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Antiproliferative effects of carbon monoxide on pancreatic cancer

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ABSTRACT

Background: Carbon monoxide, the gaseous product of heme oxygenase, is a signalling molecule with a broad spectrum of biological activities. The aim of this study was to investigate the effects of carbon monoxide on proliferation of human pancreatic cancer.
Methods: In vitro studies were performed on human pancreatic cancer cells (CAPAN-2, BxPc3, and PaTu-8902) treated with a carbon monoxide-releasing molecule or its inactive counterpart, or exposed to carbon monoxide gas (500 ppm/24 h). For *in vivo* studies, pancreatic cancer cells (CAPAN-2/PaTu-8902) were xenotransplanted subcutaneously into athymic mice, subsequently treated with carbon monoxide-releasing molecule (35 mg/kg b.w. i.p./day), or exposed to safe doses of carbon monoxide (500 ppm 1 h/day; $n = 6$ in each group).
Results: Both carbon monoxide-releasing molecule and carbon monoxide exposure significantly inhibited proliferation of human pancreatic cancer cells ($p < 0.05$). A substantial decrease in Akt phosphorylation was observed in carbon monoxide-releasing molecule compared with inactive carbon monoxide-releasing molecule treated cancer cells (by 30–50%, $p < 0.05$). Simultaneously, carbon monoxide-releasing molecule and carbon monoxide exposure inhibited tumour proliferation and microvascular density of xenotransplanted tumours ($p < 0.01$), and doubled the survival rates ($p < 0.005$). Exposure of mice to carbon monoxide led to an almost 3-fold increase in carbon monoxide content in tumour tissues ($p = 0.006$).
Conclusion: These data suggest a new biological function for carbon monoxide in carcinogenesis, and point to the potential chemotherapeutic/chemoadjuvant use of carbon monoxide in pancreatic cancer.

1. Introduction

For decades, carbon monoxide (CO) exposure has been considered a potential threat to human health, and the endogenous production of this gaseous molecule was only thought to be a waste product, a biologically inactive by-product of heme catabolism. However, it is now widely accepted that CO formed endogenously by heme oxygenase (HMOX) confers cytoprotection against tissue and cellular injury [1,2]. CO acts as a smooth muscle relaxant as well as an inhibitor of platelet aggregation *via* guanylate cyclase and cGMP generation [2]. The functional properties of CO have often been compared with nitric oxide (NO), another endogenous gaseous molecule. Indeed, CO shares a number of biological functions analogous to NO [1]. The ability of both NO and CO to act as a vasodilator and to modulate endothelial cell permeability makes it plausible that they could also play essential roles in angiogenesis. It has previously been reported that NO has a dual effect on angiogenesis, and can either promote or inhibit angiogenesis in a dose-dependent manner [3]. Although certain studies have reported that CO is pro-angiogenic [4], the similarity of CO to NO could lead to the speculation that CO might also inhibit carcinogenesis, at least partially, *via* the suppression of angiogenesis. Under stress conditions, the production of CO is increased owing to the upregulation of the stress-responsive heme oxygenase isoenzyme,

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HMOX1 (OMIM*141250) [5]. The recent discovery of transition metal carbonyls that act as CO-releasing molecules (CORMs) has provided a new impetus for the investigation of CO as a cellular messenger, as well as a potential therapeutic agent [6,7]. Both CORMs and CO at doses free from toxic side-effects have been shown to exert important biological functions in numerous model *in vitro* and *in vivo* systems, including vasodilating, antiproliferative, anti-inflammatory effects, contributing to the amelioration of many pathological conditions such as ischaemia – reperfusion injury, inflammatory bowel disease, and organ rejection (for a comprehensive review of the biological effects of CO, see Motterlini and Otterbein [7]). A wide range of CORMs/CO dosages have been tested in these models, depending on CORM type, the means of delivery, and the model used [7]. Significantly, the CO delivered was demonstrated to be non-toxic for healthy tissues, when keeping the CO haemoglobin levels within safe levels [8]. Based on these data, a CO inhalation system for human use has been developed and used in the first clinical trials [7].

Pancreatic tumours, having high mortality and recurrence rates, are an example of a tumour type in which any type of medical therapy has, at best, been only modestly effective [9]. Thus, the effective therapy for pancreatic cancer depends on the search for alternative therapeutic modalities that have the potential to inhibit multiple signalling pathways. Although pancreatic carcinogenesis is a very complex issue, with numerous intracellular pathways involved, the phosphatidylinositol-3 kinase/Akt (protein kinase B) seems to play a key role [10]. Akt activation is frequent in pancreatic cancer and correlates well with prognosis [11]; its inhibition has been reported to sensitise cancer cells to the tumour-suppressive effects of chemotherapy [12,13].

All of these facts led us to investigate the potential antiproliferative effects of CO and/or CORM-2 (a ruthenium-based, lipid-soluble CORM) on human pancreatic cancer, using experimental *in vitro* and *in vivo* models, with a special focus on the possible CO-mediated effects on Akt phosphorylation.

2. Methods

2.1. Reagents

All cell culture reagents and chemicals, and tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$) (more commonly known as CORM-2), were obtained from Sigma Aldrich (Prague, Czech Republic). The CO (500 ppm) gas mixture (20% O_2, 0.03% CO_2, remainder nitrogen) for *in vitro* studies was obtained from Linde Gas (Prague, Czech Republic).

2.2. Cell cultures

The pancreatic cancer cell lines CAPAN-2, BxPc3 (ATCC, Manassas, VA, USA), and PaTu-8902 (DSMZ, Braunschweig, Germany) were used for the *in vitro* studies. The cell lines were cultured as described previously [14]. The cell suspensions ($2 \times 10^{5-6}$ cells/ml) were used for the inoculation of individual wells in the 6-well plate. Cells were treated with either a ruthenium-based CORM (CORM-2, 50 μmol/L; this relatively high concentration was used for all *in vitro* studies because of the very short half-life of CORM-2 [7]) or its inactive counterpart, iCORM-2 (a CO-free CORM-2). The use of iCORM-2 as a control is important, since the Ru-based carrier molecule may exert some biological properties itself. Either the CORM-2 or iCORM-2 was dissolved in a solution of DMSO in PBS (final concentration of DMSO did not exceed 1%, vol/vol), on a daily basis for a period of three days. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The iCORM-2 was prepared by leaving CORM-2 at room temperature for 2 days, and flushing with nitrogen to remove the residual CO [6]. Before initiating the experiments, the release of CO from freshly diluted CORM-2 was confirmed by gas chromatography (for methodology see below). Alternatively, pancreatic cancer cells, cultured in an analogous manner to that described above, were directly exposed to CO (500 ppm) for 24 h using specific air jars (Oxoid CZ, Thermo Fisher Scientific, Prague, Czech Republic). After treatment, the cells (experiments were performed in triplicate) were washed with PBS, harvested by 0.25% trypsin, and re-suspended. Both cell growth and viability were assessed by the direct counting of trypan blue dye (0.4%) excluding cells.

To study the distribution of CO within the cells exposed to CORM, CORM-2 was incubated with PaTu-8902 pancreatic cancer cells for 75 min at 37 °C in a humidified atmosphere of 5% CO_2 in air. The iCORM-2 and 1% DMSO in PBS were used as controls. The CO concentration in the cells and media were measured as described below.

2.3. Determination of Akt phosphorylation

Akt phosphorylation in cancer cell lysates was determined by ELISA (based on anti-phospho-Akt Ser473 antibody, SuperArray Bioscience Corporation, MD, USA), after treatment of CAPAN-2 pancreatic cancer cells with CORM-2/iCORM-2 (50 mol/L) for 75 min, according to the manufacturer's instructions. Cells exposed to 1% DMSO in PBS (solvent for CORM-2) were also compared with untreated cells. Experiments were performed in hexaplets.

Simultaneously, Western blot analyses of phosphorylated Akt protein were performed on the CAPAN-2 and PaTu-8902 pancreatic cancer cells, treated in an identical manner (CORM-2/iCORM-2, 50 mol/L, 75 min incubation). The cells were lysed in Cell Lysis Buffer (Cell Signaling Technology, MA, USA), according to the manufacturer's instructions. Protein concentration was determined using a BCA assay (Thermo Scientific, IL, USA). Thirty micrograms of protein lysate were separated by SDS-PAGE electrophoresis (10% gel). The proteins were transferred to a PVDF membrane and then immunoblotted with anti-phospho-Akt (Ser473), anti-Akt, and anti-β-actin (Cell Signaling Technology, MA, USA). Antibodies were detected using Goat Anti-Rabbit IgG H&L (HRP) antibody (Abcam, UK), and analysed by ECL (LumiGLO®, Cell Signaling Technology, MA, USA). A Fusion Fx7 device and Bio-1D software (Vilber Lourmat, France) were used to quantify the signals. Results were expressed as the percentage of the total Akt level that was phospho-Akt.

2.4. In vivo tumour models

Six- to eight-week-old athymic mice (strain CD-1, Charles River WIGA, Sulzfeld, Germany) were transplanted subcutaneously with either 10^7 human CAPAN-2 or PaTu-8902 pancreatic cancer cells mixed with matrigel. Seven to ten days after tumour cell implantation, the CAPAN-2-bearing mice received a daily intra-peritoneal treatment of either CORM-2 or iCORM-2 ($n = 6$ in each group) dissolved in 1% DMSO in PBS (35 mg/kg), whereas the PaTu-8902-bearing mice were exposed for 1 h daily to either 500 ppm CO in the synthetic air or ambient air *per se*. The primary outcome of this type of *in vivo* study was the survival time; tumour progression was assessed simultaneously as well (tumour size was monitored every three days in all groups, and the tumour volume determined as described previously [15]). In an additional *in vivo* study, the animals treated with CORM-2 or iCORM-2 ($n = 6$ in each group) were sacrificed at day 14 for the quantification of the capillary density of the tumour. Finally, other sets of animals ($n = 6$ for each group) were exposed to CO (500 ppm of CO in synthetic air for 1 h a day). These animals were either sacrificed immediately after the last CO exposure, for the determination of COHb and CO content in the tumour