Combined administration of EGCG and IL-1 receptor antagonist efficiently downregulates IL-1-induced tumorigenic factors in U-2 OS human osteosarcoma cells

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Abstract. Chronic inflammation represents one of the hallmarks of cancer. Of special relevance to the malignant process is the pro-inflammatory cytokine IL-1 playing a crucial role in cancer-related inflammation. Recent observations indicate increased IL-1 levels in an animal model of human osteosarcoma, the most frequent primary malignant bone tumor in man. In patients with bone sarcomas, increased serum levels of tumor-promoting cytokines, including IL-6, IL-8 and VEGF can be found, correlating with poor overall survival. The link between cancer and inflammation makes it clear that there is a need to reduce the external factors inducing inflammation as a preventive or therapeutical measure. Therefore, in the present study the effects of anti-inflammatory IL-1 receptor antagonist (IL-1Ra) was tested alone and in combination with (-)-epigallocatechin-3-gallate (EGCG), an anti-inflammatory chemopreventive agent from green tea, on the production of IL-1-induced tumorigenic factors in U-2 OS human osteosarcoma cells. We found that IL-1Ra and EGCG downregulated IL-1-induced IL-6 and IL-8 release from U-2 OS cells by 65-85%. IL-1Ra and EGCG also reduced secretion of invasiveness-promoting MMP-2 and pro-angiogenic VEGF to 62-75% without affecting the metabolic response and caspase-3 activity. In conclusion, downregulation of IL-1-induced tumorigenic factors (IL-6, IL-8, VEGF, MMP-2) in U-2 OS by IL-1Ra and EGCG may positively affect tumor-associated inflammation and, as a consequence, lead to reduction in angiogenesis and invasiveness. This renders a combined administration of EGCG and IL-1Ra a promising approach as an adjuvant therapy in patients with osteosarcoma.

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Introduction

Osteosarcoma is the most common primary malignancy arising from the bone. It occurs most commonly in young people and affects more males than females (1). There is a predilection for the metaphyseal region of tubular long bones. It is a cancer that usually affects the large bones of the arm or leg with 65% of cases occurring around the knee (2). The 5-year survival rate for patients with osteosarcoma is at an average of 65% (3). Current treatment strategies include chemotherapy, radical resection and irradiation followed by extensive rehabilitation. Although younger patients with localized osteosarcoma benefit from this standard treatment (4), aggressive osteosarcomas respond poorly to conventional cytotoxic chemotherapy thus necessitating the search for novel therapeutical approaches. The most accepted compounds for chemoprevention in humans are naturally occurring dietary substances. Various studies have demonstrated that green tea catechins inhibit carcinogenesis and growth of established cancers at various organ sites (5,6). Many of the chemopreventive effects of green tea are mediated by its catechins, with (-)-epigallocatechin-3-gallate (EGCG) being the most abundant and powerful catechin in cancer prevention and treatment (7). The chemopreventive actions of EGCG comprise anti-oxidant activities, cell signalling modulation, apoptosis induction, cell cycle arrest as well as inhibition of matrix metalloproteinases (MMPs), urokinase-plasminogen activator, telomerase, DNA methyltransferase and proteasome (8).

Epidemiological studies indicate that inflammation serves as a potential risk factor for the development of cancer. It is generally accepted that up to 25% of human malignancies are related to chronic inflammation and to viral and bacterial infections (9). Chronic inflammation increases the risk of cancer including bone neoplasm, promotes tumor progression and supports metastatic spread (10,11). The connection between tumorigenesis and inflammation is mediated via intrinsic and extrinsic pathways (12). The intrinsic pathway is activated by various genetic alterations finally producing transformed cells which can secrete inflammatory mediators and thus can generate an inflammatory microenvironment. The extrinsic pathway is driven by inflammation or infections further increasing the risk for cancer development. Both

pathways converge in tumor cells and induce the activation of several transcription factors including NF-κB, STAT-3 and HIF-1 culminating in the formation of pro-inflammatory factors such as chemokines, cytokines and PGHS-2. These molecules recruit and activate various leukocyte populations into the tumor microenvironment. This concerted action of tumor and micromilieu results in a more pronounced generation of inflammatory mediators driving a tumor-promoting amplification loop.

Several genetic and chromosomal abnormalities as part of the intrinsic pathway have been found in osteosarcoma patients including chromosomal amplification and loss of heterozygosity, associated with poor prognosis (13,14). Additionally, mutations in the tumor suppressor proteins p53 and Rb have been implicated in the oncogenesis of osteosarcoma, enhancing the risk and thus contributing to its poorer prognosis (15,16).

Among the inflammatory mediators present in the tumor microenvironment, IL-1 acts as a crucial player in inflammation-associated carcinogenesis (17,18). Increased levels of IL-1 have been identified in several human tumor entities such as melanoma, head and neck, colon, lung and breast cancer. In an animal model of human osteosarcoma increased IL-1 levels have also been reported (19). Overall, patients harbouring IL-1-positive tumors have markedly worse prognosis (20). IL-1 is produced directly by cancer cells or by cells of the mircroenvironment. Depending on its subcellular location, different IL-1 isoforms mediate different functions. Membrane-bound IL-1α found on malignant cells induces antitumor immune responses, whereas intracellular precursors of IL-1α control homeostatic functions. In contrast, low concentrations of secreted IL-1β downregulate inflammatory responses and immune mechanisms, whereas high concentrations promote inflammation-associated tissue damage and tumor invasiveness (21). IL-1 can stimulate other cell types to produce pro-angiogenic and pro-metastatic mediators and thus plays an important role in inflammation-associated carcinogenesis (17,18). In this context, increased serum levels of tumor-promoting cytokines, including IL-6, IL-8 and VEGF, have been reported in osteosarcoma patients correlating with poor overall survival (22).

IL-1α and IL-1β exert identical agonist actions by binding to the IL-1 receptor type I (IL-1RI). A third ligand, the naturally occurring IL-1 receptor antagonist (IL-1Ra), also binds to IL-1RI without leading to its activation and competitively inhibits its activation by IL-1. IL-1RI ligation leads to the activation of intracellular signalling cascades including NF-κB (23,24) which 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 (25), and may contribute to the characteristic chemoresistance of tumor cells (26).

Since inflammatory- as well as angiogenesis- and invasiveness-promoting factors are crucially involved in the pathogenesis of osteosarcoma, there is a strong medical need to reduce these external factors as a preventive or therapeutical measure. Therefore, in this study anti-inflammatory IL-1Ra was tested either alone or in combination with the green tea-derived catechin EGCG on the production of IL-1-induced tumorigenic factors in U-2 OS human osteosarcoma cells. A combined treatment resulted in a more pronounced inhibition of tumorigenic factors rendering the combined administration of EGCG and IL-1Ra a promising approach as an adjuvant therapy in patients with osteosarcoma.

Materials and methods

Cell culture and stimulation. The human osteosarcoma cell line U-2 OS (27) was purchased from the American Type Culture Collection (#HTB-96; ATCC, Manassas, VA, USA) and cultured in McCoy's 5a medium with L-glutamine supplemented with 10% heat-inactivated fetal calf serum (all from PAA Laboratories, Pasching, Austria) in the presence of 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, 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, U-2 OS cells were seeded in 96-well microtiter plates at a density of $5x10^3$ cells/well, and after a recovery phase of at least 16 h, stimulated with or without 0.5 ng/ml of recombinant human IL-1β (PAN Biotech GmbH, Aidenbach, Germany) in the presence or absence of human recombinant IL-1Ra (R&D Systems GmbH, Wiesbaden, Germany) and (-)-epigallocatechin-3-gallate (EGCG, purity ≥95%) purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) in a time- and dose-dependent manner as indicated in the figure legends. EGCG and IL-1Ra treatment started 1 h prior to IL-1 stimulation. All treatments were conducted in the presence of 0.05 nM 2-mercaptoethanol (Sigma-Aldrich Chemie GmbH) to stabilize EGCG and quench EGCG-derived ROS.

Metabolic response and cell viability. The effect of EGCG and IL-1Ra on the metabolic response was assessed by using MTT assay as described by Mosmann (28). This assay was based on the ability of viable cells only to reduce the conversion of the water soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to an insoluble formazan. After solubilisation, the concentration was determined spectrophotometrically using the Titertek Multiscan microplate reader (Flow Laboratories, Meckenheim, Germany) at 550 nm. The assay is suitable for determining the metabolic activity/number of viable cells in proliferation, cytotoxicity or chemosensitivity assays.

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 μM EGCG and increasing concentrations (1-50 ng/ml) of IL-1Ra. 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, 10 mm $Na_2P_2O_7$) and then incubated with 25 μg/ml of the fluorogenic caspase-3 substrate N-acetyl-DEVD-7-amido-4-trifluoromethyl coumarin (Becton Dickinson GmbH, Heidelberg, Germany) in 20 mM HEPES (pH 7.5), 10% glycerol and 2 mM dithiothreitol at 37˚C for 2 h in the dark. The release of the fluorogenic AFC moiety as a measure of caspase activity was analyzed fluorimetrically using the Infinite® 200 PRO microplate reader (Tecan Group, Ltd., Männedorf, Switzerland) at an excitation/emission wavelength of 390/510 nm. Relative caspase activities were normalized to the protein content as determined by Bradford dye-binding assay (29) and compared

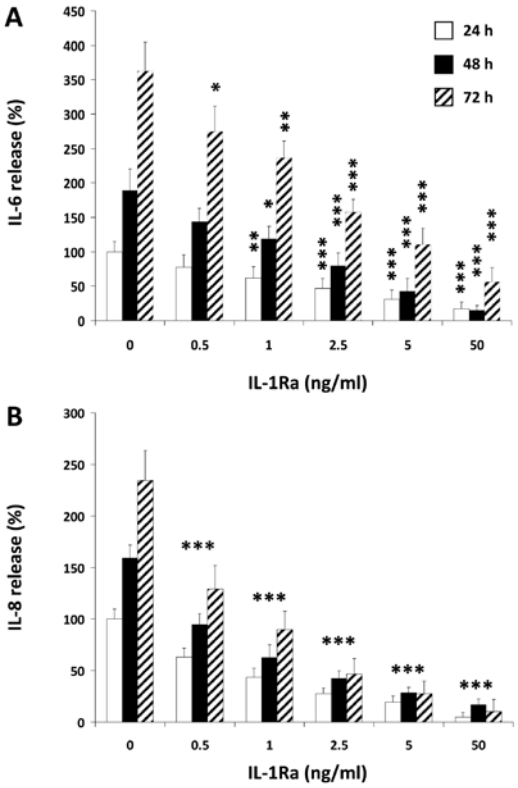

Figure 1. Effects of IL-1Ra on IL-6 and IL-8 release. Human osteosarcoma cells U-2 OS were seeded in microtiter plates and stimulated with 0.5 ng/ml of IL-1β for the indicated times in the presence of 0.5-50 ng/ml of IL-1Ra, and (A) IL-6 as well as (B) IL-8 secretion into culture supernatants was detected by sandwich ELISA. Data represent means ± standard deviation (SD) of 4 independent experiments performed in multiple replicates and were corrected by the spontaneous secretion and normalized to the metabolic response. Mediator release of the IL-1-treated sample without inhibitor after 24 h was set as 100% and corresponds to 21.9 pg/ml of IL-6 and 1134 pg/ml of IL-8, respectively. *P<0.05; **P<0.01; ***P<0.001 compared to IL-1-treated control without inhibitor at certain timepoint.

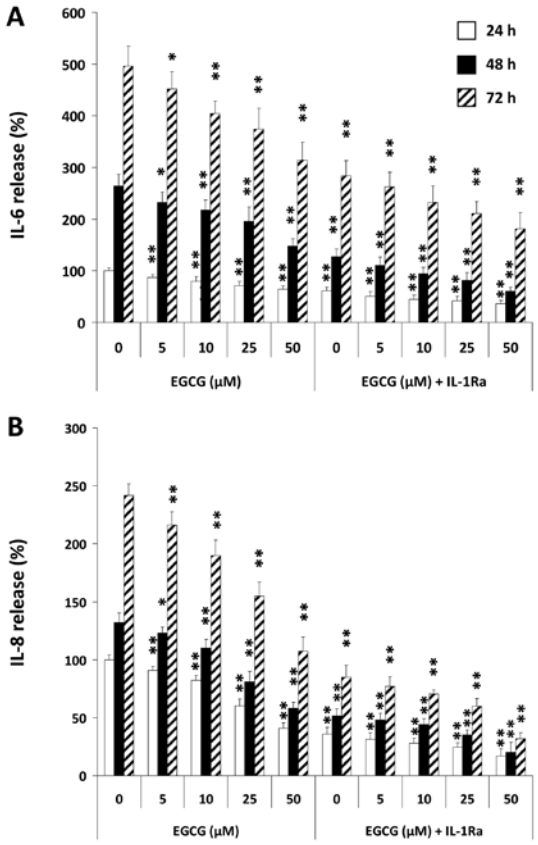

Figure 2. Cytokine response to IL-1Ra and EGCG. (A) IL-6 and (B) IL-8 were detected in culture supernatants of U-2 OS cells by sandwich ELISA after IL-1 stimulation in the presence of 5-50 μM EGCG alone or in combination with 1 ng/ml of IL-1Ra. (For more details see Fig. 1 legend). Mediator release of the IL-1-treated sample without inhibitor at 24 h was set as 100% and corresponds to 5.9 pg/ml of IL-6 and 886 pg/ml of IL-8, respectively. Data represent means ± SD of 6 independent experiments performed in multiple replicates and were corrected by the spontaneous secretion and normalized to the metabolic response. *P<0.05; **P<0.01 compared to IL-1-treated control without inhibitor at certain timepoint.

to the untreated control whose response was set as one. Etoposide (Sigma-Aldrich Chemie GmbH) at a concentration of 50 μM was used as a positive control.

Cytokine and metalloproteinase assays. Concentrations of human IL-6 and IL-8 in the culture supernatants were determined by commercially available ELISA kits (Eli-Pair; Diaclone, Besançon, France). Production of total MMP-2 was quantified by immunoassay using the corresponding Quantikine® ELISA kit purchased from R&D Systems GmbH according to the manufacturer's instructions. Secretion of VEGF into the supernatants was evaluated using the RayBio® Human VEGF EIA kit (RayBiotech, Inc., Norcross, GA, USA). All data were normalized to the metabolic response obtained by MTT assay.

Data adjustment and statistics. In order to compensate for inter-experiment variations, data were adjusted by setting the value for the untreated sample (VEGF, MMP-2) or IL-1-treated sample without inhibitor (IL-6, IL-8) as 100%. Statistical differences between mean values were analyzed using the two-tailed non-parametric Wilcoxon-Mann-Whitney test. A P-value <0.05 was considered to be statistically significant.

Results

In this study, the effect of IL-1Ra was tested alone and in combination with EGCG on the expression of IL-1-induced tumorigenic factors in U-2 OS human osteosarcoma cells. This approach acts as a model system for tumor-associated inflammation, playing a key role in carcinogenesis. In order to prevent autoxidation of EGCG and quench formation of EGCG-derived ROS, all experiments were conducted in the presence of 2-mercaptoethanol.

As an example of IL-1-induced tumorigenic factors we determined the effect of IL-1Ra and EGCG, either alone or in combination, on secretion of pro-inflammatory IL-6 (Fig. 1A and Fig. 2A) and pro-angiogenic IL-8 (Fig. 1B and Fig. 2B) by U-2 OS cells after stimulation with IL-1. We found a strong time-dependent IL-6 and IL-8 production by U-2 OS cells after IL-1 stimulation that was blocked by either IL-1Ra (Fig. 1) or EGCG (Fig. 2) in a dose-dependent manner. Since IL-1Ra inhibited the release of these molecules with an IC_{50} of ~1 ng/ml, co-incubation experiments were conducted with this IL-1Ra concentration. As demonstrated in Fig. 2, co-treatment of U-2 OS cells with EGCG and IL-1Ra reduced IL-1-induced

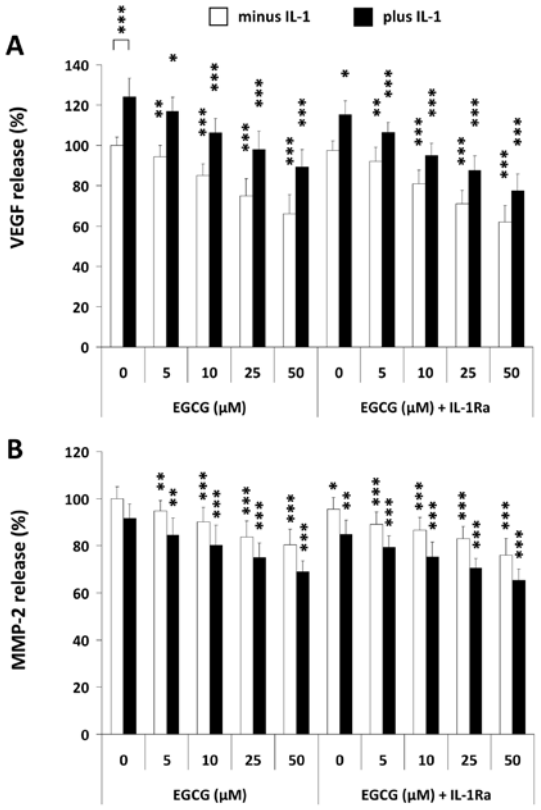

Figure 3. Effects of EGCG and IL-1Ra on VEGF and MMP-2 release. (A) VEGF and (B) MMP-2 were detected in culture supernatants of U-2 OS cells by sandwich ELISA 48 h after incubation in the absence or presence of IL-1β and different inhibitor concentrations as described in Fig. 2 legend. Mediator release of the untreated control was set as 100% and corresponds to 3250 pg/ml of VEGF and 30.3 pg/ml of MMP-2, respectively. Data represent means ± SD of 4 (VEGF) and 5 (MMP-2) independent experiments performed in quadruplicates and corrected by the metabolic response. *P<0.05; **P<0.01; ***P<0.001 compared to untreated control.

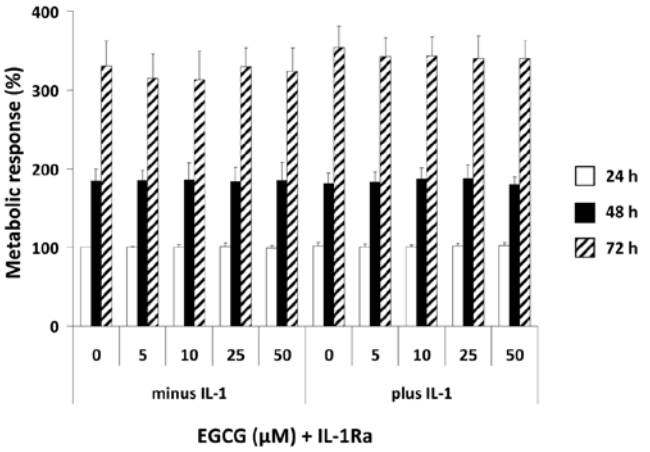

Figure 4. Effects of IL-1Ra and EGCG on the metabolic response. U-2 OS cells were stimulated with or without IL-1β as described in Fig. 2 legend followed by assessment of the metabolic response using MTT assay. Data represent means ± SD of 5 independent experiments performed at least in quadruplicates. Statistical analysis revealed no significant differences between the samples.

cytokine production in an additive manner. Co-stimulation of U-2 OS cells with 50 μM EGCG and IL-1Ra led to an inhibition of IL-1-induced IL-6 production by ~2/3 at any timepoint,

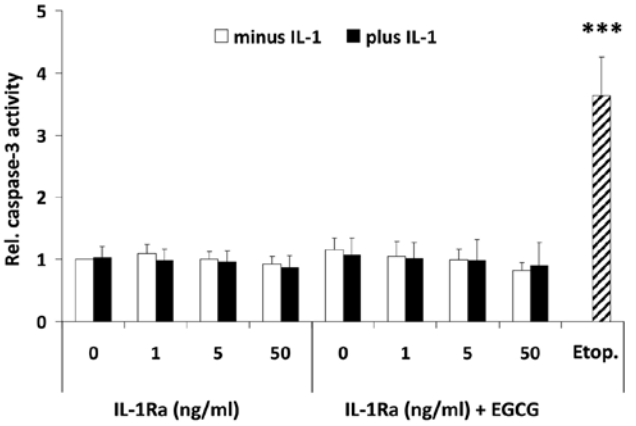

Figure 5. Activation of caspase-3 in osteosarcoma cells. U-2 OS cells were treated with 50 μM EGCG and/or increasing concentrations (1-50 ng/ml) of IL-1Ra in the presence or absence of 0.5 ng/ml of IL-1β for 24 h as indicated. Cleavage of caspase-3 substrate (N-acetyl-DEVD-7-amido-4-trifluoromethyl coumarin) in cell lysates was determined fluorimetrically. Relative caspase activities were normalized to the protein content and compared to the untreated control whose response was set as one. 50 μM etoposide (Etop.) was used as a positive control. The results shown are means ± SD of 7 independent experiments performed in duplicates. ***P<0.001 compared to control.

while IL-1-induced IL-8 release was downregulated at an average of 85%.

Increased levels of different growth factors have been previously reported in osteosarcoma including VEGF (30). We therefore tested whether U-2 OS cells constitutively secrete this pro-angiogenic cytokine and whether its secretion was altered by IL-1, IL-1Ra and EGCG, respectively. As demonstrated in Fig. 3A, the osteosarcoma 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. In contrast, co-incubation of U-2 OS cells with the agents suppressed VEGF production in an additive manner (Fig. 3A). IL-1Ra in combination with 50 μM EGCG reduced the IL-1-induced VEGF secretion as well as the spontaneous production by ~38%.

Since matrix metalloproteinases (MMPs) play an important role in the pathogenesis of osteosarcoma (31), we investigated the secretion profile of invasiveness- and angiogenesis-promoting MMP-2 by U-2 OS cells in the presence of IL-1Ra and different concentrations of EGCG, after stimulation with IL-1. It has been shown previously that MMP 2 secretion can be induced indirectly by IL-1 via IL-6 and activation of the JAK/STAT pathway (32). Our data clearly identified a strong spontaneous MMP-2 secretion by U-2 OS cells that was not augmented by added IL-1 (Fig. 3B). EGCG alone dose-dependently decreased MMP-2 secretion down to 75% of the untreated control. Also IL-1Ra single-agent treatment slightly reduced MMP-2 release. Interestingly, IL-1Ra in combination with EGCG significantly augmented EGCG-mediated decline in MMP-2 production in any case (P<0.05) (Fig. 3B).

In order to elucidate whether the effects on mediator release are related to a decline in the metabolic response, U-2 OS cells were treated with IL-1Ra and increasing concentrations of EGCG in the presence or absence of IL-1, followed by determination of the metabolic activity as evidenced by MTT assay. We

found that neither EGCG and IL-1Ra alone nor a combinatorial treatment had any effect on the metabolic response in U-2 OS cells (Fig. 4). Moreover, an effect of IL-1 on the metabolic response of U-2 OS cells could also not be observed.

To further shed light on the mechanism which causes a decrease in mediator release, U-2 OS cells were treated with the highest dose of EGCG used in this study and increasing concentrations of IL-1Ra in the presence or absence of IL-1, followed by fluorometrical determination of caspase-3 activity, indicative for apoptosis induction. Fig. 5 shows that neither treatment had any effect on caspase-3 activation, implying that the decline in mediator release is unrelated to apoptosis induction.

Discussion

The aim of the present study was to shed light on the combined effects of IL-1Ra and the green tea-derived catechin EGCG on the expression of IL-1-induced tumorigenic factors in the human osteosarcoma cell line U-2 OS (27). U-2 OS cells have been characterized to overexpress the oncoprotein Mdm-2 (33). Aberrant Mdm-2 expression can be found in a variety of human tumors of diverse tissue origin including osteosarcoma contributing to the malignant phenotype. In osteosarcoma, Mdm-2 overexpression occurs with high frequency as a result of an upregulated *MDM2* mRNA expression and translation (34).

We found that IL-1Ra and EGCG act additively in downregulating secretion of pro-inflammatory IL-6 as well as pro-angiogenic IL-8 and VEGF, thus blocking production of tumorigenic mediators in the tumor microenvironment. Since IL-1Ra and EGCG were also found to suppress export of invasiveness-promoting MMP-2 by U-2 OS cells, targeting the inflammatory network in U-2 OS cells by IL-1Ra and EGCG can be considered as a promising approach in the treatment of osteosarcoma. EGCG and IL-1Ra were used at concentrations not inducing apoptosis and not affecting the metabolic response of U-2 OS cells. Even after co-incubation with EGCG and IL-1Ra, effects on cell viability and apoptosis induction did not occur. From these findings, it can be concluded that a combinatory administration of IL-1Ra and EGCG reduces the impact of tumorigenic factors by interfering with intracellular signalling cascades such as NF-κB. It is well documented that, beside its antioxidant activities, EGCG targets the key transcription factor in tumorigenesis, NF-κB (8,35,36).

EGCG plasma concentrations achievable after oral administration of green tea extracts or catechins cover the lower micromolar range up to 60 μM (37-39). Greater oral bioavailability of free catechins in humans can be achieved when consumed in the absence of food (40). EGCG was found in our study to significantly reduce release of tumorigenic factors already at a concentration of 5 μM. We therefore speculate that EGCG will also interrupt the inflammatory network *in vivo*; however, in order to strengthen this assumption, chemoprevention studies in experimental models of human osteosarcoma will have to be performed.

IL-1Ra was found in this study to be a potent inhibitor of IL-1-induced tumorigenic factors in U-2 OS cells. In an animal model of human osteosarcoma, systemic IL-1Ra (anakinra™) dose-dependently inhibited different forms of thermal and osteosarcoma-induced hyperalgesia (19). Because of its collagenase and prostaglandin-inhibiting properties, anakinra is approved for the treatment of chronic inflammatory diseases including rheumatoid arthritis (41) and systemic onset juvenile idiopathic arthritis (42). It has also been identified to be powerful in blocking IL-1 effects in numerous pathological settings (41). With respect to cancer, anakinra was successfully used in treating the rare lymphoproliferative disorder Castleman's disease (43) and myeloma (44). Anakinra and other IL-1-blocking agents such as canakunimab™ (anti-IL-1β antibody) or rilonacept™ (construct of the two extracellular chains of IL-1RI/IL-1RAcP complex fused to the Fc segment of IgG) could thus be promising therapeutics for human metastatic diseases. The latter two agents have been approved for the treatment of the cryopyrin-associated periodic syndrome (45,46). As summarized by Dinarello (47), there are two main reasons for the use of IL-1-blocking agents in the treatment of metastatic diseases. No organ toxicities or gastrointestinal and haematological abnormalities were observed and no, unlike for TNF-blocking agents, opportunistic infections were reported except rare bacterial and upper airway infections. Due to the safety of IL-1 blockage, clinical trials are encouraged. An NIH trial of anakinra in the treatment of cutaneous melanoma is ongoing because IL-1 plays a pivotal role in angiogenesis by inducing/upregulating pro-angiogenic IL-8 and VEGF, contributing to the pathogenesis of e.g. multiple melanoma (47).

Notably, the immunomodulator mifamurtide (liposomal muramyl tripeptide phosphatidylethanolamine; MEPACT™) which has been approved in Europe for the treatment of non-metastatic osteosarcoma in combination with chemotherapy (48), has come under scrutiny because of studies suggesting an increased risk of serious adverse effects associated with its use whilst concomitantly lacking any survival benefit (49). In osteosarcoma patients, increased plasma concentrations of pro-inflammatory mediators such as IL-1, TNF and IL-6 are detected after administration of mifamurtide (50) obviously due to its macrophage/monocyte activating capacity (51). From this observation and regarding our own data, one can hypothesize that mifamurtide probably enhances the impact of tumorigenic stimuli within the tumor microenvironment thereby forcing a critical amplification mechanism in tumor-associated inflammation triggered by IL-6 and others. All these results clearly demonstrate that there is a strong medical need for the development of new concepts how such inflammatory activities working in osteosarcoma may be therapeutically targeted with novel combinations of chemopreventive drugs.

In summary, a therapeutic approach that combines the IL-1 activity inhibiting effects of IL-1Ra and the anti-angiogenic and anti-inflammatory activities of EGCG might impair the development of a malignant phenotype in osteosarcoma cells and produce a crucial additive antitumoral response compared to IL-1Ra or EGCG administered in monotherapy.

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