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Original Article

Inhibition of gastric cancer cell growth by arginine: Molecular mechanisms of action

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SUMMARY

Background & aims: Immunonutrition, containing arginine as a key component, has been shown to enhance the immune system and significantly reduce infectious complications in patients undergoing upper gastrointestinal surgery. Arginine, however, may also influence tumour cell behaviour. The aim of this study was to investigate the effects of arginine on tumour cell growth, invasion and modulation of expression of genes involved in these aspects of cell behaviour.

Methods: A human gastric cancer cell line (AGS) was grown *in vitro* and supplemented with arginine (2, 4, 8, 16 and 32 mM) for 24, 48 and 72 h. The effect of arginine on cell growth (MTT assay), apoptosis (DAPI staining), invasion (Matrigel assay), gene expression (cDNA microarray analysis and RT-PCR) and protein expression (western analysis) was determined.

Results: These studies demonstrated that arginine caused a decrease in AGS cell growth via induction of apoptosis. Whilst arginine decreased cell growth, no significant effect on the invasive potential of AGS cells was noted. Subsequent gene expression analysis demonstrated that arginine increased the expression of caspase 8, which was validated at the protein level.

Conclusions: These results suggest that that inhibition of AGS cell growth by arginine is mediated through caspase 8 activation of apoptosis.

1. Introduction

Major surgery is followed by a period of immunosuppression with a resultant increase in morbidity due predominately to septic complications. In patients with upper gastrointestinal (gastric and oesophageal) cancer undergoing surgical resection, the immunosuppression associated with surgery together with the weight loss which these patients often have (itself associated with impaired immune function), may contribute substantially to the 40% risk of major peri-operative morbidity.[1] Standard nutritional support in these patients has not been demonstrated conclusively to affect patient outcome.[2] However, nutritional supplementation with key nutrients, which modulate immune, inflammatory and metabolic pathways, offers a therapeutic modality to reduce the risk of morbidity in patients undergoing major surgery. This approach has been termed immunonutrition and such nutrient regimens usually comprise combinations of arginine, omega-3 fatty acids and nucleotides in combination with other nutrients.

Previous meta-analyses of clinical trials have shown that the use of immunonutrition in patients with critical illness, including after surgery for upper gastrointestinal cancer, modulate their immune system and results in a reduction in morbidity, infectious complications and overall hospital stay.[2–4] However, other experimental studies have shown that some of the nutrients included in immunonutritional regimens not only have immune-enhancing effects but also have the ability to modulate tumour cell growth and proliferation.

In this respect, a particular focus has been on the amino acid arginine, which has effects on the immune system as well as direct effects on tumour growth. *In vitro* studies, animal studies and clinical studies in patients with cancer, all indicate that dietary supplementation with arginine will stimulate many aspects of the immune system, including stimulation of macrophage phagocytic activity[5,6] and of natural killer cells and lymphokine-activated killer cell activity, which may play a role in preventing tumour cell dissemination.[7,8]

Arginine has also been demonstrated to modulate cell growth and proliferation in a variety of different cancer cell lines. *In vitro* studies have reported that in lung cancer cells arginine stimulates

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cell growth[9] but inhibits cell growth in a breast cancer cell line.[10] These varying effects have been confirmed in animal tumour models with both a stimulation[11] and inhibition[12] of tumour growth with arginine supplementation. Concerns, therefore, exist with regard to what effects arginine may have in patients receiving arginine-containing regimens who are undergoing surgery for cancer.

In vivo studies in patients with breast cancer revealed that three days of dietary supplementation with arginine (30 g per day orally), resulted in increased protein synthesis in tumours, together with increased expression of Ki67, used as a surrogate marker for tumour cell activation and growth.[13] The mechanisms by which this occurs are unclear but may involve direct effects on the tumour cells, modulation of hormonal[14] and growth factor production[15] and through nitrous oxide which, in addition to all of these, might itself have implications for tumour growth and tumour neovascularisation in patients receiving immunonutrition.[16,17]

The aims of this study were to evaluate the effects of arginine on the growth of a human gastric cancer cell line, AGS, in particular its effects on cell growth, apoptosis and invasive capacity and the molecular mechanisms underlying any changes.

2. Materials and methods

2.1. Cell culture

The human gastric cancer cell line (AGS), obtained from the European Collection of Animal Cell Culture (ECACC), was cultured in HAM F12 culture medium supplemented with 10% (v:v) foetal calf serum, 0.118% (w:v) sodium bicarbonate, 0.029% (w:v) glutamine, 100,000 U/l penicillin and 100 mg/l streptomycin at 37 °C in a humidified atmosphere containing 5% (v:v) carbon dioxide. The human fibrosarcoma cell line (HT1080), used as a positive control for cell invasion assay, was cultured in RPMI-1640 culture medium and the mouse fibroblast cell line (3T3), used as a negative control for cell invasion assay, was cultured in DMEM culture medium. Media were supplemented with 10% (v:v) foetal calf serum, 0.118% (w:v) sodium bicarbonate, 100,000 U/l penicillin and 100 mg/l streptomycin and cells cultured at 37 °C in a humidified atmosphere containing 5% (v:v) carbon dioxide.

2.2. Cell proliferation assay

The effect of arginine on the growth of AGS cells was determined using the 3-[4,5-dimethylthiazol]-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay.[18] AGS cells were plated in 96-well plates with a seeding density of 1.2×10^4 cells per well in HAM F12 culture medium for 24 h before the cells were treated to varying concentrations of arginine (2, 4, 8, 16 and 32 mM) for 24, 48 and 72 h. At the end of each time period, cells were treated with 50 μl (5 mg/ml) MTT dye and incubated at 37 °C for 4 h. Following this, MTT dye was removed and 200 μl dimethyl sulphoxide was added in order to solubilise the cells and dissolve the MTT-formazan crystals. The number of viable cells was determined by measuring the absorbance at 570 nm and 630 nm for each well, using a microplate spectrophotometer (DynaTech Laboratories Inc., VA, USA). All experiments were repeated eight times with six replicates per experiment.

2.3. Assessment of apoptosis

The nuclear chromatin of cells was stained with the fluorogenic compound 4′,6-diamidino-2-phenylindole (DAPI), in order to assess the morphological changes associated with apoptosis. AGS cells were treated as described previously then fixed in 0.4% (v:v) paraformaldehyde prior to attaching the cells onto glass microscope slides by cytocentrifugation at $50 \times g$ for 5 min. The slides were then stained with DAPI solution (1:500 dilution) for 10 min. Cells were visualized by fluorescent microscopy, with a blue filter (330–380 nm), to observe morphological features of apoptosis such as cell shrinkage, chromatin condensation and formation of apoptotic bodies. A minimum of 500 cells were counted per slide and the percentage of cells displaying apoptotic characteristics was expressed as an apoptotic index (AI). This was defined as the proportion of apoptotic cells compared with the total number of cells, expressed as a percentage. All the experiments were repeated three times.

2.4. Cell invasion assay

The effect of arginine on the invasive potential of AGS cells was assessed using the BD Biocoat™ Tumour Invasion System. The system consists of a BD Falcon FluroBlok™ insert plate with an 8 μm pore PET membrane uniformly coated with BD Matrigel™ Matrix. Cell invasion was quantified by pre-labelling cells with fluorescent dye and measuring the fluorescence of invading cells with a fluorescent plate reader. AGS cells, HT 1080 cells (positive control, invasive cell line) and 3T3 cells (negative control, non-invasive cell line) were labelled *in situ* with 10 μg/ml DiI (Invitrogen, Paisley, UK) for one hour after which the cells were re-suspended in serum-free medium. Into the upper compartment AGS cells, at 5×10^4 cells/well, were added in varying concentrations of arginine (2, 4, 8, 16 mM), in triplicate, along with positive and negative controls. Into the lower compartment media containing 5% (v:v) foetal calf serum, acting as a chemo-attractant, was added. Both the Matrigel™-coated tumour invasion plate and the uncoated migration plate were incubated for 24 h, at which stage the fluorescence of invaded cells was quantified using a FL 600 fluorescence plate reader (BioTek, Vermoth, USA) at excitation and emission wavelengths of 530 nm and 590 nm, respectively. The data was expressed as the mean fluorescent units of cell invasion divided by the mean fluorescent units of cell migration and expressed as a percentage. Experiments were repeated in triplicate.

2.5. cDNA microarray analysis

Total RNA was isolated from AGS cells, treated with and without arginine, using Trizol reagent (Invitrogen, Paisley, UK), according to the manufacturer's instructions, and purified using the RNeasy Mini Kit (Qiagen, West Sussex, UK). The resulting RNA was electrophoresised, to check its integrity, using a 1% (w:v) agarose gel and in addition the purity and quantity of the RNA was confirmed by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer. Gene expression of cancer pathway genes was determined using the Cancer Pathway Finder gene expression arrays (Super Array Inc, Maryland, USA), according to the manufacturer's instructions. Gene-specific biotin-labelled cDNA probes were synthesised using 2.5 μg total RNA with gene-specific primers for reverse transcription using 200 U MMLV reverse transcriptase (Promega, Southampton, UK) and 40 U RNase inhibitor (Promega, Southampton, UK). Prior to hybridization, the cDNA membrane array was prehybridized at 60 °C for 2 h in the supplied hybridization buffer (Super Array Inc, Maryland, USA) supplemented with denatured salmon sperm DNA (Invitrogen, Paisley, UK). The cDNA probe was then denatured by heating at 94 °C for 5 min and quickly cooled on ice before hybridization with the cDNA membrane array, at 60 °C overnight. The following morning the membrane was washed accordingly and the hybridization signals were detected using the CPD Star chemiluminescent detection kit (Super Array Inc, Maryland, USA). The Fluor S phosphorimager (Biorad, Hertfordshire, UK) was used to scan the cDNA membrane array and the