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The cooked meat-derived mammary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine promotes invasive behaviour of breast cancer cells

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ABSTRACT

The cooked meat derived genotoxic carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) induces cancer of the colon, prostate and mammary gland when fed to rats. Epidemiology studies link these tumours to a Western diet and exposure to heterocyclic amines such as PhIP. We have shown that PhIP is also potently estrogenic and have proposed that this hormonal activity contributes to its target site carcinogenicity. We now postulate that the estrogenic properties of PhIP influence metastatic potential. We have used an *in vitro* assay for cell invasion based upon digestion and migration through a reconstituted basement membrane model. Zymography and immunoblotting were used to confirm PhIP-mediated changes associated with induction of the invasive phenotype. Treatment of the mammary cancer cell lines MCF-7 and T47D with PhIP induces cells to digest and migrate through a reconstituted basement membrane. The response was dose dependent, observed at sub-nanomolar concentrations of PhIP and was inhibited by the antiestrogen ICI 182,780. The PhIP-induced invasive phenotype was associated with expression of cathepsin D, cyclooxygenase-2 and matrix metalloproteinase activity. These findings emphasise the range and potency of the biological activities associated with this cooked meat product and mechanistically support the tissue-specific carcinogenicity of the chemical.

1. Introduction

It is well documented that dietary factors play a crucial role in the development of human cancer. Since the consumption of meat is positively correlated with human cancer (Gerhardsson de Verdier et al., 1991), the carcinogenic heterocyclic amines (HCA) formed during the cooking of meat have been proposed as candidate etiological agents of diet-associated neoplastic disease. One such HCA, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), induces cancer of the colon, prostate and mammary gland of rats (Ito et al., 1991), tumours that are strongly associated with a Western diet (Sinha et al., 2000; Willett, 1994). Exposure to PhIP is difficult to avoid because of its presence in many commonly consumed cooked meats, particularly chicken, beef and fish (Felton and Knize, 1991; Murray et al., 1993). PhIP has also been identified in cigarette smoke, diesel exhaust particles and incineration ash (Manabe et al., 1991), suggesting that it may be a widespread environmental pollutant. Key to assessing the role PhIP may play in the etiology of human cancer is understanding the mechanisms by which it exerts its effects.

The genotoxicity and mutagenicity of PhIP have been studied extensively. Like many carcinogens, PhIP itself is not genotoxic, but requires metabolic activation to a direct acting mutagenic species. The major pathway for bioactivation involves *N*-hydroxylation catalysed by cytochrome P450, followed by esterification of the *N*-hydroxy intermediate (Holme et al., 1989; Zhao et al., 1994). The activated PhIP attacks and covalently binds DNA, primarily at the C-8 position of guanine to form the N^2-(deoxyguanosin-8-yl)-PhIP adduct (Buonarati et al., 1990). The formation of such DNA adducts is considered to be a precursor to mutation.

While the genotoxicity of high-dose activated PhIP has been characterised comprehensively, few studies have been carried out regarding the effects exerted by physiologically relevant concentrations of the compound. In terms of daily intakes, Layton et al. (1995) estimated that total HCA intake ranged from <1 to 17 ng/kg body weight (b.w.) per day. Compared to the concentrations found in the human diet, the majority of experimental studies have used PhIP at much higher doses, since PhIP's mutagenic potential is essentially not measurable at these lower concentrations.

It is well documented that17β-oestradiol (E2) has an important influence on the development of breast cancer. In addition to its pro-proliferative role, the hormone stimulates the metastatic potential of cells through its high-affinity binding to the estrogen receptor (Albini et al., 1986) and increases the ability of breast can-

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cer cells to form tumours and distant metastases *in vivo* (Shafie and Liotta, 1980). In addition, antiestrogens such as tamoxifen can limit the metastatic potential of cells. Taken together, these findings indicate that E2 has an important role in promoting cell invasion and metastasis of breast cancer.

In vivo cancer is defined not just by the growth rate of tumour cells, but additionally by the breakdown of tissue organisation and by cell invasion. Therefore, tumour progression is critically dependent on the degradation of extracellular membrane (ECM) proteins. The expression of certain enzymes by tumour cells may lead to the metastatic phenotype, presumably by breaking down ECM barriers. Since PhIP has estrogenic activity in terms of cell proliferation and the ability to induce E2-dependent proteins (Lauber et al., 2004; Lauber and Gooderham, 2007), it was hypothesised that PhIP may also have the ability to influence cancer cell invasion. This was investigated using *in vitro* invasion assays, by examining the expression of proteins involved in cell migration and by looking at the ability of PhIP to modulate the activity of the gelatinase enzyme, matrix metalloproteinase-9 (MMP-9).

2. Materials and methods

2.1. Chemicals and cell lines

MCF-7 and T47D cells were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK). Roswell Park Memorial Institute (RPMI) 1640 media, Minimal Essential Medium (MEM), foetal bovine serum (FBS), L-glutamine, penicillin/streptomycin were purchased from Invitrogen (Paisley, UK). PhIP was obtained from Toronto Research Chemicals (Toronto, Canada) and the antiestrogen ICI 182,780 from AstraZeneca (Macclesfield, UK). BD BioCoat™ Matrigel™ Invasion Chambers were bought from BD Biosciences (Oxford, UK). Rabbit polyclonal anti-COX-2 (H-62) and anti-cathepsin D (C-20) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Dextran coated charcoal stripped FBS was prepared as previously described (Lauber et al., 2004). Hybond ECL nitrocellulose membrane was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other reagents were purchased from Merck-BDH (Lutterworth, UK) and were of AnaLar grade. Amicon Centriprep units were purchased from Amicon (Gloucestershire, UK). MMP-9 recombinant protein was purchased from CN Biosciences (Nottingham, UK). All other chemicals and reagents were obtained from Sigma (Poole, UK).

2.2. Invasion assay

Numerous studies have used the mammary cancer cell lines MCF-7 and T47D for migration and invasion studies (Arima et al., 2008; Guo et al., 2008; Litzenburger et al., 2009; Naik et al., 2008; Nouhi et al., 2006). MCF-7 cells were routinely maintained in MEM supplemented with 1% non-essential amino acids, 10% FBS, 2 mM L-glutamine and 100 IU/ml penicillin/100 μg/ml streptomycin in an incubator maintained at 5% CO_2 and 37 °C. T47D cells were maintained in RPMI 1640 supplemented with 5% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

Invasion assays were carried out by a method based on previously described work (Albini et al., 1986; Cos et al., 1998) using 8 μm-pore size Falcon cell culture inserts coated with Matrigel. MCF-7 or T47D cells were suspended in phenol red-free MEM or RPMI supplemented with 5% dextran-coated charcoal-stripped serum (DCCSS) and plated at 8×10^4 cells/filter in 24-well plates. Media containing 10% FBS and 1 μg/ml fibronectin as a chemoattractant was added to the lower chamber. At the beginning of the assay, PhIP (10^{-7} M to 10^{-11} M) or E2 (10^{-8} M) was added to the upper compartments, in the presence or absence of the antiestrogen ICI 182,780. Experiments were also undertaken to examine the effects of the structurally related cooked meat-derived HCA 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) (10^{-6} M to 10^{-10} M) on T47D cell invasion. After incubation for 72 h at 37 °C, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The cells that had transversed the Matrigel membrane (invading cells) and attached to the lower surface of the filter were fixed and stained with haematoxylin and eosin staining. The filters were then mounted onto glass slides and photographed. Invading cells were quantified by counting the cells visible in 10 randomly selected microscopic fields (×40) per membrane.

2.3. Zymography

Substrate gel zymography of the activity of MMP-9 from MCF-7 conditioned medium was performed based on a method described by Wolczynski et al. (2001). This technique involves the electrophoresis of secreted protease enzymes through discontinuous polyacrylamide gels containing enzyme substrate. Subconfluent MCF-7cells were inoculated at 5×10^5 cells per well in six-well plates in 2 ml of experimental medium (phenol red-free MEM supplemented with 5% DCCSS). After 2 days, the cells were washed with PBS and the media was replaced. Cells were then treated with E2 (10^{-8} M) or PhIP (10^{-6} M to 10^{-10} M) in triplicate for 24 h. Control cells were incubated with a media containing an equivalent amount of ethanol vehicle without the test compound. The MCF-7 conditioned medium was carefully removed from the wells and concentrated ~40 fold by centrifugation in Amicon Centriprep 30 concentrators at 6000 rpm for 1 h. The samples were then mixed with an equal volume of 2× non-reducing sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.00625% bromophenol blue) and stored at −20 °C until required.

Thawed samples (20 μl) were loaded without boiling into the wells of a polyacrylamide (7.5%, w/v) gelatine (0.2%, w/v) gel. Molecular weight protein marker (5 μl) and the recombinant MMP-9 positive control (0.1 μg/lane) were loaded into additional wells. Gels were electrophoresed (120 V at 4 °C in 25 mM Tris–HCl, pH 8.3, 192 mM glycine, 0.1% SDS) for 1 h, until the bromophenol blue marker dye reached the bottom of the gel. After electrophoresis SDS was removed from the gel by washing for 3 × 10 min in 2.5% Triton X-100 solution to allow any MMPs present to renature. The gel was rinsed in distilled water and then incubated for 20 h at 37 °C in low salt collagenase buffer (50 mM Tris–HCl, pH 7.6, 10 mM $CaCl_2$, 50 mM NaCl, 0.02% Brij 35). After incubation, the gel was stained with a solution of 0.25% Coomassie blue R250, 40% methanol and 10% acetic acid for 20 min at room temperature and destained with 40% methanol, 10% acetic acid until the bands of lysis became clear. Relative band intensities were quantified using Scion Image Beta 4.02 software (Scion Corporation, MD, USA).

2.4. Immuno blotting

MCF-7 cells were placed in serum-free medium for 24 h prior to treatment. The cells were then transferred to phenol red-free MEM supplemented with 5% DCCSS and treated with compounds for 24 h. Lysates were prepared from the treated cells using RIPA buffer (50 mM Tris–HCl pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA pH 7.4, 1 mM sodium fluoride and 50 μg/ml leupeptin) and incubated at 4 °C for 15 min and 15 μg of each protein sample were separated on a 10% SDS-polyacrylamide gel. The proteins in the gel were electrotransferred onto nitrocellulose membranes in transfer buffer (192 mM glycine, 25 mM Tris base and 20% (v/v) methanol, pH 8.3). Non-specific binding sites were blocked by incubation of the membranes in blocking buffer (5% non-fat powdered milk, 0.05% Tween 20 in PBS) for 30 at room temperature. Membranes were incubated with rabbit anti-cathepsin D or rabbit anti-COX2 antibody at a dilution of 1:200, overnight at 4 °C in blocking buffer. The following day, unbound antibody was removed by washing the membrane in PBST (PBS containing 0.5% Tween 20) for 3 × 10 min. After washing, membranes were incubated in horseradish peroxidase-conjugated anti-rabbit antibody (1:10,000 dilution in blocking buffer) for 1 h at room temperature. Membranes were washed again and target protein bands were visualised using Supersignal™ chemiluminescent reagent.

3. Results

3.1. Invasion assay

In concordance with other studies, E2 enhanced the ability of both MCF-7 and T47D cells to invade through Matrigel membrane (Cos et al., 1998; Meng et al., 2000). When PhIP was added to the upper compartment of the invasion chamber, the number of both MCF-7 and T47D cells that invaded the Matrigel membrane increased as compared with that of the control group (Fig. 1A and B). This increase in invading cell number was PhIP dose-dependent (Fig. 2). Concurrent treatment with the antiestrogen ICI 182,780 inhibited the number of cells migrating through the Matrigel in both PhIP and E2-treated cells. These results concur with those of Bliss et al. (1996), who found that ICI 182,780 inhibited E2-mediated mammary cell invasion through Matrigel membrane.

Treatment of T47D cells with MeIQx did not result in increased invasion through the Matrigel membrane (Fig. 1C), compared to the vehicle control.

3.2. Zymography

Zymographic analysis of the culture medium conditioned by MCF-7 cells revealed a 92-kDa band corresponding to pro-MMP-9. In E2 stimulated MCF-7 cells there was an increase in the activity of pro-MMP-9 (Fig. 3A and B), which is in good agreement with the findings of previous studies (Wolczynski et al., 2001). Similarly, MCF-7 cells displayed a 2-fold increase in the secretion of MMP-9