and serum p21.[46] PAH-DNA adducts in the peripheral lymphocytes of these workers were found to correlate with levels of ambient exposure (average adducts = 1.08 fmol/ng in the exposed *v* 0.14 fmol/ng in the controls). No p21 abnormalities were identified in the controls, but one of the exposed workers had elevated expression (>5-fold) of serum p21.[46] Follow-up will be necessary to determine whether this person is indeed at risk to develop pulmonary malignancy.

In another cohort of 16 hazardous waste workers with mixed respiratory exposures to carcinogens (including PAHs), abnormal levels of serum p21 were detected in three persons.[47] In an age-sex-race matched control cohort of workers, two were found to have abnormal elevation of serum p21, but both of these workers were heavy cigarette smokers; all nonsmoking controls were negative.[48] Follow-up will be necessary to determine whether the positive workers will develop pulmonary malignancy. One of the positive persons subsequently developed a premalignant neoplasm of the colon, and on removal of the lesion, his serum pattern of p21 expression reverted to normal, suggesting that the p21 biomarker may be useful for carcinogenic monitoring of other (nonpulmonary sites).[49]

To address the usefulness of this approach for monitoring pulmonary carcinogenesis in exposed occupational cohorts, a follow-up study is under way. Banked serum specimens have been collected annually over years on workers with respiratory exposure to carcinogens. A certain number of these workers subsequently developed lung cancers. The samples are being assayed for p21 and other oncoproteins to examine the time course of expression and the potential for predicting subsequent development of disease. Preliminary findings on this cohort indicate that nine of the 46 developed cancers of the respiratory tract. Of these nine persons, seven were found to be positive for elevated serum p21, and, on average, their serum specimens were positive 14 months before the time of clinical diagnosis. The two workers with negative tests who subsequently developed lung cancer had their last blood samples drawn more than 2 years before the time of diagnosis; no samples were available from the time of diagnosis (P. W. Brandt-Rauf et al, work in progress). This suggests that p21 may be a useful early marker of preclinical carcinogenic pulmonary response in persons with respiratory exposure to chemical carcinogens.

Conclusions

Evidence is accumulating for an important role of oncogenes and oncoproteins in chemical carcinogenesis. In particular, it seems likely that the *ras* oncogene and its p21 oncoprotein play some part in pulmonary carcinogenesis induced by chemicals of occupational and environmental concern such as PAHs. Thus, *ras*/p21 may serve as a useful biomarker for cancer monitoring in cohorts with such chemical exposures. The detection of such markers may prove useful for early intervention and prevention. For example, in persons with altered serum p21, radiolabeled monoclonal antibodies may be useful for localization of small lesions that may be amenable to surgical cure. This approach has been demonstrated for localization of lung cancers using monoclonal antibodies to the protein product of another oncogene (c-*myc*).[50] For positive persons with no localizable lesion, it may be possible to develop anti-p21 specific chemoprophylactic agents that could be used to prevent the development of clinical lesions. Studies on the development of prototypes are under way by J. E. Carucci et al,[51] G. Lee et al,[52] and D. L. Chung, P. W. Brandt-Rauf, R. B. Murphy, et al (unpublished data).

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