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EGCG downregulates IL-1RI expression and suppresses IL-1-induced tumorigenic factors in human pancreatic adenocarcinoma cells

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ABSTRACT

Human pancreatic cancer is currently one of the fifth-leading causes of cancer-related mortality with a 5-year survival rate of less than 5%. Since pancreatic carcinoma is largely refractory to conventional therapies, there is a strong medical need for the development of novel and innovative therapeutic strategies. Increasing evidence suggests an association of carcinogenesis and chronic inflammation. Because IL-1 plays a crucial role in inflammation-associated carcinogenesis, we analyzed the biological effects of IL-1 and its modulation by the chemopreventive green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) in the human pancreatic adenocarcinoma cell line Colo357. Proinflammatory IL-6 and PGHS-2 as well as proangiogenic IL-8 and VEGF were induced by IL-1, whereas the secretion of invasion-promoting MMP-2 remained unaffected. IL-1 responsiveness and constitutive MMP-2 release in Colo357 were downregulated by EGCG in a dose- and time-dependent manner. Moreover, EGCG reduced cell viability via induction of apoptosis in Colo357. Since EGCG effects on cytokine production precede reduction in cell viability, we hypothesize that these findings are not only a result of cell death but also depend on alterations in the IL-1 signaling cascade. In this context, we found for the first time an EGCG-induced downregulation of the IL-1RI expression possibly being caused by NF-κB inhibition and causative for its inhibitory action on the production of tumorigenic factors. Thus, our data might have future clinical implications with respect to the development of novel approaches as an adjuvant therapy in high-risk patients with human pancreatic carcinoma.

1. Introduction

Human pancreatic cancer is one of the most fatal among all solid malignancies. Despite advances in surgery and radiation oncology, no significant improvement of the overall survival was achieved. Evidence is now emerging that inflammation may be an important precursor in pancreatic carcinogenesis [1]. Current epidemiological data support an association of pancreatic carcinoma with the sporadic and hereditary forms of chronic pancreatitis. Patients with hereditary pancreatitis and cigarette smokers have a considerably increased risk for pancreatic carcinoma as compared to the general population [2,3].

The pathogenesis of chronic pancreatitis involves expression of the transcription factor NF-κB by epithelial cells in response to damaging events, such as infection or necrosis which can be caused by extensive tissue autodigestion in the pancreas mediated by digestive enzymes released from the zymogen granules [4]. Activation of NF-κB represents an early inflammatory response leading to the secretion of proinflammatory cytokines, such as IL-1β and TNF-α [5]. Concomitant activation of endothelial cells and recruitment of activated macrophages to the site of damage enhances cytokine release and induces expression of acute phase genes such as *IL8* and *PGHS2* [6,7]. The proinflammatory PGHS-2, formerly known as COX-2, is an inducible enzyme expressed only in response to stimuli such as mitogens, cytokines, growth factors or hormones. The products of the PGHS-2 enzyme are prostanoids, which are key mediators of inflammation. PGHS-2 is upregulated in many cancers and involved in cellular proliferation, antiapoptotic activity, angiogenesis, and increase of metastasis [8]. Expression of both, acute phase genes and adhesion molecules on endothelial cells serves as a signal for the recruitment of leukocytes to the site of tissue damage. Moreover, an enhanced

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production of prostaglandins mediated by PGHS-2 augments vasopermeability leading to a more pronounced recruitment of leukocytes [9]. Leukocytes are the main source of reactive nitrogen species (RNS) and reactive oxygen species (ROS) acting as chemical effectors in inflammation-driven carcinogenesis [7].

The proinflammatory cytokine IL-1 plays a crucial role in inflammation-associated carcinogenesis and tumor progression [10,11]. IL-1 comprises a family of closely related molecules, IL-1α and IL-1β, not only affecting inflammation, immunity and haematopoiesis but also carcinogenesis, tumor invasiveness and tumor/host interactions. IL-1 is produced directly by cancer cells or by the cells of the mircroenvironment such as stromal and endothelial cells as well as infiltrating cells resulting in the formation of proangiogenic and prometastatic mediators [12]. In pancreatic cancer IL-1 confers chemoresistance via upregulation of PGHS-2 [13] and promotes angiogenesis during tumor progression [14].

IL-1α and IL-1β exert identical agonist actions by binding to the IL-1 receptor type I (IL-1RI). After ligation, IL-1RI associates with the IL-1 receptor accessory protein (IL-1RAcP) which activates intracellular signal transduction cascades. This complex recruits a number of intracellular adapter molecules [15,16] to activate signal transduction pathways such as AP-1, p38MAPK, JNK and NF-κB. In particular NF-κB provides a mechanistic link between inflammation and cancer and is a major factor controlling the ability of both, preneoplastic and malignant cells, to resist apoptosis-based tumor surveillance mechanisms. NF-κB might also regulate tumor angiogenesis and invasiveness [5], and may contribute to the characteristic chemoresistance of pancreatic tumor cells [17].

The present limited options for the management of pancreatic cancer and its increasing incidence necessitate the search for novel preventive approaches for this disease. One approach is related to chemoprevention, a means of cancer management by which the occurrence of the disease can be entirely slowed, blocked, or reversed by the administration of one or more naturally occurring and/or synthetic compounds. The most accepted compounds for chemoprevention in humans are naturally occurring dietary substances. Many studies on animal models have demonstrated that tea and tea polyphenols exhibit cancer preventive activities in a variety of organs (reviewed by Ref. [18]). However, the role of tea consumption on the risk of human cancer still remains a matter of debate. Green tea exhibits the most beneficial effects for health, and it is commonly accepted that many of the chemopreventive effects of green tea are mediated by polyphenols. Among the green tea polyphenols, (−)-epigallocatechin-3-gallate (EGCG) is the most abundant and active constituent in affecting experimental carcinogenesis [19]. Based on different approaches pleiotropic effects of the compound were reported including modulation of cell signaling, inhibition of telomerase, DNA methyltransferase and the proteasome, induction of apoptosis and cell cycle arrest as well as antioxidant activities [19–21].

In the current study we investigated the effects of EGCG on the expression of IL-1-induced tumorigenic factors in human pancreatic adenocarcinoma cells. Beside its inhibitory effect on the production of tumorigenic factors, we found for the first time an EGCG-induced downregulation of the IL-1RI expression that might be causative for its inhibitory action on the production of tumorigenic mediators. Thus, our data give further insight into the pleiotropic action of EGCG as a useful tool for adjuvant therapy in human pancreatic carcinoma.

2. Materials and methods

2.1. Cell culture and treatment

After three i.p. passages through SCID/beige mice, a master cell bank (total, 1×10^9 cells) was established from the human pancreatic adenocarcinoma cell line Colo357 [22]. Identical aliquots (5×10^6) of this fast-growing (doubling time increased from 40 to 24 h) and highly metastatic variant were frozen in liquid nitrogen. After thawing, Colo357 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 1 mM sodium pyruvate (all from PAA Laboratories, Pasching, Austria) in the presence of 100 IU/ml penicillin and 100 μg/ml streptomycin (GIBCO, Grand Island, NY, USA). The cells were maintained under standard cell culture conditions at 37 °C in a 5% CO_2 humidified atmosphere. For stimulation experiments, Colo357 cells were seeded in 96-well microtiter plates at a density of 7.5×10^3 cells per well and, after a recovery phase of at least 16 h, stimulated with or without 1 ng/ml of recombinant human IL-1β (Pan Biotech, Aidenbach, Germany) in the presence or absence of 50–200 μM (−)-epigallocatechin-3-gallate purchased from Sigma–Aldrich GmbH (Steinheim, Germany) for the indicated times. EGCG is the major constituent within the group of flavanols, characteristic polyphenolic compounds also known as catechins, found in the leaves of the tea plant *Camellia sinensis*. EGCG treatment started 1 h prior to IL-1 stimulation. All treatments were conducted in the presence of 10 mM N-acetylcysteine (NAC; Sigma–Aldrich GmbH) to stabilize EGCG and quench EGCG-derived ROS.

2.2. Cell growth and viability

The effect of EGCG and IL-1 on the viability of cells was assessed by the CellTiter 96® AQueous non-radioactive Cell Proliferation Assay (Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. This assay is based on the ability of viable cells only to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium (MTS) to formazan by mitochondrial dehydrogenases and thus suitable for determining the metabolic activity/number of viable cells in proliferation, cytotoxicity or chemosensitivity assays.

2.3. Analysis of apoptosis

For determination of chromatin condensation Colo357 cells were seeded in chamber slides at a density of $2.5 \times 10^4/cm^2$ and stimulated with or without 1 ng/ml of IL-1β in the presence or absence of 50–200 μM EGCG. As a positive control cells were treated with 1 μM paclitaxel (Sigma–Aldrich GmbH) for the indicated time. After washing, cells were fixed for 2 min in methanol/acetone (1:1) at room temperature (RT). Thereafter, 4′,6-diamidino-2-phenylindole (DAPI, Carl Roth GmbH, Karlsruhe, Germany), a DNA-binding blue fluorescent dye at a concentration of 1 μg/ml, was perfused into the culture chambers to incubate the cells for 2 min in the dark. The cells with stained nuclei were visualized and photographed using an Olympus IX 70 fluorescence microscope. Apoptotic cells were morphologically defined by nuclear shrinkage and by chromatin condensation or fragmentation

2.4. Determination of caspase-3 activity

Caspase-3 activity was measured after a 24-h incubation period with or without IL-1β in the presence or absence of 50–200 μM EGCG. Harvested cells were lyzed with caspase lysis buffer (10 mM Tris/HCl, 10 mM sodium phosphate buffer, pH 7.5, 130 mM NaCl, 1% Triton X-100 and 10 mm $Na_2P_2O_7$) and then incubated with 25 μg/ml of the fluorogenic substrate Ac-DEVD-AFC (Becton Dickinson GmbH, Heidelberg, Germany) in 20 mM Hepes (pH 7.5), 10% glycerol and 2 mM dithiothreitol at 37 °C for 2 h. The release of AFC as a measure of caspase-3 activity was analyzed fluorimetrically using the Varioskan Flash spectral scanning

multiplate reader (Thermo Fisher Scientific, Hudson, NH, USA) at an excitation/emission wavelength of 390/510 nm. Relative caspase activities were normalized to the protein content and compared to the untreated control whose response was set as 100%. Again, paclitaxel was used as a positive control.

2.5. Protein extraction and Western blotting

For immunodetection of antigens cells were harvested and lyzed in lysis buffer consisting of 25 mM Tris/HCl, 270 mM sucrose, 1% CHAPS, 1% Triton X-100, 0.01% SDS, 0.001% mercaptoethanol pH 7.5 supplemented with 5 mM EGTA, 1 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM benzamidine, 1 mM $NaVO_3$, and 100 μM PMSF. The cleared protein lysates were separated by 12.5% SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes. After blocking with 10% Roti®-Block (Carl Roth GmbH) in TBST (TBS/0.1% Tween-20/5% BSA), blots were incubated with appropriate concentrations of monoclonal or polyclonal antibodies for 1 h at RT or overnight at 4 °C in TBST. Primary antibodies used were rabbit anti-human PGHS-2 (Acris Antibodies, Herford, Germany), mouse anti-human IL-1RI (R&D Systems GmbH, Wiesbaden, Germany), and rabbit anti-human GAPDH from LabFrontiers Co. Ltd. (Seoul, Korea). After washing, membranes were incubated for 1 h at RT with DyLight 488-conjugated rabbit anti-mouse IgG (Dianova, Hamburg, Germany) and HRP-conjugated goat anti-rabbit IgG, respectively (Cell Signaling Technology, Boston, MA, USA). The presence of the proteins was revealed using the LumiGLO® chemiluminescent substrate (Cell Signaling Technology). For densitometric analysis films were scanned, and the intensity of the corresponding bands was quantified using Kodak 1D Image Analysis Software and standardized to GAPDH.

2.6. Immunodetection of IL-1RAcP

Quantification of the low abundance IL-1RAcP protein was performed using the tyramide signal amplification kit (Invitrogen, Darmstadt, Germany) according to the instructions of the manufacturer with minor modifications. Adherent cell layers of Colo357 were fixed with 3.7% formaldehyde in PBS for 20 min, rinsed and permeabilized with 0.1% Triton X-100 for 5 min at RT. Cells were incubated with 1% H_2O_2 for 60 min to quench endogenous peroxidase activity and then blocked with 1% blocking reagent in PBS for 60 min prior to incubation with mouse anti-human IL-1RAcP (Abnova, Taipei City, Taiwan) diluted in 1% blocking reagent in PBS for 1 h at RT. After rinsing, HRP-conjugated goat anti-mouse IgG was applied for 60 min, and the cells were incubated with Alexa Fluor 488 dye in freshly prepared amplification buffer supplemented with 0.0015% H_2O_2 for 10 min at RT. Fluorescence of the labelled cells was measured using the Tecan infinite 200 microplate reader (Männedorf, Switzerland) at an excitation/emission wavelength of 485/535 nm.

2.7. RNA extraction and quantitative RT-PCR

Total RNA was extracted from cultured Colo357 cells using TriFast® Reagent (PEQLAB Biotechnologie GmbH, Erlangen, Germany) as instructed by the manufacturer. RNA quantity was assessed by UV spectrophotometry. Reverse transcription was performed with 1 μg of RNA and oligo(dT) primer using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Real-time PCR was performed as described previously [23] using primer pairs as given in Table 1. PCR was carried out as a TaqMan method using the Bio-Rad CFX96 cycler (Bio-Rad Laboratories, Munich, Germany) and white 96-well reaction plates (Biozym, Hessisch Oldendorf, Germany).

2.8. Cytokine and metalloproteinase assays

Concentrations of human IL-6 and IL-8 in the culture supernatants were determined by commercially available ELISA kits (Elipair, Diaclone, Besançon cedex, France). Production of total MMP-2 was quantified by immunoassay using the corresponding Quantikine® ELISA kit purchased from R&D Systems GmbH (Wiesbaden, Germany) according to the manufacturer's recommendation. Secretion of VEGF into the supernatants was evaluated using the RayBio® Human VEGF EIA kit (RayBiotech, Inc., Norcross, GA, USA).

2.9. Sequence analysis of putative promoter regions

Nucleotide sequences 5000 bp upstream of the transcription start sites were identified by DNA BLAT using the UCSC Genome Browser (UCSC Genome Bioinformatics Group, Santa Cruz, CA, USA) at http://genome.ucsc.edu/cgi-bin/hgBlat and analyzed for potential transcription factor binding sites using MatInspector Release professional 8.0 software (Genomatix Software GmbH, Munich, Germany).

2.10. Detection of NF-κB activity in transiently transfected Colo357 cells

4×10^5 Colo357 cells were seeded in 60-mm culture dishes, cultured overnight until adherence and then transiently transfected with effectene™ reagent (Qiagen, Hilden, Germany) according to the manufacturer's instruction. For measurement of NF-κB activation, Colo357 cells were transfected with 1 μg of the firefly luciferase reporter plasmid pGL3-5xNF-κB harboring five NF-κB binding sites [24]. As an internal control, Colo357 cells were co-transfected with 25 ng of pRL-TK vector (Promega, Mannheim, Germany) encoding *Renilla* luciferase under control of the herpes simplex virus thymidine kinase (HSV-TK) promoter leading to moderate expression of *Renilla* luciferase. The total amount of DNA

Table 1
Sequences for primers and probes used in real-time PCR assays.

Gene/accession no.	Primer/probe	Amplicon (bp)
RPLP0/NM_001002	f: 5′-GGG AAT GTG GGC TTT GTG TTC-3′ r: 5′-TGG CAC AGT GAC TTC ACA TGG-3′ p: 5′-CAA TGG CAC CAG CAC GGG CAG CA-3′	123
IL1RI/NM_000877	f: 5′-AGG TTC TGG AAG AAT GTC AGG TAC -3′ r: 5′-AGC CTC TCT TTG CAG TTT CTC C-3′ p: 5′-ATG CCA GTC CAG CGA CGG TCA CCT -3′	108
ILRACP/NM_134470	f: 5′-AGG ACC GGG ACC TTG AGG AG -3′ r: 5′-ATA GTT GCC AGT GTC ATT GAG GAG-3′ p: 5′-TTC CGC CTC CCC GAG AAC CGC AT -3′	113

f: forward; r: reverse; p: probe; *RPLP0*, ribosomal phosphoprotein large P0.

in all transfections was kept constantly by adding empty vector DNA. After 12 h medium was removed and the transfectants were serum-starved for 18 h in RPMI 1640/0.1% FCS plus supplements. After starvation, transfected cells were stimulated for 12 h with 1 ng/ml of IL-1β in the presence or absence of 100 μM EGCG or remained unstimulated. Treatments were conducted in the absence of NAC since the reducing agent is known to inhibit NF-κB [25]. Expression of both, firefly and *Renilla* luciferase was detected using the dual luciferase reporter assay system (Promega) as described by the manufacturer. The luciferase activity was measured in at least triplicate cultures for 10 s using the Mini Lumat LB 9506 luminometer (EG&G Berthold, Bad Wildbad, Germany).

2.11. Statistical analysis

Statistical differences between mean values were analyzed using the two-tailed non-parametric Mann–Whitney test. A p value of less than 0.05 was considered statistically significant.

3. Results

In this study, the effect of (−)-epigallocatechin-3-gallate, a promising chemopreventive agent in green tea, was tested on the expression of IL-1-induced tumorigenic factors in the human pancreatic adenocarcinoma cell line Colo357. This approach mimicks tumor-associated pancreatic inflammation as a key player in pancreatic malignancy. Since EGCG has been found to promote cytotoxicity selectively in cancer but not normal cells [26], we analyzed the effect of the catechin in the presence or absence of IL-1 on viability of Colo357. As given in Fig. 1, cell viability was reduced in a time- and dose-dependent manner by EGCG. EGCG concentrations of 200 μM led to a significant reduction in cell viability at any time point, whereas lower concentrations affected cell viability only after 48 h. An effect of IL-1 on the metabolic response of Colo357 could not be observed.

As examples of IL-1-induced tumorigenic factors we determined secretion of proinflammarory IL-6 and proangiogenic IL-8 by Colo357 after stimulation with IL-1. We found a strong time-dependent IL-6 and IL-8 production by Colo357 after IL-1 stimulation (Fig. 2). Furthermore, Colo357 showed a high spontaneous IL-8 secretion that could be further enhanced by an average factor of 5.3 after stimulation with IL-1 (data not shown). The constitutive IL-8 production remained unaffected by EGCG (not shown). IL-1-induced cytokine production was inhibited by EGCG in a dose-dependent manner. From 100 μM upwards EGCG significantly reduced IL-1-mediated IL-6 secretion at any time point, whereas 50 μM of the polyphenol only diminished secretion at 72 h (Fig. 2A). The same observation was made for IL-8 except that 50 μM EGCG did not alter IL-1-induced IL-8 release by Colo357 during the observation period (Fig. 2B).

In addition, increased levels of different growth factors are reported in pancreatic cancer including VEGF. We therefore tested whether Colo357 constitutively secret this proangiogenic cytokine and whether its secretion was altered by IL-1 and EGCG. As demonstrated in Fig. 3A, the pancreatic adenocarcinoma cells constitutively secrete high amounts of VEGF that can be further enhanced in the presence of IL-1. EGCG was found to downregulate both, the constitutive as well as the IL-1-mediated VEGF secretion in a dose-dependent manner. Under conditions not affecting metabolic activity (≤100 μM) EGCG reduced the IL-1-induced VEGF secretion by a maximum of 75% and the spontaneous production by 63%. 200 μM EGCG, which affected cell viability in Colo357 only marginally at this early time point (Fig. 1), completely inhibited VEGF secretion.

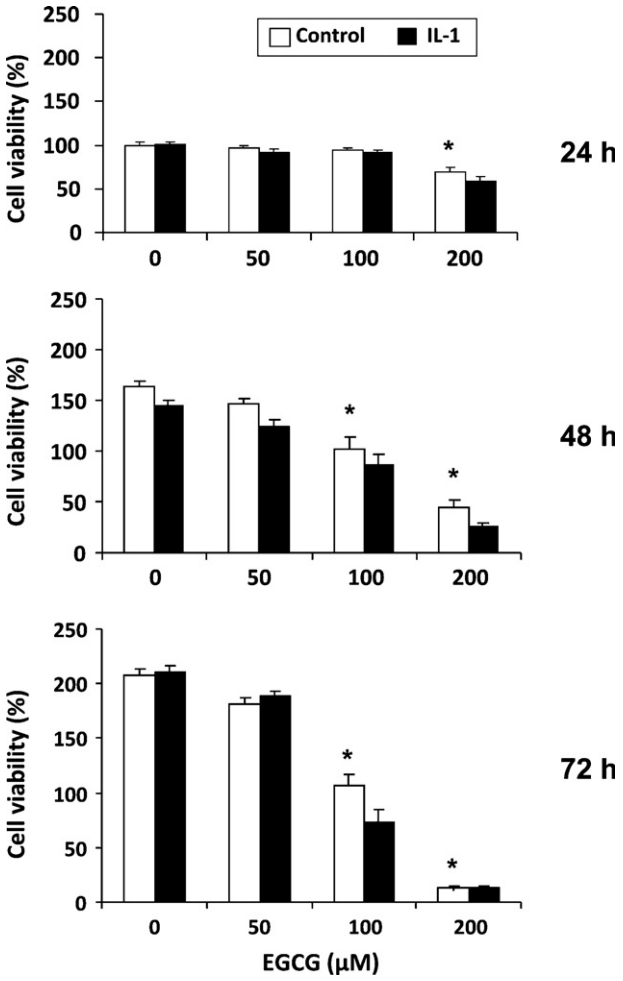

Fig. 1. Effects of EGCG and IL-1 on Colo357 cell viability. Human pancreatic adenocarcinoma cells Colo357 were seeded in microtiter plates and stimulated with or without 1 ng/ml of IL-1β for the indicated times in the presence or absence of 50–200 μM EGCG supplemented with 10 mM N-acetylcysteine. Viability of cells was assessed by the CellTiter 96® AQueous non-radioactive Cell Proliferation Assay. Data represent means ± SEM from four separate experiments performed in multiple replicates. Viability of the untreated control at 24 h was set as 100%. $^*p < 0.05$ compared to control.

Since MMP play an important role in the pathogenesis of pancreatic cancer, we investigated the secretion profile of invasiveness- and angiogenesis-promoting MMP-2 in the presence or absence of IL-1 and different concentrations of EGCG. It was shown previously that MMP-2 secretion can be induced indirectly by IL-1 via IL-6 and activation of the JAK/STAT pathway [27]. Our data clearly identified a strong spontaneous MMP-2 secretion by Colo357 that could not be increased by IL-1 (Fig. 3B). EGCG was found to inhibit the MMP-2 release in a dose-dependent manner. 100 μM EGCG reduced the MMP-2 export by approximately 60% whereas 200 μM blocked the MMP-2 secretion by 72%.

In many malignancies the expression of proinflammatory PGHS-2 is upregulated. We therefore investigated a putative modulatory effect of EGCG on PGHS-2 expression. For this purpose Colo357 cells were treated with a concentration of the catechin not affecting metabolic activity in the presence or absence of IL-1, and the expression of the proinflammatory enzyme was detected by Western blotting. We found that Colo357 constitutively expresses PGHS-2 and that this expression is enhanced 3-fold in the presence of IL-1 (Fig. 4). EGCG significantly reduced both, constitutive and IL-1-induced PGHS-2 amounts.

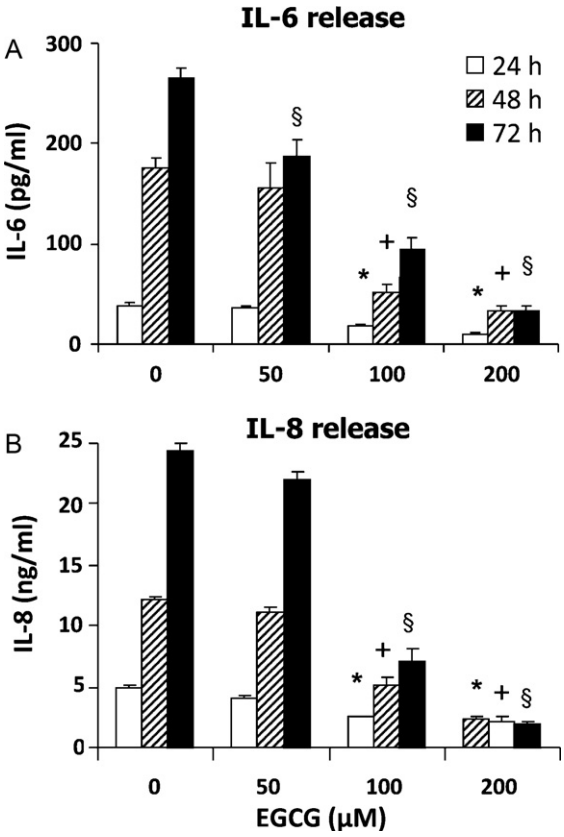

Fig. 2. Cytokine response to EGCG. Colo357 cells were stimulated as given in the legend to Fig. 1. Release of IL-6 (A) and IL-8 (B) into supernatants was detected by sandwich ELISA. Data represent means ± SEM of four independent experiments performed in multiple replicates and were corrected by the spontaneous secretion. $^{*,+,\S}p < 0.05$ compared to IL-1 stimulation without inhibitor.

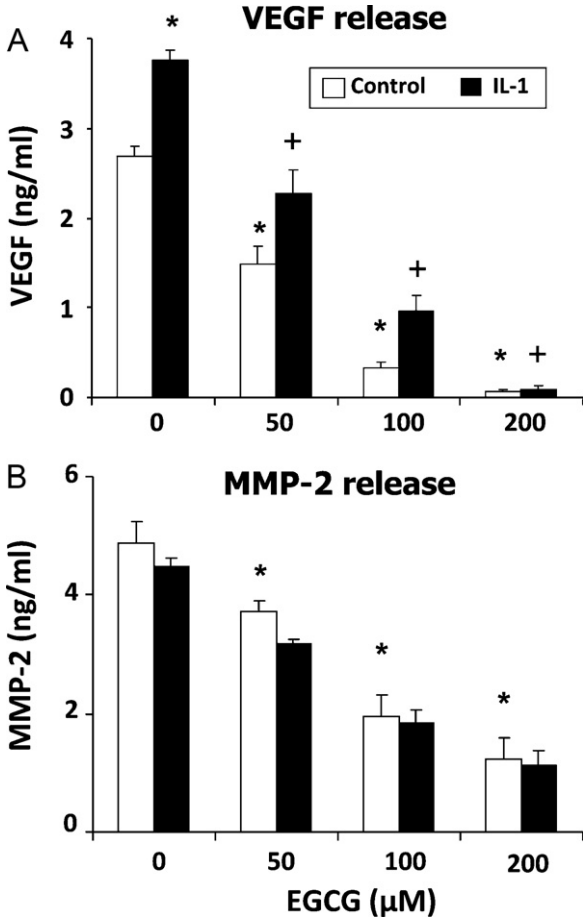

Fig. 3. Effects of EGCG and IL-1 on VEGF and MMP-2 release. VEGF (A) and MMP-2 (B) were detected in culture supernatants of Colo357 by sandwich ELISA 24 h after IL-1 stimulation. For details see legend to Fig. 1. Data represent means ± SEM from four individual experiments performed in quadruplicates. $^{+}p < 0.05$ compared to IL-1 stimulation without inhibitor. $^{*}p < 0.05$ compared to negative control.

Our data clearly demonstrate an antiproliferative effect of EGCG on Colo357 cells (Fig. 1). To further examine the mechanism of proliferation inhibition and to determine whether DNA degradation into nucleosome-sized fragments occurred following EGCG and IL-1 treatment, DAPI staining was performed. For this purpose Colo357 cells were treated with 50–200 μM EGCG in the presence or absence of IL-1 for 24 h. As a positive control we used paclitaxel. The results are shown in Fig. 5. Cells treated with EGCG displayed dose-dependently condensed and fragmented nuclei typical of apoptosis. IL-1 alone did not affect the integrity of nuclei. Co-treatment of Colo357 cells with EGCG and IL-1 led to a slight reduction in the number of fragmented nuclei. In order to investigate the effect of EGCG and IL-1 on apoptosis induction in Colo357 in more detail, we determined caspase-3 activity under the same experimental conditions. Fig. 6 shows that EGCG induced a dose-dependent increase in caspase-3 activity that became significant from a concentration of 100 μM upwards. Additionally, EGCG-mediated caspase-3 activation was inhibited significantly by IL-1.

The mechanisms for the cancer-preventive activity of EGCG include the modulation of several signal transduction pathways thereby altering the expression of genes promoting inflammation, tumor growth and invasiveness. Since EGCG was shown in this study to suppress IL-1-induced tumorigenic factors in Colo357, we tested a putative modulatory effect of the catechin on the expression of the IL-1 receptor complex in the presence or absence of IL-1. As given in Fig. 7A, EGCG downregulated *IL1RI* mRNA under conditions not affecting the metabolic response, whereas *IL1RACP* mRNA remained unaffected (Fig. 7B). Of note, this suppressive

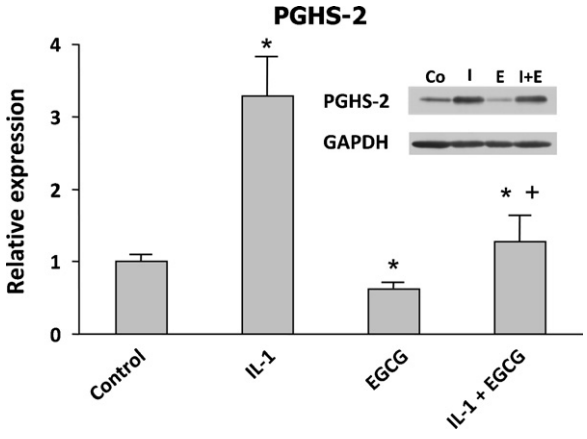

Fig. 4. Detection of PGHS-2 by Western blotting. Colo357 cells were treated for 24 h with 1 ng/ml of IL-1β, 100 μM EGCG or a combination of both, respectively. After preparation of whole-cell lysates, proteins were separated by 12.5% SDS-PAGE and visualized by Western blotting. Data were quantified by densitometric analyses and standardized to the GAPDH response. Values are expressed as means ± SEM and were derived from five separate experiments. The Western blot depicted represents one typical experiment of a series of at least five similar ones with comparable results. $^{*}p < 0.05$; $^{**}p < 0.01$ compared to control; $^{+}p < 0.05$ compared to IL-1 stimulation.

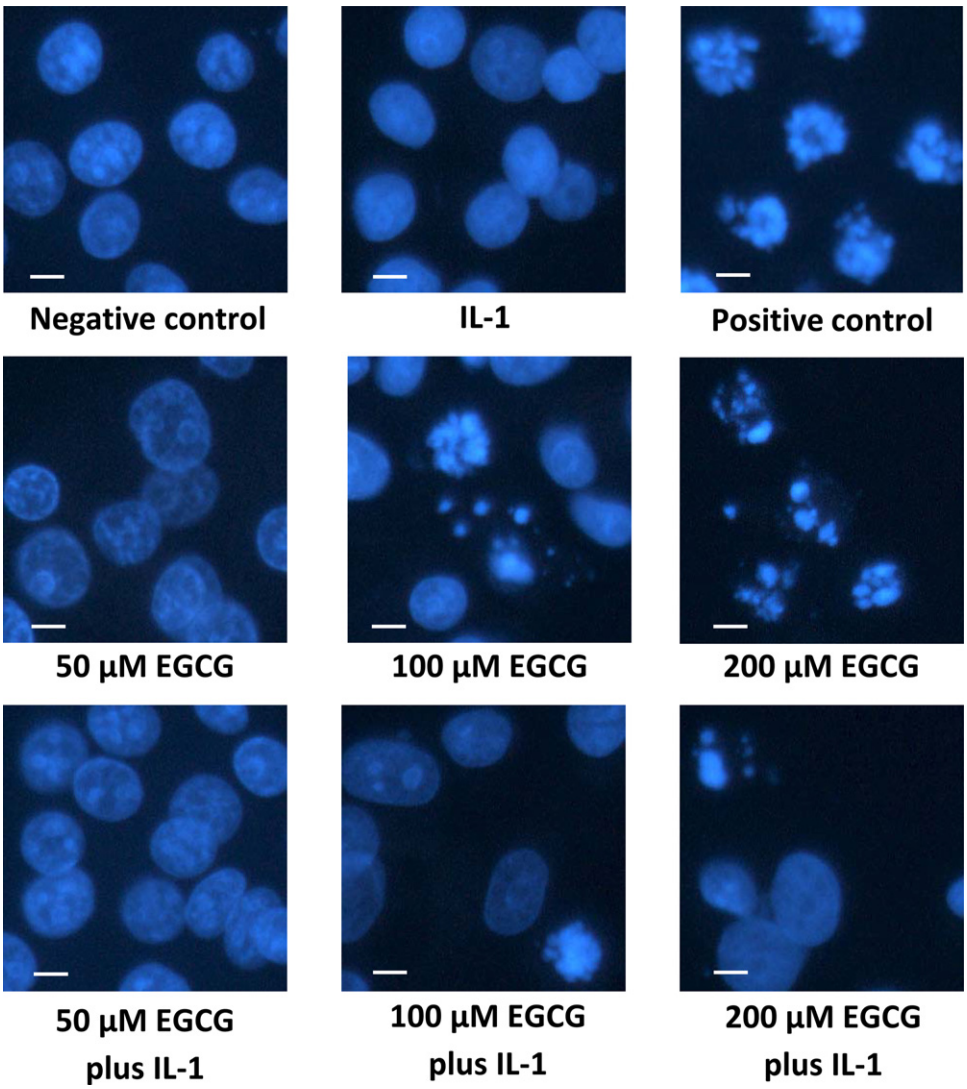

Fig. 5. Assessment of nuclear integrity by DAPI-staining. Colo357 cells were treated with 50–200 µM EGCG supplemented with 10 mM N-acetylcysteine in the presence or absence of 1 ng/ml of IL-1β for 24 h as indicated. 1 µM paclitaxel functioned as positive control. After harvesting, cells were fixed in methanol/acetone and incubated with 500 ng/ml of DAPI. Nuclear morphology was examined by fluorescence microscopy. Data depicted represent one typical experiment of a series of at least three similar ones with comparable results. Scale bars correspond to 10 µm.

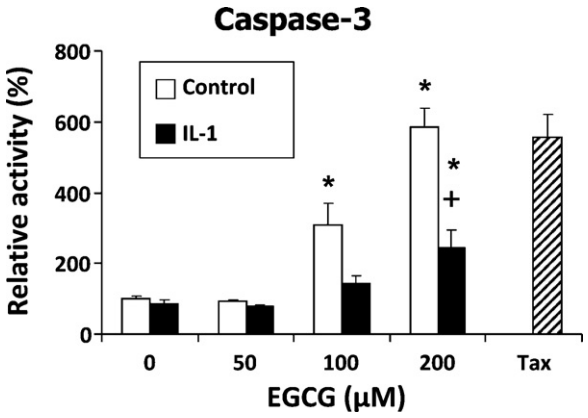

Fig. 6. Activation of caspase-3 in pancreatic adenocarcinoma cells. Colo357 cells were treated as described in the legend to Fig. 5. Enzymatic activity of caspase-3 was determined by adding caspase reagent. Relative caspase activities were normalized to the protein content and compared to the untreated control whose response was set as 100%. Again, paclitaxel was used as a positive control. The results shown are means ± SEM of four independent experiments performed in triplicates. $^{*}p < 0.05$ compared to untreated control; $^{+}p < 0.05$ compared to unstimulated sample.

effect of EGCG was more pronounced at apoptotic concentrations (≥200 µM) of the catechin (data not shown). At the protein level neither IL-1 or EGCG nor a combinatorial treatment showed any effect on IL-1RAcP, as shown by indirect immunofluorescence (data not shown). In contrast, treatment of Colo357 with IL-1 led to an increase of IL-1RI. While EGCG was unable to affect the constitutive IL-1RI presence, it markedly diminished IL-1-mediated IL-1RI amounts (Fig. 8).

It is well documented that the pleiotropic action of EGCG involves inhibition of the transcription factors NF-κB and AP-1. We therefore performed a sequence analysis of the putative promoter regions in *IL1RI* and *IL1RACP*. Our analysis revealed that the region spanning 100 nucleotides upstream of the transcription start sites contains neither a TATA box nor a CAAT box. Moreover, the *IL1RI* putative promoter region harbors 13 NF-κB-like binding sites and eight AP-1-like motifs, whereas for *IL1RACP* one NF-κB and four AP-1 consensus binding sites were found. To further validate our data we examined the effect of IL-1 and EGCG on activation of NF-κB. For this purpose, Colo357 cells were co-transfected with the luciferase reporter plasmid pGL3-5xNF-κB as well as a *Renilla* luciferase reporter construct and analyzed for the effect of EGCG and IL-1 on NF-κB

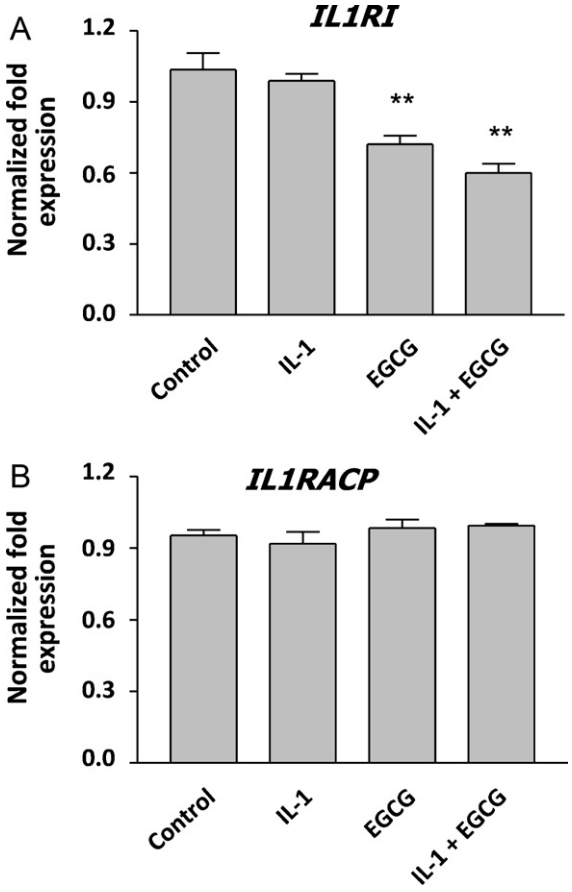

Fig. 7. Effects of EGCG and IL-1 on *IL1RI* and *IL1RACP* mRNA expression. Colo357 cells were treated for 24 h as given in the legend to Fig. 4, and mRNA expression of *IL1RI* (A) and *IL1RACP* (B) was analyzed by quantitative RT-PCR. Data represent means ± SEM from 5 to 7 separate experiments and were normalized to the *RPLP0* response. $^{**}p < 0.01$ compared to control.

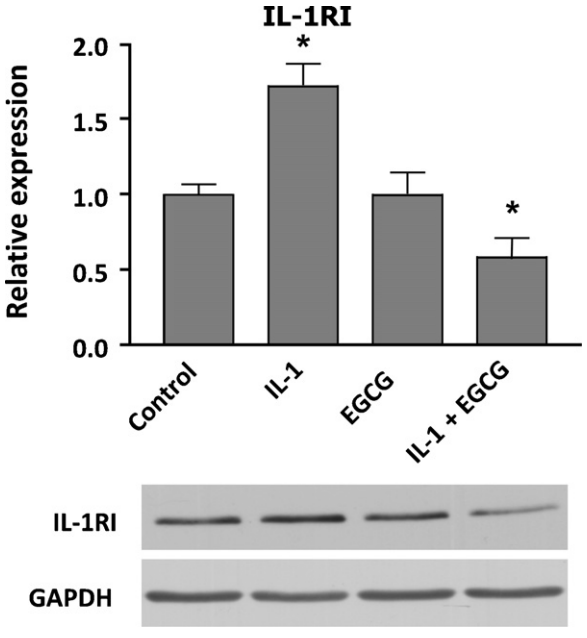

Fig. 8. Effects of EGCG and IL-1 on IL-1RI presence. Colo357 cells were treated for 24 h as given in the legend to Fig. 4. Data were quantified by densitometric analyses and standardized to the GAPDH response. They are expressed as means ± SEM and were derived from four separate experiments. Expression of the untreated controls was set as one. The Western blot depicted represents one typical experiment of a series of at least four similar ones with comparable results. $^{*}p < 0.05$ compared to control.

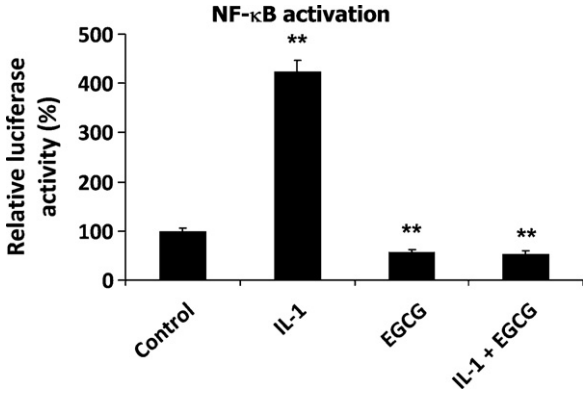

Fig. 9. Activation of NF-κB in pancreatic adenocarcinoma cells. Colo357 cells were transiently transfected with 1 μg of luciferase reporter plasmid pGL3-5xNF-κB together with 25 ng of pRL-TK control vector constitutively expressing the *Renilla* luciferase reporter gene as described under Section 2. 12 h after transfection, cells were serum-starved for 18 h, and stimulated with 1 ng/ml of IL-1β for 12 h in the presence or absence of 50 μM EGCG without NAC. After stimulation the luciferase activity was calculated as a measure of NF-κB activation and corrected for the transcription efficiency. Data represent means ± SEM of six experiments. $^{**}p < 0.01$ compared to control.

activation. As shown in Fig. 9, EGCG was able to reduce both, constitutive as well as IL-1 inducible activity of NF-κB.

4. Discussion

The proinflammatory cytokine IL-1 plays a pivotal role in inflammation-associated pancreatic tumorigenesis. In the present study we aimed to analyze the biological effects of IL-1 on the expression of tumorigenic factors in the human pancreatic adenocarcinoma cell line Colo357 and its modulation by the chemopreventive green tea polyphenol EGCG. EGCG was found to downregulate the expression of proinflammatory IL-6 and PGHS-2 after stimulation with IL-1 confirming previous results of different groups. The upregulated expression of these factors by IL-1 together with the prominent IL-1-independent secretion of MMP-2 results in an accumulation of tumorigenic mediators in the microenvironment of the tumor, maintaining inflammation and representing growth factors for malignant cells. We also detected a constitutive expression of proinflammatory PGHS-2 by the pancreatic adenocarcinoma cells that is further enhanced in the presence of IL-1 confirming previous findings of our group and others [6,23]. The constitutive production of PGHS-2 and its key product PGE_2 in tissues and microenvironment of pancreatic carcinomas accounts for an enhanced malignancy of pancreatic tumor cells leading to inhibition of apoptosis, increase in cell proliferation, induction of angiogenesis as well as invasion of malignant cells into surrounding nerve cords [6,28,29]. PGHS-2 also induces the expression of MMP-2 [30–32] as well as proinflammatory IL-6 and IL-1 via PGE_2 [33]. PGHS-2-mediated effects on growth, angiogenesis, invasiveness and metastasis are augmented by the IL-1-induced upregulation of the enzyme by forcing the progression of a positive amplification loop triggered by PGE_2 and IL-6. VEGF secretion was also found to be upregulated by IL-1 in Colo357. Under normoxic conditions, the expression of this proangiogenic molecule is mainly regulated directly by HIF-1 that can be activated by proinflammatory stimuli such as IL-1 and TNF-α via PI3K and NF-κB [34,35] or indirectly by IL-6 and PGE_2 in an autocrine manner [36,37]. Thus, the amplification triggered by IL-6 and PGE_2 enhances the impact of inflammatory stimuli within the tumor environment. Since EGCG downregulates the secretion of both molecules, the catechin is thereby able to suppress a critical amplification mechanism in tumor-associated inflammation.

EGCG also downregulates the IL-1-induced IL-8 and VEGF secretion as well as the constitutive MMP-2 production in Colo357 affecting not only the proangiogenic potential of the tumor cells but also proliferation, metastasis, invasiveness and tumor-associated inflammation possibly by blocking inflammatory cell recruitment. A recent xenograft mouse model of human pancreatic cancer demonstrated that EGCG inhibits pancreatic cancer growth, invasion, metastasis and angiogenesis implying an association of the EGCG-mediated downregulation of proangiogenic and metastasis-promoting factors with the reduction in tumor growth and the development of a malignant phenotype that might play a crucial role in pancreatic carcinogenesis [38]. Beside its inhibitory function on the production of tumorigenic factors EGCG was found in our study to induce apoptosis in Colo357. EGCG-induced apoptosis seems to occur via inhibition of NF-κB and direct interaction between EGCG and antiapoptotic members of the Bcl-2 family [39]. Unlike in pancreatic beta cells, IL-1-induced NF-κB activation in tumor cells potentiates the expression of antiapoptotic molecules [5]. Since IL-1 has been described as an autocrine growth factor for malignant cells [7], one can hypothesize that the inhibitory effect of IL-1 on EGCG-mediated caspase-3 activation in Colo357 is based on NF-κB activation thus compensating for the catechin-mediated inhibition of the transcription factor finally leading to induction of apoptosis. The protective effect of IL-1 on EGCG-mediated apoptosis induction highlights the critical role of inflammation in tumorigenesis. Beside its impact in angiogenesis, metastasis, invasiveness and inflammation IL-1 functions as a growth and survival factor for pancreatic adenocarcinoma cells. As a consequence, EGCG reduces viability of Colo357 in a time- and dose-dependent manner. However, EGCG-mediated reduction in cell viability was secondary to cytokine production and caspase-3 activation. Therefore, we looked at alterations in intracellular signaling cascades as a cause. At the protein level, EGCG decreased IL-1-mediated IL-1RI whereas IL-1RAcP remained unaffected although EGCG downregulated *IL1RI* mRNA expression in the presence or absence of IL-1. This may be explained by the observed IL-1-induced mRNA stabilization in nonmyeloid cells [40] brought about by IRAK-1. Thus, Colo357 appeared to respond to IL-1 by augmenting the amount of ligand-binding IL-1RI. Considering the immunomodulatory, proangiogenic and tissue remodelling properties of IL-1, one can hypothesize that modulation of pancreatic adenocarcinoma cell responsiveness towards IL-1 via regulation of its own receptor might represent a critical regulatory mechanism of IL-1-mediated production of tumorigenic factors in human pancreatic carcinogenesis.

Sequence analyses of the putative promoter region of both genes identified an excess of NF-κB and AP-1 consensus binding sites in *IL1RI* compared to *IL1RACP*. Keeping in mind that the pleiotropic action of EGCG involves inhibition of NF-κB and AP-1, we propose that the EGCG-induced *IL1RI* mRNA downregulation might be caused, at least in part, by diminishing transcriptional activity of these factors. This is supported by our finding of EGCG-mediated inhibition of NF-κB activation in Colo357. However, closer functional analysis of proximal/distal promoter elements will have to be done.

As demonstrated in this study EGCG was able to block constitutive and IL-1-dependent NF-κB activation as well as production of tumorigenic factors in Colo357. Inhibition of constitutive activity of NF-κB in highly metastatic Colo357-L3.3 cells *in vitro* as well as *in vivo* was found to lead to a reduction in tumor growth, angiogenesis and metastasis by impairing the production of proangiogenic IL-8 and VEGF [41]. The presence of constitutively active NF-κB has now been demonstrated in most cancer tissues including pancreatic cancer correlating with poor clinical outcome [42]. Moreover, most chemopreventive agents including nutraceuticals derived from different sources have the potential to suppress both, constitutive and inducible NF-κB activation pathways [43] rendering them useful tools in blocking chronic inflammation, the seventh hallmark of cancer.

In line with other groups our data clearly suggest an immunoregulatory circuit linking IL-1 signaling and pancreatic tumorigenesis. We were able to identify tumorigenic factors in Colo357 which are downregulated by EGCG breaking the described fatal amplification loop. The catechin can be considered as a natural agent which prevents or at least delays the development of a malignant phenotype in pancreatic tumor cells under proinflammatory conditions such as chronic pancreatitis.

Several studies concerning the use of green tea or green tea polyphenols exist with contradicting results, *e.g.* Bettuzzi et al. [44] reported on the chemopreventive potential of EGCG in human prostate cancer. In this study, the dose of 200 mg green tea polyphenols three times a day did not produce adverse effects. Moreover, a population-based cohort study in Japan on 100,507 individuals revealed that high green tea consumption may be inversely associated with postmenopausal thyroid cancer risk in women, but positively associated with premenopausal thyroid cancer risk [45]. Finally, in a large Japanese cohort with 77,850 participants EGCG failed to decrease the risk of pancreatic cancer in humans [46]. Due to liver toxicity of green tea extract-based supplements at high concentrations, high doses should be handled with care [47].

Concerning a possible liver toxicity a promising approach in targeting pancreatic cancer therapeutically might be the combination of low-dose chemopreventive drugs with complementary mechanisms such as EGCG plus established chemotherapeutics or selective PGHS-2 inhibitors [48]. Such combined administration should positively affect the balance between risk and benefit in fighting the interplay of tumor-associated pancreatic inflammation and carcinogenesis in high-risk patients with pancreatic neoplasia. In this context, the PGHS-2-specific inhibitor celecoxib and EGCG have been found to act synergistically in inhibiting cellular growth and inducing apoptosis in a xenograft model of human prostate cancer [49].

Some concern had been raised about the relevance of the EGCG-dependent drug targets for the anticancer actions of EGCG as many observations had been made *in vitro* using drug concentrations that largely exceeded plasma concentrations due to the low oral bioavailability of EGCG in humans [50]. Plasma concentrations achievable after oral administration of green tea extracts or catechins are still a matter of debate covering the lower micromolar range up to 60 μM [51–53]. Greater oral bioavailability of free catechins in humans can be achieved when consumed in the absence of food [54]. After oral administration, the highest levels of EGCG were found in organs of the gastrointestinal tract which have direct contact to tea catechins [55] thus enabling strong anticancer actions in the pancreas. In line with that assumption, the antineoplastic EGCG effects will not only be observed *in vitro* but also in the *in vivo*-situation (chemoprevention or treatment) at clinically relevant plasma concentrations. Nevertheless, the biological efficacy of EGCG can be further enhanced when encapsulated in PEG nanoparticles leading to a 10-fold dose advantage both in cell culture system and *in vivo* settings in an animal model of human prostate cancer [56]. These findings strongly suggest that the effects of EGCG discovered *in vitro* are also relevant for drug-efficacy *in vivo*. To clarify whether our findings are transferable to the *in vivo* situation chemoprevention approaches with experimental animal models of human pancreatic cancer will have do be done in a separate study.

Taken together, the green tea polyphenol EGCG exerts pleiotropic effects with respect to inflammation and chemoprevention in

pancreatic adenocarcinoma cells Colo357. EGCG functions as an antiproliferative compound in Colo357 which reduces the proinflammatory, proangiogenic and invasive potential of the cells under proinflammatory conditions. Aggressive chemotherapy often results in a negative selection towards highly aggressive tumor clones. Regarding an efficient intervention in pancreatic carcinogenesis the use of additional therapeutic agents with more than one target specificity should be taken into consideration. On the basis of the presented work we propose naturally occurring phytochemicals like EGCG as promising candidates to supplement standard therapeutic concepts.

Conflict of interest

The authors declare no conflicts of interest or financial interests.

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