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ORIGINAL ARTICLE

Nitric oxide (NO) expression in co-culture of colon tumour spheroids with normal cells after incubation with interleukin-1β (IL-1β) and/or camptothecin (CPT-11)

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Abstract: The present study was performed to assess the influence of IL-1β and/or camptothecin (CPT-11) on nitric oxide (NO) secretion by co-cultures of 3 human colon adenocarcinoma cell lines: HT29, LS180 and SW948, derived from different grades of tumours (Duke's grade) cultivated as spheroids with normal human colon epithelium (CCD 841 CoTr), myofibroblasts (CCD-18Co) and endothelial cells (HUVEC). Tumour cell spheroids in monoculture produced higher amounts of NO than normal cells. In co-cultures, the level of the radical decreased compared to the sum of NO produced by tumour and normal cell monocultures. IL-1β non-significantly induced NO production in colon tumour cell spheroids and normal cell monolayers, but significantly induced the radical production in co-culture of low grade HT29 tumour cell spheroids with normal cells. CPT-11 used alone limited NO production, while in combination with IL-1β it increased the level of the radical. IL-1β and CPT-11, dependent on whether they are added separately or jointly, differentially modulate NO in monocultures of colon tumour spheroids or normal cells and their co-cultures.

Key words: colon tumour spheroids, co-culture, interleukin-1β, camptothecin, nitric oxide

INTRODUCTION

Colon adenocarcinoma is one of the most common fatal malignancies in western countries. NO is a short-lived molecule, pivotal for numerous physiological functions and the pathogenesis of various diseases. It is generated via the oxidation of the terminal guanide group of L-arginine to L-citruline by various NADPH-dependent enzymes called NO synthases (NOS). In tumour biology, however, NO play a dual role – both facilitatory and inhibitory – depending on microenvironmental conditions. Under particular circumstances, NO signalling may potentate tumour growth and invasiveness, but in contrast, the molecule at higher concentrations can also be cytotoxic to tumour cells, exerting antioxidant or anti-angiogenic properties decreasing tumour development and metastasis [1-3]. In laboratory studies, the role of NO should be analysed with regard as to whether monocultures of tumour cells or composed co-culture systems are used.

The aim of the present study was to evaluate the effects of IL-1β and/or CPT-11 on NO secretion in co-cultures of human colon adenocarcinoma cells derived from tumours of different Duke's grade cultivated as tumour spheroids with normal colon epithelium, myofibroblasts and endothelial cells.

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MATERIALS AND METHODS

Cell culture. Human colon adenocarcinoma cell lines HT29 (ATCC No. HTB-38), derived from a grade I tumour, LS180 (ATCC No. CL-187), from a grade II tumour, and SW948 (ATCC No. CCL-237), from a grade III tumour, were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) (Gibco™, Paisley, UK) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere with 5% CO_2.

Human normal colon myofibroblasts CCD-18Co (ATCC No. CRL-1459) and normal epithelial cells CCD 841 CoTr (ATCC No. CRL-1807) were cultured in RPMI 1640 + DMEM (1:1) medium (Sigma) supplemented with 10% FCS at 37°C (CCD-18Co) or 34°C (CCD 841 CoTr) in a 5% CO_2/95% air atmosphere.

Human normal umbilical vein vascular endothelium HUVEC (ATCC No. CRL-1730) was cultured in CS-C medium (Sigma) supplemented with 75 μg/ml endothelial cell growth factor (ECGF) (Sigma) and 10% FCS in a humidified atmosphere with 5% CO_2.

Preparation of tumour cell spheroids. Tumour cell spheroids were prepared by the liquid overlay method, as described previously [4]. Briefly, the tumour cell suspension (200 μl) at a density of 1×10^5 cells/ml in RPMI 1640 medium, supplemented with 10% FCS, was plated on 1% agarose-coated 96-multiwell culture plates (2×10^4 cells/well). After incubation for 4 days at 37°C in a humidified atmosphere with 5% CO_2, the cells formed spheroids.

Co-culture of tumour spheroids with normal cell monolayers. The tumour spheroids were harvested with glass pipettes from the agarose-coated microplates and transferred into a Petri dish filled with warm RPMI 1640 medium. After 5 min. washing, 5 spheroids each were transferred onto confluent myofibroblast or colon epithelium or HUVEC monolayers (1×10^5 cells/ml) in 24-well tissue culture plates in RPMI 1640 medium, supplemented with 2% FCS, and incubated at 37°C in a humidified atmosphere with 5% CO_2. Parallel experiments were performed with tumour spheroids or normal cell monolayers alone as culture controls. After 12-h culture, supernatants and cell lysates were collected and stored at -80°C until further estimation.

Co-cultures of tumour spheroids with colon epithelial cells or myofibroblasts reflected the early stages of tumour development, while interactions with endothelium resembled tumour cell dissemination via blood circulation.

Exposure of cells to camptothecin (CPT-11) and IL-1β. After 24-h incubation of the cells in RPMI 1640 with 10% FCS, the medium was discarded and fresh RPMI 1640 containing 2% FCS and CPT-11 (1 μg/ml) (MP Biomedicals, Inc., Eschwege, Germany) and/or IL-1β (2 ng/ml) (Strathmann Biotec GmbH, Hamburg, Germany) were added.

The incubation with the mentioned substances was performed for 6h. Thereafter, the culture medium was changed, and the cells incubated in a new culture, but without the addition of CPT-11 and IL-1β, for another 6h at 37°C/5% CO_2. Culture supernatants were then selected and stored at -80°C for no longer than 3 months.

Nitric oxide (NO) measurement. Nitrate, a stable end product of NO, was determined in culture supernatants by a spectrophotometric method based on the Griess reaction. Briefly, 100 μl of supernatant was plated in 96-well flat-bottomed plates in triplicate and incubated with 100 μl of Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)ethylenediamine dihydrochloride) **(Sigma)** in 3% H_3PO_4 (POCH, Gliwice, Poland) at room temperature for 10 min. The optical density was measured at 550 nm using a microplate reader. A standard curve was performed using 0.5-25 μM sodium nitrite ($NaNO_2$) for calibration.

Statistical analysis. Results are presented as means ± SD of 3 independent experiments. The data were analyzed using one-way analysis of variance ANOVA, followed by Bonferroni's multiple comparison *post-hoc* test. Differences of $p\leq0.05$ were considered significant.

RESULTS

Nitric oxide (NO) production in co-cultures treated with IL-1β and/or CPT-11. Tumour cells produced higher, grade-dependent amounts of NO than normal cell monocultures. In the co-cultures (Fig. 1) of tumour spheroids with normal colon epithelium and myofibroblasts, the NO level did not change significantly compared to the normal cell monocultures. A significant reduction was detected in the co-culture of SW948 tumour cell spheroids with 18Co cells. Interestingly, in tumour cells co-cultured with myofibroblasts, NO decreased in a tumour grade-dependent manner. However, when tumour spheroids were co-cultured with endothelial cells, the amounts of NO slightly increased above the level detected in the endothelial monoculture (Fig. 2a).

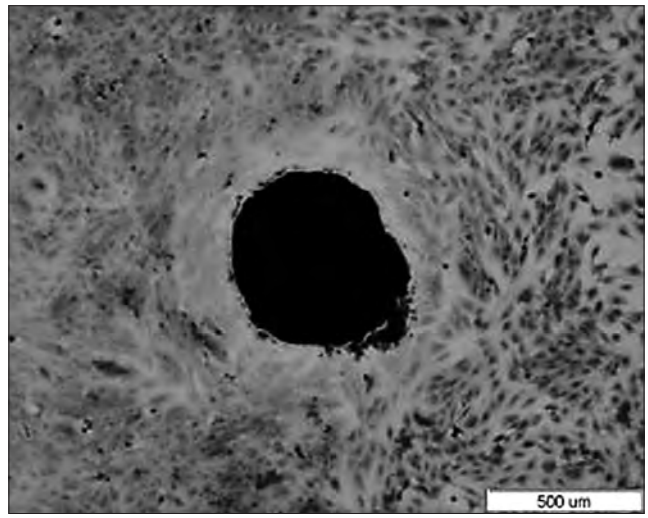


Figure 1 Co-culture of human colon carcinoma cell spheroids (HT29 cell line) with human normal colon epithelium (CCD 841 CoTr cell line). Cells were stained by the May-Grünwald-Giemsa method. Magnification 40x. Bar 500 μm.

The addition of IL-1β non-significantly increased NO production in the tumour and normal cell monocultures. NO level was significantly increased in co-cultures of low grade HT29 colon tumour spheroids with normal cell monolayers after the addition of IL-1β, but no significant changes were detected in the remaining co-cultures (Fig. 2a).

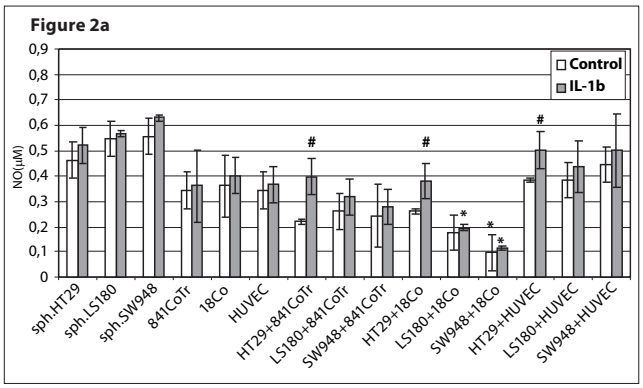
The addition of CPT-11 did not cause a significant reduction in the NO level in the analyzed monoculture- and co-culture models except the LS180 +18Co combination (Fig. 2b).

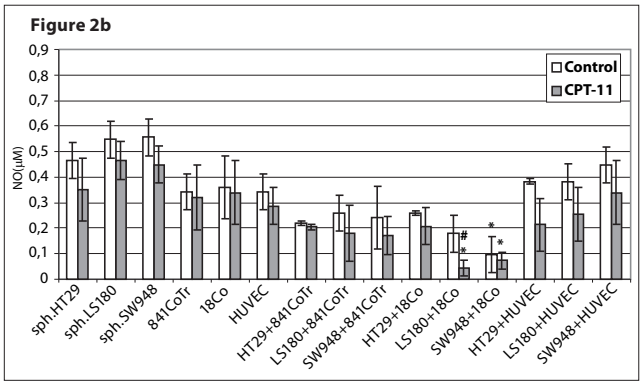
On the other hand, the combination of IL-1β plus CPT-11 increased NO production by the monocultures of normal cells (18Co and HUVEC). Moreover, exposure to CPT-11+IL-1β significantly increased NO level in co-cultures of SW948+841CoTr, HT29+18Co, and tumour spheroids with HUVEC. However, IL-1β and CPT-11 administered simultaneously had no effect on NO production by tumour cells cultivated alone (Fig. 2c).

DISCUSSION

It was shown that in co-cultures IL-1β increased but CPT-11 decreased NO production when used alone, while in combination it increased the radical level. There is substantial evidence indicating that NO is an endogenous tumour growth stimulator; a promoter of metastasis inducing migration, invasion and the angiogenic capacities of tumour cells; it is also an enhancer of protooncogene expression and an inhibitor of apoptosis [1, 2]. On the other hand, NO has been reported to inhibit carcinogenesis. This molecule may be toxic to tumour cells and thereby decrease tumour growth and metastasis by inhibiting angiogenesis or enhancing differentiation and apoptosis [1]. This paradox of NO action seems to depend on the local intracellular concentration of this factor (a 1-2 times increase of NO above optimal concentration may be toxic to cells), the cell type and other cofactors, such as oxidative stress and inflammation [5].

IL-1β is a pro-inflammatory factor of which the most relevant properties are the initiation of cyclooxygenase-2





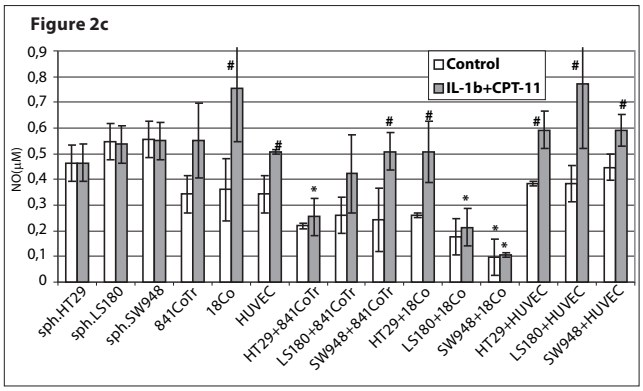


Figure 2 Nitric oxide (NO) production in co-cultures of colon carcinoma cell spheroids with normal colon epithelial cells, normal myofibroblasts and normal endothelial cells during 6 h of incubation with IL-1β (a), CPT-11 (b), or IL-1β plus CPT-11 (c). Analysis performed using the Griess method. Tumour cells produced higher, grade-dependent amounts of NO compared to the normal cell monocultures. In co-cultures of tumour spheroids with normal colon epithelium and myofibroblasts, the NO level decreased compared to the normal cell monocultures. The combination of IL-1β plus CPT-11 and IL-1β added alone increased NO production by the normal cell monocultures and the co-cultures of normal cells with tumour spheroids, while CPT-11 administered alone decreased NO production.
* p≤0.05 – co-culture of tumour/normal cells compared to an appropriate monoculture of normal cells.
# p≤0.05 – culture of tumour and/or normal cells treated with IL-1β and/or CPT-11 compared to an appropriate non-treated culture.

(COX-2) and nitric oxide synthases (NOS). In consequence, cells exposed to IL-1β produce NO which is an important mediator in many (patho)physiological processes including inflammation [6, 7]. This pro-inflammatory cytokine also induces NO production in a variety of tumour cell types [8]. However, as shown in the presented study, the level of NO production may depend not only on tumour cell type, but also on the grade of the tumour. On the other hand, CPT-11 reduced NO production in our study. Yin et al. [9] revealed that SN-38, the active metabolite of irinotecan (CPT-11), significantly down-regulated (iNOS) expression and NO production in human squamous cell carcinoma of the head and neck. Therefore, the therapeutic activity of the CPT-11 consists not only in the inhibition of the nuclear enzyme topoisomerase I (topo I) and a modification of DNA topology, but also in the inhibition of other processes, e.g. neoangiogenesis through down-regulation of NO production.

Interestingly, when IL-1β was added together with CPT-11 to the co-cultures, stimulation of NO release was observed. This may suggest that after IL-1β-stimulated inflammation and NO production, chemoprevention using CPT-11 is not sufficient to control tumour development. Therefore, appropriate treatment for inflammation should be further explored for the chemoprevention of human cancers [10].

In conclusion, IL-1β and CPT-11, depending on whether they are added separately or together, differentially modulate NO in monocultures of colon tumour spheroids or normal cells and their co-cultures. The NO production may also depend on the grade of the tumour and type of normal cells which were applied. Moreover, the level of NO, as a mediator of inflammation, may be taken into consideration before CPT-11 application.

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