Expression of cyclin D1/2 in the lungs of strain A/J mice fed chemopreventive agents

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Male strain A mice were fed a diet containing chemopreventive agents. After 1 and 3 weeks on the diets, lung nuclear fractions were examined for expression of cyclin D1/2 with western blot analysis. In animals fed a diet containing a mixture of myoinositol and dexamethasone, a treatment found previously to be effective in preventing the development of tobacco smoke-induced lung tumors in A/J mice, cyclin D1/2 expression was reduced to 30–40% of control levels. A similar decrease in cyclin D1/2 expression was found when animals were fed either myoinositol or dexamethasone alone. Paradoxically, tobacco smoke by itself had a similar effect on cyclin D1/2 expression. On the other hand, several agents that had been previously found not to be effective against tobacco smoke carcinogenesis [phenethyl isothiocyanate, 1,4-phenylenebis(methylene)selenoisocyanate, N-acetylcycteine, aceftalsaliclycic acid, δ-limonene and beta carotene] did not decrease cyclin D1/2 expression after 1 or 3 weeks of feeding. It was concluded that expression of cyclin D1/2 might be a potentially useful marker in the identification of chemopreventive agents for tobacco smoke and could be of some help in the evaluation of their effects.

Introduction

Loss of cell cycle control is a key event in the progression from normal to cancerous tissue. Cyclins and the cyclin-dependent kinases (CDKs) are essential cell cycle regulators in normal cells. In cancer cells, their function is often overridden. Modulation of their activity might conceivably play an important role in cancer chemoprevention and therapy (1). Cyclin D1, a checkpoint control protein that acts at the midpoint of the transition from G1 to S, has received particular attention. In many human tumors the protein is over-expressed. It has been postulated that drugs capable of inhibiting cyclin D1 expression could be useful in cancer chemoprevention (2,3). It has recently been shown in a rat mammary tumor model that tamoxifen, a proven chemopreventive agent, decreases expression of cyclins D1 and E (4). Another putative chemopreventive agent, epigallocatechin-3-gallate, decreases cyclin D1 expression in human breast and epidermoid carcinoma cells (5,6). Studies with transformed human bronchial epithelial cells have shown that a third group of chemopreventive agents, retinoids, markedly reduce expression of cyclins D and E and of cdk2 and of cdk4, presumably due to increased proteolysis at the post-translational level. On the other hand, expression of p27 was eventually increased, whereas p21 was somewhat decreased and p16 did not change (7–9).

During the last few years, we have conducted a series of studies in which we examined the effects of chemopreventive agents on the development of tobacco smoke-induced lung tumors in strain A/J mice (10–13). In these studies we found that a combination of myoinositol and dexamethasone, fed in the diet, proved to be an effective chemopreventive regimen. On the other hand, several other agents such as phenethyl isothiocyanate (PEITC), 1,4-phenylenebis (methylene) selenoisocyanate (pXSC), green tea, aspirin, N-acetylcycteine (NAC) or δ-limonene, although previously having been shown to be effective against selected constituents of tobacco smoke, were largely ineffective when tested against the full complex mixture of cigarette smoke. It was therefore of interest to examine cyclin D1 expression in the lungs of animals fed successful or ineffectual chemopreventive diets and to see whether cyclin D expression could serve as a biomarker of effect.

Materials and methods

Animals

Male strain A/J mice, 6–8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). Randomly chosen animals were sent to the Comparative Pathology Laboratory, UC Davis, for a standard rodent health surveillance screen. No evidence for infectious disease (pathogenic agents) or presence of parasites or ova in pelage and cecum were reported. Histopathology was not processed as no significant lesions were noted. Serology was negative for mouse hepatitis virus, Sendai virus, Reovirus type 3, pneumonia virus, parvo, extromelia and mycoplasma pulmonis. The animals were housed, four to a cage, on Teklad bedding in polypropylene cages with tightly fitting wire screen lids. At all times during the experiment, including during smoke exposure, water and the test diets were provided ad libitum.

Materials

Anti-cyclin D1/2, clone 5D4 was purchased from Upstate Biotechnology (Lake Placid, NY) and anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA). PEITC, NAC, acetylsaliclycic acid (ASA), myoinositol, dexamethasone, δ-limonene and corn oil were obtained from Sigma Chemical Co. (St Louis, MO). The AIN-76A test diet was purchased in powdered form from Dyets (Bethlehem, PA) and consisted of 20% casein, 0.3% L-methionine, 15% corn starch, 55% sucrose, 5% cellulose, 3.5% mineral mix, 1% vitamin mix and 0.2% choline bitartrate. The organoselenium compound pXSC was synthesized according to the method originally described by El-Bayoumy et al. (14). Purity and spectral characteristics were the same as described before (12). Test diets containing the chemopreventive agents were prepared fresh every other week by adding the appropriate amounts of the ingredients plus 50 ml of corn oil/kg of diet. The diets were mixed thoroughly in a Hobart blender and stored at 4°C until used. All other materials were of the highest obtainable grade.

Tobacco smoke exposure

Mice were exposed to a mixture of 89% cigarette sidestream and 11% mainstream smoke generated from burning Kentucky 1R4F reference cigarettes in the exposure system as described before (15,16). Exposure was for 6 h a day, 5 days a week to an average concentration of 130 mg total suspended particulate matter (TSP) per cubic meter air.

Western blot analysis

All animals were killed by pentobarbital overdose. Protein from lungs was prepared as described by Sabourin et al. (17). Lungs were homogenized gently

Abbreviations: ASA, acetylsaliclycic acid (aspirin); CDK, cyclin-dependent kinases; NAC, N-acetylcycteine; PEITC, phenethyl isothiocyanate; pXSC, 1,4-phenylenebis(methylene)selenoisocyanate; TSP, total suspended particulates.
with a teflon-glass homogenizer in buffer consisting of 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 1.0 M EDTA, 1.0 M Na3VO4, 1.0 mM dithiothreitol, 1.0 mM phenylmethylsulfonide fluoride, 25 µg/ml aprotinin, 25 µg/ml leupeptin and 7 µg/ml pepstatin. The homogenate was centrifuged at 14,000 g for 15 min and protein concentration measured by using the Bio-Rad assay (Bio-Rad, Richmond, CA). Fifty to 100 µg of protein were mixed with loading buffer (187.5 mM Tris buffer, pH 6.8, 6% SDS, 30% glycerol and 0.3% bromphenol blue). Each gel was loaded with four experimental nuclear extracts, each one prepared by pooling the lungs of two animals and with four different control extracts (representing two pooled lungs each). After loading for 5 min, samples were electrophoresed on 10% SDS–PAGE. Proteins were electroblotted to a polyvinylidene difluoride membrane (PVDF Bio-Rad), blocked in 5% milk in TBST (150 mM NaCl, 10 mM Tris–HCl pH 8.0, 0.1% Tween-20) for 1 h at room temperature and incubated with the monoclonal antibodies in blocking buffer overnight at 4°C. The membrane was rinsed in TBST for 30 min and then incubated with HRP-linked secondary antibody in TBST for 1 h at room temperature. Super Signal West Pico Chemoluminescent substrate was used to visualize the protein bands and intensity of the signals were captured on film. Densitometric analysis of the protein bands was done using AMBIS Imaging Software (AMBIS Image Acquisition and Analysis, Version 4.0; AMBIS, San Diego, CA). The same PVDF membrane was probed for actin by first incubating the membrane in standard stripping buffer (2% SDS, 62.5 mM Tris–HCl, 100 mM β-mercaptoethanol, pH 6.8) for 30 min at 50°C followed by two TBST washes, blocking with 5% milk in TBST for 1 h at room temperature and reprobing with actin antibody. Visualization was performed as described above and the optical density of each protein band was normalized with respect to the actin protein band.

Immunocytochemistry

Procedures described previously were followed (18). Briefly, the lungs were fixed by inflation for 1 h with 1% parafomaldehyde, removed into and stored in 70% ethanol, embedded in paraffin, cut into 5 µm thick sections with a rotary microtome and placed on Superfrost/Plus glass slides. The sections were deparaffinized, rehydrated in decreasing concentrations of ethanol and treated with 3% hydrogen peroxide to eliminate endogenous peroxidase activity. Non-specific binding sites were blocked with rabbit or goat serum in phosphate-buffered saline. The tissue was incubated at room temperature with the appropriate dilutions of antibody for 60 min at room temperature. After washing, the sections were incubated with an avidin–biotin peroxidase complex at room temperature for 30 min using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and color was be developed with 3,3′-diaminobenzidine tetrahydrochloride as substrate.

Data analysis

For each gel, the mean densitometric value was calculated from the four control lanes. All densitometric values (individual experimental and individual control lanes) were then expressed as percentage of the mean of controls. Experimental and control data were calculated as mean ± SE and comparisons between treated and control animals were analyzed with Welch’s alternate test, using InStat software (GraphPad Software, San Diego, CA). A P value of 0.05 or less was considered to be significant.

Results

In the first experiment, male strain A/J mice were fed diets containing a mixture of myoinositol and dexamethasone or either agent alone. It had been found in previous experiments that a diet containing the two agents at concentrations used did not produce signs of toxicity (11). For each treatment, one group of animals was killed after 1 week on the diet and another group after having been fed the diet for 3 weeks. Age-matched animals that had been fed the control diet alone were killed at the same time. The lungs from two animals were pooled. Nuclear fractions were prepared and extracts analyzed for cyclin D1/2 expression as described in Materials and methods. For each gel, four lanes were run with four different nuclear fractions from treated animals and the other four lanes contained different nuclear extracts from control animals. Optical densities were normalized with respect to the actin protein band and then calculated as percentage of the corresponding control values on the same gel. The results are shown in Table I and representative gels in Figure 1. Within 1 week, the mixture of myoinositol–dexamethasone had produced a marked decrease in the expression of cyclin D1/2. Myoinositol or dexamethasone alone also produced a similar reduction, initially of lesser magnitude. However, after 3 weeks on the diet, each of these agents alone had reduced cyclin D1/2 expression almost to the same extent as the complete mixture.

In the next experiment mice were exposed for 5 weeks to a mixture of 89% cigarette sidestream smoke and 11% mainstream smoke, generated by burning Kentucky 1R4F cigarettes in our inhalation exposure facility. The average concentration of TSP was 130 mg/m3, for CO 385 p.p.m. and for nicotine 13.8 mg/m3. Exposed under these conditions, mice show initially some reduced weight gain and even some weight loss which, however, is fully reversible once the animals are removed from the tobacco smoke atmosphere (12,16). In the
present experiment, smoke-exposed animals, together with animals kept in a chamber ventilated with air, were killed at 1, 3 and 5 weeks after smoke exposure for determination of cyclin D1/2 expression in lung nuclear fractions. After week 5, the remaining animals were removed from the smoke atmosphere and placed into chambers ventilated with air. Half of the animals were now fed a diet containing myoinositol–dexamethasone and the other half was given control diet. After 1 and 3 weeks on the diets, lungs were analyzed for cyclin D1/2 expression.

The results of this experiment are presented in Figures 2 and 3. As long as the animals were exposed to tobacco smoke, cyclin D1/2 expression was significantly reduced when compared with values found in the lungs of air-exposed animals. One week after removal from tobacco smoke, there was a significant increase in the expression of the cell cycle regulatory protein, but after 3 weeks levels had fallen somewhat, although not significantly, below control values. However, in the animals fed the chemopreventive diet of myoinositol and dexamethasone, cyclin D1/2 expression remained significantly depressed. Immunohistochemical analysis of selected lungs showed nuclear staining for cyclin D1/2. It was found in some cuboidal epithelial cells, presumably type II epithelial cells as well as in some interstitial cells in the alveolar region. Compared with lungs from control animals (Figure 4a), fewer cells were stained positive in the lungs of animals exposed to tobacco smoke (Figure 4b). When the animals were removed from smoke, the number of positively staining cells appeared to return to normal (Figure 4c), but fewer cells stained positively in the lungs of the animals fed the myoinositol–dexamethasone diet (Figure 4d). Nuclear staining was also observed in a few cells of the small airways. No attempt at morphometric quantification of the lung sections was made.

We also examined the effects on cyclin D1/2 expression in animals fed a variety of other chemopreventive agents for either 1 or 3 weeks. In previous experiments, none of the agents tested had been shown to be effective against tobacco smoke, although all of them were highly effective against selected constituents of tobacco smoke. Fed at concentrations used in the present study, these agents did not produce signs

Fig. 2. Expression of cyclin D1/2 in the lungs of animals initially exposed to tobacco smoke (TS) for 5 weeks and then removed into air. At this time, half of the animals were fed a diet containing myoinositol–dexamethasone (Myo-Dexa). Each value represents the mean ± SE from four to eight lanes (nuclear extracts from two animals per lane), expressed as percent of control extracts run on the same gel. Values significantly different from controls (P < 0.05) are labeled with an asterisk (*).

Fig. 3. Representative gels from animals exposed for 1 week (1 wk TS) or 5 weeks (5 wk TS) to tobacco smoke. Animals then were removed into air and fed either control diet (1 wk post TS) or a diet containing myoinositol–dexamethasone (1 wk post TS plus Myo/Dexa). Lung nuclear extracts were analyzed with western blot for cyclin D1/2 (35 kDa). Each band was obtained by pooling the lungs of two animals. The three left lanes in the top two gels or the four left lanes in the bottom two gels represent treated animals and the lanes on their right are from corresponding controls fed AIN-76A alone.

Fig. 4. Lung sections stained with anti-cyclin D1/2 antibody (a) from a control animal, (b) from an animal exposed for 5 weeks to tobacco smoke, (c) from an animal removed for 1 week from tobacco smoke and (d) from an animal removed from tobacco smoke, but now fed a diet containing myoinositol–dexamethasone.
Table II. Expression of cyclin D1/2 in lung nuclear fraction of mice fed diets containing chemopreventive agents*  

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration in diet</th>
<th>After 1 week on dieta</th>
<th>After 3 weeks on dietb</th>
<th>Effective against</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA</td>
<td>300 mg/kg</td>
<td>117 ± 12% (12)</td>
<td>115 ± 10% (4)</td>
<td>No</td>
<td>(11)</td>
</tr>
<tr>
<td>Beta carotene</td>
<td>5 g/kg</td>
<td>305 ± 70% (8)*</td>
<td>120 ± 25% (4)</td>
<td>No</td>
<td>Unpublished observations</td>
</tr>
<tr>
<td>t-limonene</td>
<td>6.3 g/kg</td>
<td>84 ± 7% (8)</td>
<td>103 ± 30% (4)</td>
<td>No</td>
<td>(12)</td>
</tr>
<tr>
<td>NAC</td>
<td>2 g/kg</td>
<td>149 ± 24% (11)</td>
<td>67 ± 14% (8)</td>
<td>No</td>
<td>(10)</td>
</tr>
<tr>
<td>PEITC</td>
<td>500 mg/kg</td>
<td>160 ± 35% (8)</td>
<td>97 ± 20% (8)</td>
<td>No</td>
<td>(10,12)</td>
</tr>
<tr>
<td>pXSC</td>
<td>20 mg/kg (10 p.p.m. Sc)</td>
<td>216 ± 25% (4)*</td>
<td>107 ± 36% (8)</td>
<td>No</td>
<td>(12)</td>
</tr>
</tbody>
</table>

*Animals were fed the diets at concentrations indicated for 1 or 3 weeks. Nuclear extracts were prepared from the pooled lungs of two animals each and analyzed for cyclin D1/2 expression by western blot analysis. Samples from animals fed control diet only were run on each gel in parallel.

Data are expressed as percentage of corresponding controls run on the same gel (mean ± SE, number of assays in brackets). Average control values were 100 ± 16%. Values significantly different from control values (P < 0.05) are labeled with an asterisk (*).

Discussion

In normal, immortalized and transformed human bronchial epithelial cells, retinoids decreased the amount of cyclin D1 protein, presumably through post-translational proteolysis. As a consequence, overall cell growth declined. Carotenoids had no such effect (7,9). While cis-retinoic acid has shown some limited beneficial effects in clinical trials, beta carotene was ineffective and even might have enhanced the development of lung cancer in current smokers (19,20). The in vitro studies with human bronchial cells might therefore have correctly predicted the diverging effect of retinoids and carotenoids. In addition, the experiments offer a mechanistic basis for chemopreventive action. It may be concluded, as was postulated before (3), that decreased expression of cyclin D1 is related to chemopreventive action.

In principle, the results of the present in vivo investigations seem to agree with this general inference that, so far, was mostly drawn only from in vitro studies. The decreased expression of cyclin D1/2 in animals treated with a combination of myoinositol and dexamethasone, as well as in animals treated with either agent alone, probably reflects a potential decrease in cell proliferation. Glucocorticoids are known to interfere with the growth of lung tumors in strain A/J mice (21,22). In human cultured smooth airway muscle cells, dexamethasone produces a G1 block in cell cycle progression, presumably due to decreased expression of cyclin D1 (23). As far as myoinositol, the unphosphorylated stereoisomer of inositol, is concerned it is present in many foods and is a major component in rice bran (24). Although its exact mechanism of chemopreventive action remains to be established, there is enough evidence to ascertain that control of cell division may be a central pathway (25). Our observations support this view and will require further investigations into possible mechanisms of action.

The work by Wattenberg et al. (26–28) has established that a combination of myoinositol–dexamethasone, as well as either agent alone, is an effective chemopreventive agent against lung tumors in mice induced by tobacco smoke-specific carcinogens. Both agents represent a small group of agents that are true suppressing agents, i.e. agents that have chemopreventive activity even when given once carcinogen exposure has ceased (26). Dose–response relationships for dietary myoinositol in a NNK-benzo[a]pyrene lung tumor model have recently been documented (29). In a tobacco smoke-induced lung cancer model, only the combination of myoinositol and dexamethasone has been studied (11,12). It is entirely conceivable that either agent alone might also be an effective chemopreventive treatment against tobacco smoke carcinogenesis. This could have some practical implications. Glucocorticoids have the disadvantage of being able to bring about undesired side effects when administered over a long period of time, such as would be necessary for chemoprevention. Attempts to circumvent...
this problem have been made through topical delivery of synthetic glucocorticoids in the hope that systemic toxicity can be kept to a minimum (28,30). Chemoprevention could be accomplished with the use of inhalers. On the other hand, myoinositol has no known toxicity and has been given to people over weeks in doses from up to 10–20 g/day without any apparent side effects (31,32). Dietary supplementation with myoinositol, should it found to be effective in an animal model of tobacco smoke carcinogenesis, could thus be considered as a useful chemopreventive agent in current smokers or in smokers who have recently quit smoking where it might further reduce their risk to develop lung cancer.

Of the other chemopreventive agents evaluated, none of them decreased expression of cyclin D1/2 in the lungs. This is in agreement with the fact that none of these agents had been found previously to modify tobacco smoke-induced tumor development in A/J mice (13). Beta carotene produced an initial over-expression of cyclin D1/2; although the effect was transient, it might be related to the evidence for the proliferative response found in the lungs of ferrets fed beta carotene for prolonged periods of time (33,34). No explanation is available for the temporary increase in cyclin D1/2 expression caused by pXSC. As far as NAC is concerned, it has recently been reported that prolongation of the G1 phase could be one possible mechanism in the chemopreventive action that has been demonstrated in several animal systems (35,36). Although there are many compelling mechanistic reasons for NAC to be an effective chemopreventive agent, it has so far not been found to effective against lung tumors induced by tobacco smoke in strain A/J mice; the reason for this—possible strain differences—remains to be elucidated (37). Human trials have so far been disappointing (38). These observations raise the question whether correlation between cyclin D1 expression and chemopreventive action will apply for all agents and under all circumstances.

An unexpected finding was the discovery of substantial and sustained, albeit reversible, decreased cyclin D1/2 expression in the lungs of mice exposed to tobacco smoke. We have reported before that tobacco smoke is capable of interference with the growth of chemically induced lung tumors (16). Also, as long as mice are kept in tobacco smoke, lung tumor growth is effectively curtailed, and tumors appear to grow only once the animals have been removed from the smoke atmosphere (13,39). One possible mechanism to account for this observation is that tobacco smoke is capable of inducing apoptosis. This was shown in human cells maintained in vitro, including pulmonary macrophages (40,41), in hair follicle cells of the skin (42) and in epithelial cells lining the airways of tobacco smoke-exposed mice and rats (43). Cytotoxicity might represent an additional mechanism, as could the observed reduction in the expression of cyclin D1/2. However, the data conflict with previous studies that showed initially increased cell proliferation in nasal epithelia, conducting airways and alveolar regions of A/J mice exposed to similar concentrations of tobacco smoke for up to 20 weeks, with no significant rebound phenomenon observed following cessation of smoke exposure (16,44). These discrepancies as well as the exact mechanisms by which tobacco smoke, as long as present, appears to interfere with tumor development remain to be further elucidated.

In conclusion, the observed correlations between decreased cyclin D1/2 expression and effective/non-effective chemoprevention might make the monitoring of cyclin D1/2 expression a good biomarker of effect for chemopreventive activity. In animal studies, the marker could be used to help dose setting and as a general screening tool for putative new chemopreventive agents. Finding an effect on cyclin D1/2 expression in comparatively short-term experiments might be an encouraging sign for further and more involved bioassays. In human chemoprevention trials, quantitative assessment of cyclin D expression might be an additional tool to estimate possible efficiency of chemopreventive regimen, provided the study protocol already foresees that biopsies of human respiratory tract tissue become available.

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