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Green tea polyphenols-induced apoptosis in human osteosarcoma SAOS-2 cells involves a caspase-dependent mechanism with downregulation of nuclear factor-κB

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Abstract

Development of chemotherapy resistance and evasion from apoptosis in osteosarcoma, a primary malignant bone tumor, is often correlated with constitutive nuclear factor-κB (NF-κB) activation. Here, we investigated the ability of a polyphenolic fraction of green tea (GTP) that has been shown to have antitumor effects on various malignant cell lines to inhibit growth and induce apoptosis in human osteosarcoma SAOS-2 cells. Treatment of SAOS-2 cells with GTP (20–60 μg/ml) resulted in reduced cell proliferation and induction of apoptosis, which correlated with decreased nuclear DNA binding of NF-κB/p65 and lowering of NF-κB/p65 and p50 levels in the cytoplasm and nucleus. GTP treatment of cells reduced IκB-α phosphorylation but had no effect on its protein expression. Furthermore, GTP treatment resulted in the inhibition of IKK-α and IKK-β, the upstream kinases that phosphorylate IκB-α. The increase in apoptosis in SAOS-2 cells was accompanied with decrease in the protein expression of Bcl-2 and concomitant increase in the levels of Bax. GTP treatment of SAOS-2 cells also resulted in significant activation of caspases as was evident by increased levels of cleaved caspase-3 and caspase-8 in these cells. Treatment of SAOS-2 cells with a specific caspase-3 inhibitor *Ac-Asp-Glu-Val-Asp-CHO* (Ac-DEVD-CHO) and general caspase inhibitor *N-benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl ketone* (Z-VAD-FMK) rescued SAOS-2 cells from GTP-induced apoptosis. Taken together, these results indicate that GTP is a candidate therapeutic for osteosarcoma that mediates its antiproliferative and apoptotic effects *via* activation of caspases and inhibition of NF-κB.

Keywords: Green tea; Osteosarcoma; Nuclear factor-κB; Apoptosis; Caspases

Introduction

Osteosarcoma, a primary malignant bone tumor, is most common in children and adolescents, accounting for 0.3% of pediatric-cancer-related deaths in the United States (Herzog, 2005). Osteosarcoma is thought to be derived from osteoblasts which secrete bone matrix. Despite aggressive multimodal therapy, this devastating tumor often acquires drug resistance and metastasizes (Marina et al., 2004). Therefore, our goal is to identify agents that could promote apoptosis of osteosarcoma cells which do not exert any toxic effects on normal cells.

Green tea polyphenols (GTP) has received much attention over the last few years as cancer chemopreventive and chemotherapeutic agent (Adhami et al., 2004; Shimizu et al., 2005). These dietary polyphenols have shown to possess antitumor effects in several malignant cell lines including breast, skin, liver, pancreas, lung, prostate and bladder (Adhami et al., 2004; Shimizu et al., 2005; Cooper et al., 2005a). Furthermore, it has been shown that GTP possesses anti-inflammatory, anti-oxidant, anti-clastogenic and anti-mutagenic activities in a variety of malignant cells and preclinical models of cancer (Cooper et al., 2005b; Crespy and Williamson, 2004). Green tea polyphenols have shown differential effects in inhibiting cell growth, causing cell cycle arrest and inducing apoptosis in cancer cells without affecting normal cells (Ahmad et al., 2000; Chen et al., 1998).

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GTP-mediated effects include inhibition of various kinases *viz.* MAPK, protein kinase B/AKT, (Siddiqui et al., 2004); loss of mitochondrial transmembrane potential (Nakazato et al., 2005); modulation of Bax and Bcl-2 family members (Baliga et al., 2005), induction of p21/WAF1 and p27/SDI1, inhibition of cyclin D1-associated pRB (Liberto and Cobrinik, 2000), activation of caspases (Qanungo et al., 2005) and inhibition of various growth factors including IGF-1, VEGF and FGF; and inhibition of serine proteases and matrix metalloproteinases (Annabi et al., 2002), critical for cancer progression.

The members of Rel/nuclear factor (NF)-κB family form hetero- and homodimers and control the expression of a number of genes that regulate cell survival, proliferation, immune response and apoptosis (Gilmore, 2003). In unstimulated cells, NF-κB is sequestered in the cytoplasm as heterodimers, composed of p50/c-Rel and p50/p65 subunits, bound by IκB-α, thus preventing their translocation into nucleus. In response to various stimuli, IκB-α subunit is phosphorylated by an upstream kinase, IKK-α, at serine residues 32 and 36, triggering ubiquitination and degradation of IκB-α by the 26S proteosome. This signal facilitates the release and translocation of the NF-κB heterodimer into the nucleus, where it binds with specific DNA motifs in the promoter regions of target genes and activates their transcription (Vermeulen et al., 2002; Pahl, 1999). The diverse signals (several hundred described so far) that can trigger the NF-κB activation highlight its pivotal role in several biological processes including neoplastic progression and possess high activity in transformed and malignant cells (Liu et al., 2001). NF-κB has been shown to be constitutively activated in most types of human cancer including breast, colon, skin, lung, esophagus, pancreas, prostate and gliomas and plays a critical role in the regulation of cell survival, proliferation and apoptosis (Sovak et al., 1997; Lind et al., 2001; Bell et al., 2003; Mukhopadhyay et al., 1995; Wang et al., 1999; Nair et al., 2003; Tselepis et al., 2002; Suh et al., 2002). In recent years, NF-κB has emerged as a major therapeutic target in cancer because of its ability to cause chemotherapy resistance and evasion from apoptosis (Yamamoto and Gaynor, 2001; Garg and Aggarwal, 2002). Studies have shown that the inhibition of constitutively active NF-κB leads to reversion of malignancy in human osteosarcoma cells (Andela et al., 2002). Therefore, sustained inhibition of NF-κB may be a rational strategy for effective management of this disease. Non-toxic agents that have the ability to inhibit NF-κB activity may be ideal candidates as therapeutics for osteosarcoma. Since human osteosarcoma SAOS-2 cells possess high constitutive levels of activated NF-κB and are resistant to chemotherapy and apoptosis, we investigated whether GTP has the potential to induce apoptosis along with its mechanism of action. Our results demonstrate that GTP is a candidate therapeutic agent for osteosarcoma that mediates its antiproliferative and apoptotic effects *via* activation of caspases and inhibition of NF-κB.

Materials and methods

Cell lines and reagents. Human osteosarcoma SAOS-2 cells were a kind gift of Dr. Brian Johnstone, Department of Orthopedics, Case Western Reserve University. Green tea extract was obtained from Mitsui Norin Co. (Polyphenon-E®, Tokyo, Japan) and is subsequently referred to as GTP. Polyphenon-E contains epicatachin-3-gallate (EGC) 6.4%, epicatechin (EC) 10.7%, epigallocatechin-3-gallate (EGCG) 63%, gallocatechin-3-gallate (GCG) 2.0%, epicatechin-3-gallate (ECG) 6.1%, catechin-3-gallate (CG) 0.1%, gallocatechin-3-gallate (GCG) 0.2% and catechin-3-gallate (CG) 1.1%. McCoy's 5A modified medium and penicillin–streptomycin were purchased from Cellgro (Mediatech Inc. Herndon, VA). Fungizone and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Antibodies against anti-Bax (Cat.# sc-493), anti-Bcl-2 (Cat.# 7382), anti-caspase-8 (Cat.# sc-7890), anti-IκB-α (Cat.# sc-1643), anti-IKK-α (Cat.# sc-7182), anti-IKK-β (Cat.# sc-7330), anti-NF-κB/p65 (Cat.# sc-800), anti-NF-κB/p50 (Cat.# sc-8414), anti-phospo-IκB-α (Cat.# sc-8404) and anti-PARP (Cat.# sc-8007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3 (Cat.# 9662) and cleaved anti-caspase-3 (Cat.# 1996) antibodies were obtained from Cell Signaling Technologies, (Beverly, MA). Horseradish-peroxidase-conjugated secondary antibodies were purchased from either Pierce Biotechnology (Rockford, IL) or Cell Signaling Technologies (Beverly, MA). Propidium iodide and MTT (3-(4,5-2-yl]-2,5diphenyl tetrazoliumbromide) were purchased from Sigma Chemical Co. (St. Louis, MO). Pan-caspase inhibitor (Z-VAD-FMK) was obtained from R&D Systems (Minneapolis, MN), and specific caspase-3 inhibitor (Ac-DVD-CHO) was obtained from Calbiochem (San Diego, CA).

Cell culture and treatments. Human osteosarcoma SAOS-2 cells were grown in McCoy's 5A modified medium containing 10% heat inactivated fetal bovine serum (FBS), 1% penicillin–streptomycin and 1% fungizone in a tissue culture incubator at 37 °C with 5% CO_2. GTP was dissolved in PBS, and the solution was filter sterilized with 0.02 μm syringe filter. Cells (70–80% confluent) were treated with desired concentrations of GTP.

Cell proliferation assay. Cell growth inhibition was determined by MTT-based cell proliferation assay. Briefly, SAOS-2 cells (5.0×10^4/well) were plated in 96-well culture plates for 24 h in complete culture medium, which was replaced by fresh media. Different concentrations of GTP were added (10–80 μg/ml), and the cells were incubated at 37 °C for 16, 24 and 48 h. Two hours prior to termination, 20 μl of MTT reagent (5 mg/ml in sterile PBS) was added to each well and incubated at 37 °C. At the end of incubation, cells were washed twice with PBS, formazone crystals were solubilized in 100 μl of DMSO, and the optical density was read at 540 nm.

Preparation of cell lysates and immunoblot analysis. After 24 h of treatment, the medium was aspirated and the cells washed with ice-cold PBS. The cells were suspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 and 1% SDS) supplemented with protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF and 1 mM DTT) and phosphatase inhibitors (1 mM β-glycerophosphate, 1 mM Na_3VO_4), placed over ice for 30 min and passed through a 21G needle to break up the cell aggregates. Cell lysates were cleared by centrifugation at 14,000×*g* for 15 min at 4 °C, and the supernatant was either used immediately or stored at −80 °C. For assessment of protein activation of caspases, total cell lysates were prepared without adding the protease inhibitor. Cytoplasmic and nuclear fractions were prepared as previously described (Shukla and Gupta, 2004). Protein content of each sample was determined by using the BioRad protein assay (BioRad Laboratories, Hercules, CA) according to manufacturer's protocol.

For immunoblot analysis, 25 μg of protein was resolved over 4–20% gradient acrylamide gel and transferred to a nitrocellulose membrane (BioRad). Blots were blocked (5% non-fat dried milk, 2% Tween 20 in 20 mM TBS, pH 7.6) for 2 h at room temperature and then probed with appropriate primary and secondary antibodies. Immunoreactive proteins were visualized by chemiluminescence (Pierce Biotechnology Rockford, IL). Images were captured, and the intensities of the protein bands were analyzed using the Alpha Innotech Imaging Systems and the values are expressed in arbitrary optical density units.

Quantitative assessment of apoptosis. The extent of apoptosis induced by GTP treatment in SAOS-2 cells was quantified by using the Cell Death Detection ELISA^{Plus} kit (Roche Diagnostic, Indianapolis, IN). This sandwich

assay quantifies histone-complexed DNA fragments present in the cytoplasm of cells undergoing apoptosis. The intensity of color developed corresponds to the number of apoptotic cells in the sample. Briefly, SAOS-2 cells (2×10^4 cells/well) were cultured in 24-well plate with 5% CO_2 at 37 °C and GTP treatment at a dose of 10–60 μg/ml was provided for 24 h. Cells were collected after completion of GTP treatment and resuspended in 400 μl of kit supplied lysis buffer for 30 min. The cellular lysates were centrifuged at 200×*g* for 10 min, and 20 μl (1×10^3 cells equivalent) and the supernatant (cytoplasmic lysate containing nucleosome) were incubated with monoclonal anti-histone and anti-DNA-POD for 2 h at room temperature. The immunocomplex was exposed to ABTS substrate for 10 min. Color development was monitored spectrophotometrically at 405 nm. The enrichment factor (total amount of apoptosis) was calculated by dividing the absorbance of the sample by the absorbance of the controls without treatment.

Electrophoretic mobility shift assay. After treatment of SAOS-2 cells with desired doses of GTP, nuclear fractions were prepared and electrophoretic mobility shift assays were performed with a gel shift assay kit (Pierce). Briefly, 10 μg of nuclear extract was incubated with 1 pmol of biotin labeled NF-κB oligonucleotide for 30 min at 37 °C. The DNA–protein complex formed was resolved on 6% DNA retardation gel, transferred to a nylon membrane. After transfer was completed, DNA was cross-linked to the membrane at 120 mJ/cm^2 using a UV cross-linker. The biotin end-labeled DNA was detected using the streptavidin–horseradish peroxidase conjugate and LightShift™ chemiluminiscent substrate according to manufacturer's instruction.

Statistical analysis. The enrichment factor for cell death detection and MTT assay for cell growth inhibition are expressed as mean ± SE. The significance values between the control and treated groups were calculated by using the Student's *t* test, and *p* value less than 0.05 was considered as significant.

Results

GTP treatment reduces the viability of human osteosarcoma SAOS-2 cells

To ascertain the effect of GTP on the viability of SAOS-2 cells, we performed MTT assay. As shown in Fig. 1, treatment of cells with 10–80 μg/ml of GTP resulted in dose- and time-dependent inhibition of cell growth compared to control group. The cell growth inhibitory effect was more pronounced at 48 h post-GTP treatment as compared to 16 and 24 h. Compared to controls, significant inhibition in cell growth was observed at the GTP doses of 60 and 80 μg/ml for 16 and 24 h ($P < 0.001$). In contrast, at lower dose (20 μg/ml), significant inhibition in cell growth was recorded at 48 h post-GTP treatment ($P < 0.001$).

GTP treatment induces apoptosis in human osteosarcoma SAOS-2 cells

To assess whether the inhibition of cell viability was due to GTP-induced apoptosis, human osteosarcoma SAOS-2 cells were treated with different concentrations of GTP (10–60 μg/ml) for 24 h and the induction of apoptosis was analyzed by probing the Western blots for PARP cleavage in control and treated cells. GTP treatment (20–60 μg/ml) induced PARP cleavage in a dose-dependent manner with higher concentrations being more effective (Fig. 2A). We further evaluated time-dependent induction of PARP cleavage using a dose of 40 μg/ml of GTP. Low level of PARP cleavage was detected in SAOS-2 cells treated for 12 h and was significantly enhanced in cells treated for 24 and 48 h, respectively (Fig. 2A). In our study, a low intensity band reactive with anti-PARP antibodies was also observed in untreated cells probably due to the spontaneous degradation product of PARP recognized by this antibody. The cross-reactivity of this antibody has also been observed in previous studies (Wainstein et al., 1994).

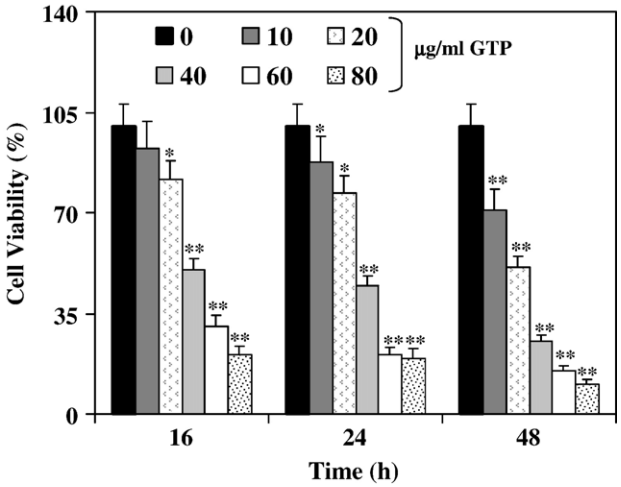

Fig. 1. Effect of GTP treatment on cell viability in human osteosarcoma SAOS-2 cells. Cell growth inhibition was studied by using the MTT-based cell proliferation assay. Cells were treated with specified doses of GTP for 16, 24 and 48 h. The values shown here are mean ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.001$, compared to control.

Next, we quantified the extent of apoptosis after GTP treatment in SAOS-2 cells by an ELISA method. As shown in Fig. 2B, GTP treatment to SAOS-2 cells resulted in the induction of apoptosis. A significant dose-dependent increase in the proportion of apoptotic cells was observed in treated groups, compared to the control group. These results are in agreement with the pervious data on PARP cleavage observed at the same doses used in this experiment.

GTP treatment inhibits the DNA binding activity of NF-κB in human osteosarcoma SAOS-2 cells

Next, we correlated the above results with decrease in the DNA binding activity of NF-κB/p65 by EMSA. As shown in Fig. 3A, GTP treatment of SAOS-2 cells resulted in a dose-dependent decrease in the NF-κB/p65 DNA binding activity which was significantly inhibited at doses of 40 and 60 μg/ml. Treatment of SAOS-2 cells with lower doses (10 and 20 μg/ml) was less effective and showed partial inhibition of NF-κB/p65 DNA binding (Fig. 3A).

GTP treatment inhibits constitutive NF-κB activity in human osteosarcoma SAOS-2 cells

In the next series of experiments, we first checked the basal level of NF-κB/p65 and NF-κB/p50 in these cells (data not shown) and then assessed the effect of GTP on the levels of these proteins. Treatment of SAOS-2 cells with GTP at doses of 10–60 μg/ml for 24 h resulted in a dose-dependent inhibition of constitutive NF-κB/p65 and NF-κB/p50 protein expression in both cytoplasmic and

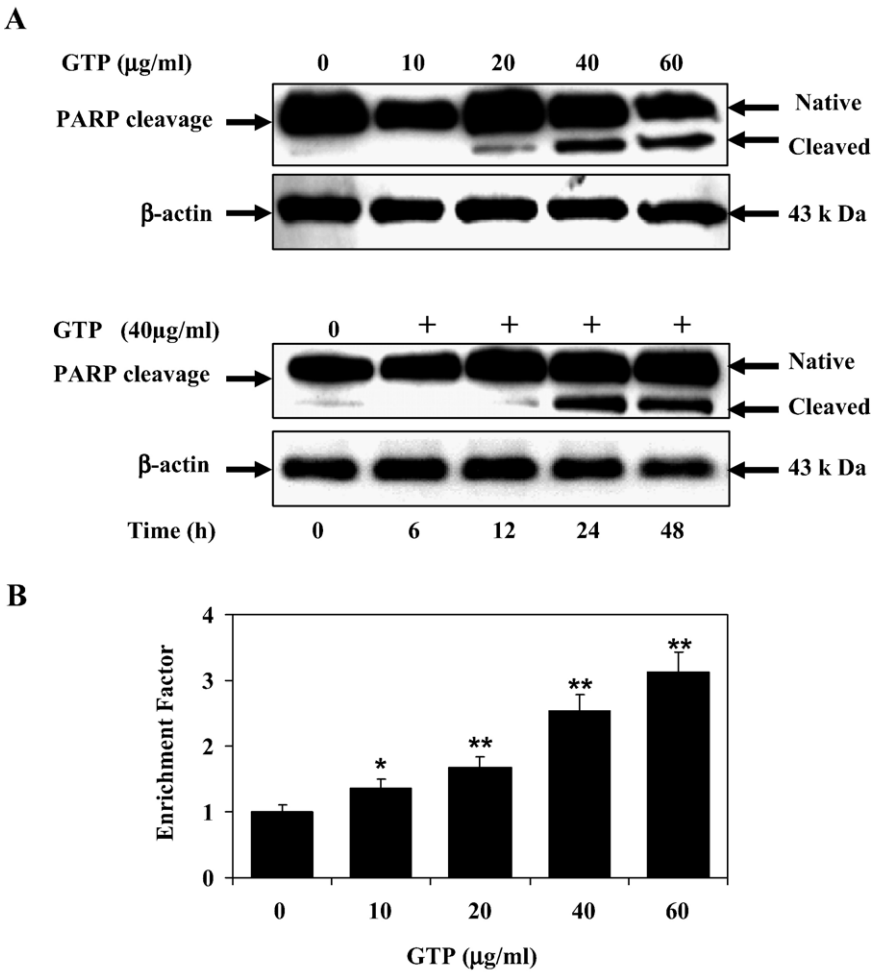

Fig. 2. Dose- and time-dependent induction of PARP cleavage by GTP in human osteosarcoma SAOS-2 cells. (A) Cells were treated with specified doses of GTP for 24 h, and for the assessment of time-dependent effects of GTP on PARP, cleavage cells were treated with a dose of 40 μg/ml for 6, 12, 24 and 48 h (lower panel). The data shown are representative of three independent experiments with similar results. (B) Frequency of apoptotic cells was quantified by cell death ELISAPLUS kit. Cells were treated with specified doses of GTP for 24 h and the values expressed as enrichment factor. *$P < 0.05$ and **$P < 0.001$, compared to control.

nuclear fractions (Fig. 3B). At doses of 20, 40 and 60 μg/ml of GTP, 10%, 30% and 80% inhibition of NF-κB/p65 in cytoplasm was detected whereas 56%, 60% and 64% inhibition of NF-κB/p65 was observed in the nucleus (Fig. 3B). Similar results were observed after GTP treatment for NF-κB/p50 where cells treated with the 60 μg/ml GTP completely abolished its expression in both cytoplasmic and nuclear fraction, respectively (Fig. 3B). At doses of 20 and 40 μg/ml of GTP, 20% and 40% inhibition of NF-κB/p50 was detected in cytoplasm whereas 30% and 60% inhibition was observed in the nucleus (Fig. 3B).

GTP treatment inhibits NF-κB activity by inhibiting the phosphorylation of IκB-α in human osteosarcoma SAOS-2 cells

Next, we examined the cytoplasmic levels of total and phosphorylated IκB-α after GTP treatment. As shown in Fig. 4A, treatment of SAOS-2 cells with GTP showed no effect on the protein expression of IκB-α but inhibited its phosphorylation. Compared with vehicle control, 10%, 30%, 69% and 98% inhibition in the cytoplasmic level of phospo-IκB-α was observed at 10, 20, 40 and 60 μg/ml doses of GTP, respectively. These results suggest that GTP inhibits NF-κB activation *via* inhibition of phosphorylation of IκB-α.

GTP treatment inhibited IKK-α and IKK-β in human osteosarcoma SAOS-2 cells

Next, we studied the effect of GTP treatment on IKK expression, the upstream kinase involved in phosphorylation of IκB-α. As shown in Fig. 4B, GTP treatment of SAOS-2 cells at a dose of 60 μg/ml completely inhibited both IKK-α and IKK-β protein expression. However, at a dose of 40 μg/ml, only partial inhibition of IKK-α (69%) and IKK-β (58%) expression was observed in these cells.

GTP treatment induces apoptosis through modulation in Bax/Bcl-2 ratio in human osteosarcoma SAOS-2 cells

Antiapoptotic protein Bcl-2 has been associated with cell survival and inhibits programmed cell death, whereas increase in proapoptotic protein Bax results in apoptosis (Vermeulen et

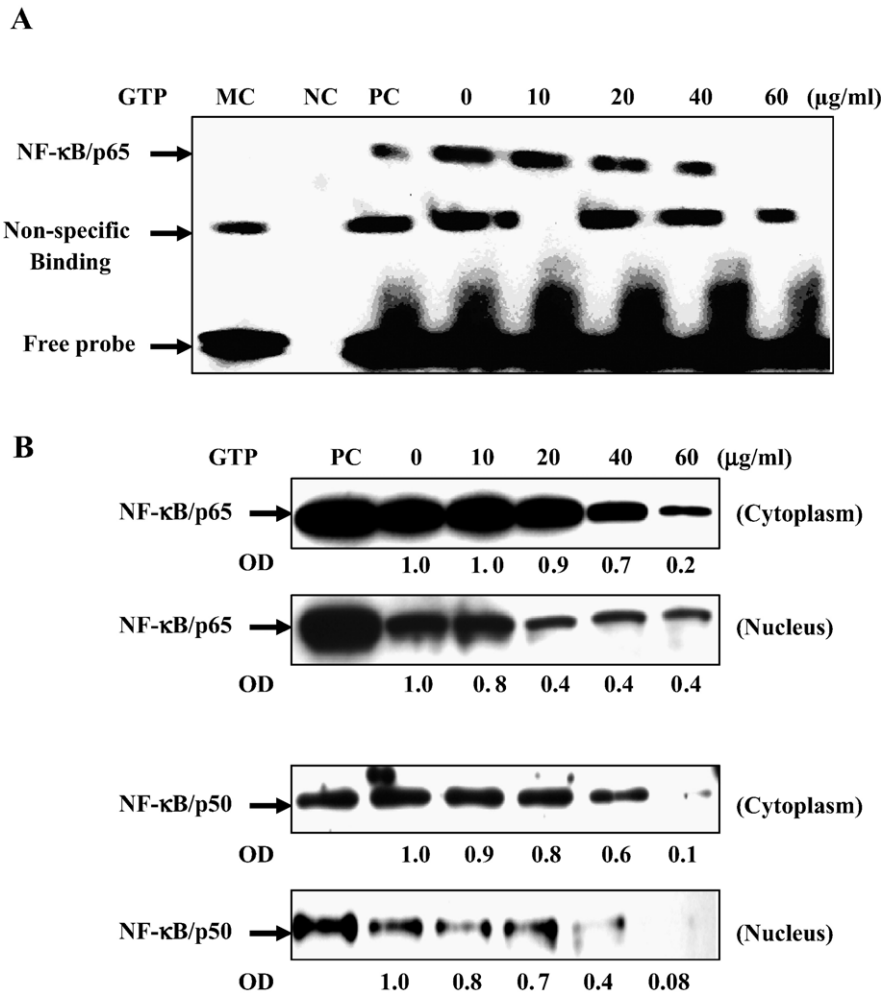

Fig. 3. Effect of GTP on NF-κB activity in human osteosarcoma SAOS-2 cells. (A) NF-κB DNA binding activity, as performed by EMSA. The cells were treated with indicated doses of GTP, and 10 μg of nuclear protein was incubated with biotinylated NF-κB oligonucleotides (MC, mutated control; NC, negative control where NF-κB oligos were run without nuclear lysate and PC represents kit supplied positive control). (B) Cytoplasmic and nuclear expression of NF-κB/p65 and NF-κB/p50. The cells were treated with indicated doses of GTP for 24 h, nuclear and cytoplasmic fractions were prepared as described in Materials and methods, and the blots were probed with an antibody specific for NF-κB/p65 or NF-κB/p50 isoforms. Data shown below the blots represent fold modulation in the protein expression normalized to β-actin or β-tubulin. The results shown here are representative of three independent experiments with similar results (PC HeLa cells stimulated with TNF-α were used as positive control).

al., 2002; Pahl, 1999). As determined by immunoblot analysis, treatment of SAOS-2 cells with GTP resulted in a dose-dependent increase in the protein expression of Bax after 24 h of treatment, as shown by the intensity of the bands shown below the blot (Fig. 5A). The protein expression of Bcl-2 was correspondingly downregulated with increasing concentration of GTP treatment. It has been suggested that the ratio of Bax/Bcl-2 protein expression plays a determinant role in transducing the apoptotic signal (Baliga et al., 2005). As shown in Fig. 5A, the ratio of Bax/Bcl-2 was increased in favor of apoptosis.

GTP treatment activates caspases in human osteosarcoma SAOS-2 cells

We next performed immunoblot analysis for the detection of caspases in SAOS-2 cells. As shown in Fig. 5B, GTP treatment of SAOS-2 cells resulted in a dose-dependent increase in the level of cleaved caspase-3 (17 kDa), with concomitant decrease in the 32 kDa pro-form. Similarly, a dose-dependent increase in the active form of caspase-8 (18 kDa) was observed after GTP treatment compared to untreated control group.

Next, we investigated whether inhibition of caspases inhibits GTP-induced apoptosis in SAOS-2 cells. For these studies, cells were pretreated with the specific caspase-3 inhibitor DEVD-CHO (0. 5 μM) for 2 h followed by GTP treatment. As shown in Fig. 6, GTP-mediated apoptosis was blocked in cells pretreated with DEVD-CHO. Similar results were obtained when cells were pretreated with pan-caspase inhibitor Z-VAD-FMK (20 μM) for 2 h. These results indicated that the activation of caspases is a requirement for the GTP-mediated apoptosis in human osteosarcoma SAOS-2 cells.

Discussion

In this study, we have demonstrated the anti-proliferative effects of GTP against human osteosarcoma SAOS-2 cells.

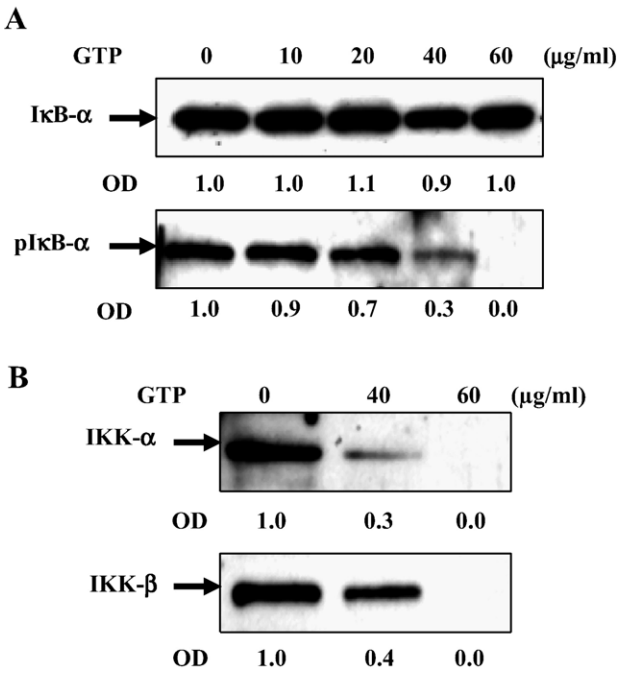

Fig. 4. Effect of GTP on IκB and IKK kinases in human osteosarcoma SAOS-2 cells. (A) Total IκB-α and phospo-IκB-α protein expression and (B) IKK-α and IKK-β expression in cells treated with specified concentrations of GTP for 24 h and subjected to gel electrophoresis followed by immunoblot analysis and chemiluminescence detection as described in Materials and methods. Data shown below the blots represent fold modulation in the protein expression normalized to β-actin. The results shown here are representative of three independent experiments with similar results.

Recent studies indicate that green tea polyphenols exert inhibitory effects on the activity of several enzymatic and metabolic pathways of relevance to the development and progression of cancer (Cooper et al., 2005b; Crespy and Williamson, 2004; Ahmad et al., 2000; Chen et al., 1998; Siddiqui et al., 2004; Nakazato et al., 2005; Baliga et al., 2005; Liberto and Cobrinik, 2000; Qanungo et al., 2005; Annabi et al., 2002; Roy et al., 2003; Singh et al., 2002). These dietary polyphenols, by virtue of their ability to selectively induce apoptosis in cancer cells and not in non-cancer cells, represent a potential agent for further development as therapeutics. Several studies in the past have reported that green tea polyphenols induce apoptosis in various cancer cells in vitro and in in vivo animal models (Cooper et al., 2005a, 2005b; Crespy and Williamson, 2004). Apoptosis, a discrete way of cell death, is different from necrotic cell death, and its induction is regarded as an ideal strategy to eliminate damaged cells (Brown and Attardi, 2005; Sun et al., 2004). Agents which can modulate apoptosis may be able to affect the steady-state cell population and can be useful in the management and therapy of cancer. In recent years, some plant-derived dietary agents have shown to inhibit cell growth and induce apoptosis in numerous cancer cell types (Surh, 2003). Here, for the first time, we investigated whether GTP has ability to impart antiproliferative effects on osteosarcoma cells and its molecular mechanism using SAOS-2 cells. Our data demonstrated that GTP was effective in inhibiting the growth and causing apoptosis of SAOS-2 cells.

The NF-κB signal transduction pathway has been implicated in several solid tumors and other hematological malignancies (Sovak et al., 1997; Lind et al., 2001; Bell et al., 2003; Mukhopadhyay et al., 1995; Wang et al., 1999; Nair et al., 2003; Tselepis et al., 2002; Suh et al., 2002). Aberrant NF-κB activation has been demonstrated to offer resistance to numerous solid tumors against a variety of anticancer agents (Pahl, 1999; Yamamoto and Gaynor, 2001). Several findings have shown that NF-κB activation provides cell survival signal and protects malignant cells from apoptosis (Arlt et al., 2003; Haefner, 2002). It was shown that inhibition of NF-κB activity in carcinoma cell lines could dramatically reduce cell growth and metastatic properties in vivo (Huang et al., 2001). In addition, inhibition of NF-κB activity by dominant negative approach results in reversion of malignancy in human osteosarcoma cells (Andela et al., 2002). We show that GTP effectively decreased NF-κB DNA binding activity and its nuclear translocation, which correlated with growth inhibition and apoptosis in human osteosarcoma SAOS-2 cells. Studies have shown that NF-κB activation was induced by various agents including TNF, H_2O_2, PMA, hypoxia and growth factors (Karin and Greten, 2005). In response to most of these stimuli, NF-κB activation proceeds sequentially through activation of IKK, phosphorylation at serines 32 and 36 of IκB-α ubiquitination and finally degradation of IκB-α and the release of NF-κB (Gilmore, 2003; Karin and Greten, 2005). This in turn activates a number of survival genes that are regulated by NF-κB, including genes encoding Bcl-2 like proteins (Bcl-2, Bcl-$_{XL}$, Nr13), inhibitors of apoptosis proteins (cIAP1, cIAP2) and others like PAR4 (Gilmore, 2003; Vermeulen et al., 2002; Pahl, 1999). Our study indicates that GTP inhibits NF-κB activation by suppressing IKK activation and increasing phosphorylation of IκB-α in SAOS-2 cells. This effect mediates a decrease in Bcl-2 protein expression and an increase in the levels of Bax, shifting the Bax/Bcl-2 ratio in favor of apoptosis. This inhibition of NF-κB proapoptotic and antiproliferative activities by green tea polyphenols could provide specific and causative links to inhibition of osteosarcoma cell growth and survival.

Caspases are part of a family of cysteine proteases that have been shown to be involved in many forms of apoptosis (Lavrik et al., 2005). Previously, our laboratory has shown that activation of caspases was associated with the induction of apoptosis in chondrosarcoma cells (Islam et al., 2000). The downstream protease caspase-3 has been defined as a key executioner involved in the apoptosis induction by various stimuli (Lavrik et al., 2005; Islam et al., 2000). In our study, GTP activated both the caspase *viz*. caspase-3 and 8. Furthermore, treatment of SAOS-2 cells with specific caspase-3 inhibitor DEVD-CHO and general caspase inhibitor Z-DEVD-FMK rescued SAOS-2 cell from GTP-induced apoptosis, indicating that activation of caspases correlated with induction of apoptosis. In addition, it has been demonstrated that suppression of NF-κB activation by caspase inhibitors could block apoptosis in malignant cells (Neuzil et al., 2001). These findings point to a critical role of NF-κB and an essential role of caspases in apoptotic cell death. Recently, it has been shown that epigallocatechin-3-gallate, the major

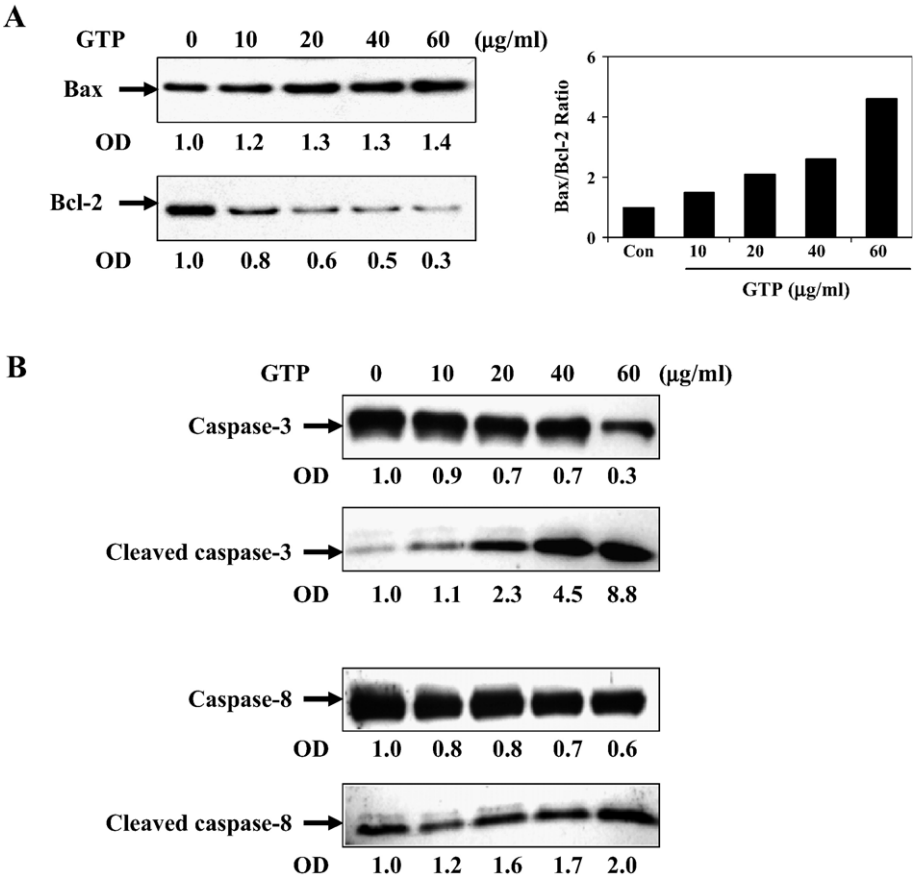

Fig. 5. Effect of GTP on the protein expression of (A) Bax, Bcl-2 and their ratio and (B) caspase-3 and caspase-8 in human osteosarcoma SAOS-2 cells. The cells were treated with specified concentrations of GTP for 24 h and subjected to gel electrophoresis followed by immunoblot analysis and chemiluminescence detection as described in Materials and methods. Data shown below the blots represent fold modulation in the protein expression normalized to β-actin. Data shown are representative of three independent experiments with similar results.

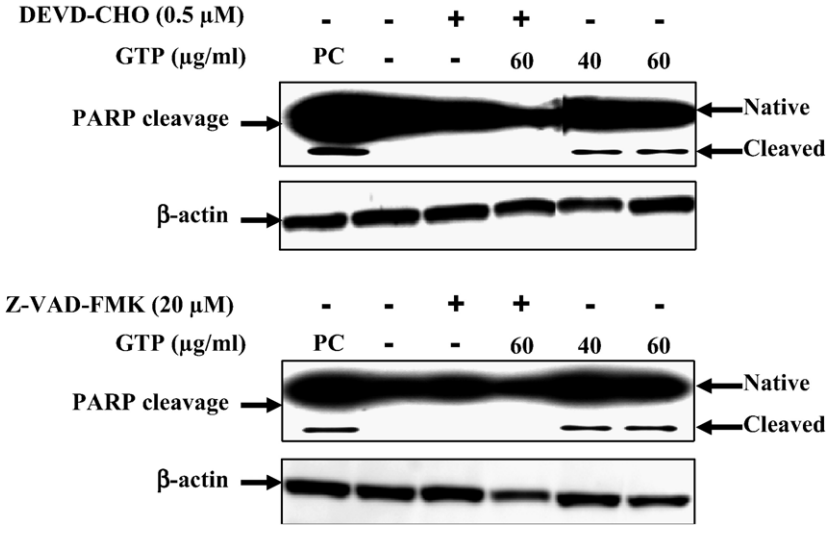

Fig. 6. Effect of caspase inhibitors on GTP-induced PARP cleavage in human osteosarcoma SAOS-2 cells. The cells were pretreated with caspase-3 inhibitor DEVD-CHO at a dose 0. 5 μM and with general caspase inhibitor Z-VAD-FMK for 2 h at a dose 20 μM (lane 3) and post-treated with GTP (60 μg/ml) for 24 h (lane 4). After treatment, cell lysates were subjected to gel electrophoresis followed by immunoblot analysis and chemiluminescence detection as described in Materials and methods. PC represents company supplied positive control which is a total cell lysate of HUT Human lymphoma cell line (Lane 1).The data shown here are representative of three independent experiments with similar results.

polyphenolic constituent of green tea, causes activation of caspases which is involved in the depletion of NF-κB/p65 subunit in cytoplasm (Gupta et al., 2004). The observed inhibition of NF-κB/p65 and NF-κB/p50 protein expression in the cytosolic fraction after GTP treatment and the presence of active caspases indicate that the depletion of NF-κB may lead to the loss of transactivation domains, thereby driving the SAOS-2 cells towards apoptosis. However, more detailed studies are needed to prove these findings.

Plant-derived anticancer agents have provided novel prototypes for drug design. The concentration of green tea polyphenols used to induce apoptosis in our studies may not be physiologically achievable in humans. Because concentrations that induce antitumor effects in vitro and in vivo often differ, further experiments are needed to delineate these findings in preclinical models of osteosarcoma. In conclusion, novel findings of this study are that green tea polyphenols are potent inducers of apoptosis in human osteosarcoma SAOS-2 cells via activation of caspase-3 and inhibition of NF-κB. Based on these findings, it is tempting to suggest that use of green tea polyphenols alone or in combination with other conventional chemotherapeutics may be an improved strategy for treatment of osteosarcoma.

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