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The Effect of TNFα on the PGE_2-Induced Apoptosis in Gastric Carcinoma Cell

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PGE_2에 의한 위암세포의 세포자연사(Apoptosis) 유도과정에서의 TNFα의 영향

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박민선 · 민병례 · 김규찬[1] · 남명진[1]

Prostaglandin E_2 (PGE_2)가 위암세포주인 AGS의 성장에 미치는 영향을 관찰하고, 이때 확인된 성장억제가 세포자연사에 의한 것인가를 알아보았다. 또한 TNFα의 처리가 PGE_2의 세포성장억제 효과를 촉진시키는 사실이 TNFα에 의한 arachidonic acid (AA) metabolism의 활성화와 연관성이 있는가를 증명하고자 하였다.

AGS 세포주에 다양한 농도의 PGE_2를 첨가한 후 시간별 세포의 성장을 MTT assay로 측정하였고, 위상차 현미경으로 세포모양을 관찰하였다. 세포자연사는 DNA fragmentation과 RT-PCR에 의한 *c-myc* 유전자의 발현변화로 확인하였다. 한편 TNFα에 의한 AA metabolism의 변화는 [^{14}C]-AA로 표지된 AGS에 TNFα를 처리하고 시간별로 배양한 후 방출되는 AA metabolite의 방사능을 측정하였다.

AGS 세포성장은 PGE_2 처리농도와 시간에 비례하여 억제되었고, 농도가 높아질수록 세포간 접착력이 소실되어 바닥으로부터 분리되었다. 10 μg/ml의 농도로 처리하였을 때 48시간 후부터 DNA ladder가 확인되었으며, 처리시간에 비례하여 *c-myc*의 발현이 증가되는 것을 확인하였다. 특히 AGS 세포는 PGE_2를 단독으로 처리했을 때보다 TNFα와 같이 처리하였을 때 성장이 더욱 억제되었고, TNFα를 처리한 AGS 세포는 대조군에 비하여 AA metabolite를 더 많이 방출하였다.

PGE_2에 의한 AGS 세포의 성장 억제 효과는 세포자연사에 의한 것으로 사료되며, 이때 수반하여 나타나는 *c-myc*의 시간별 발현 증가 역시 세포자연사와 관련이 있는 것으로 추정된다. 특히 PGE_2를 단독으로 처리했을 때보다 TNFα를 같이 처리하였을 때 세포의 성장이 더욱 억제되었는데, AGS 세포에서도 TNFα에 의하여 AA metabolite의 방출이 촉진되는 것으로 확인되었기 때문에 이때 생성된 AA metabolite가 exogenous PGE_2의 성장억제효과를 촉진한 것으로 추정된다.

Key Words: AGS cell, Apoptosis, [^{14}C]-Arachidonic acid, Prostaglandin E_2, TNFα

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INTRODUCTION

Eicosanoids are important autacoids that are known to regulate a wide range of physiological processes in gastrointestinal epithelia including the secretion of fluid and electrolytes, mucosal blood flow and cell proliferation.[1,2] Many eicosanoids have been shown to affect the growth of tumor cell lines. For example, certain members of the prostaglandin(PG)A_2, PGE_2 and PGD_2 series (including Δ^{12}-PGJ_2, the ultimate metabolite of PGD_2) are potent inhibitors of growth for certain culture tumor cells.[3~5] These PGs exert their cytotoxic effects via endogenous cytotoxic pathway in L1210 murine leukemia cells.[6] But the exact mechanism by which these PGs initiate the cellular events resulting in cell death is not fully understood. PG of the E series plays an important role in the control of the immune response in normal and pathological conditions.[7] For example, PGE_2 is known to down-regulate selected immune function among which T cell mitogenesis and IL-2 production.[8] And PGE_2, as well as other intracellular cAMP elevation agents, have been shown to introduce apoptosis in B lymphocytes[9] and thymocytes.[10] And Nakamura *et al*. also showed significant suppressive effect on the growth of the human gastric cancer cell line, KATOIII.[11]

Tumor necrosis factor α (TNFα) is a 157- amino acid-long polypeptide with a molecular mass of 17,000.[12,13] Besides macrophages, it is produced by a wide variety of other cell types in response to highly diverse stimuli, and especially plays pivotal roles in triggering and reinforcing inflammatory responses. Indeed, TNFα can act on monocytes/macrophages themselves to further induce the production of other inflammatory cytokines such as IL-1, IL-6 and IL-8.[14,15] TNFα also induces the production of prostaglandins and oxygen radicals in monocytes/macrophages.[16,17] Zhang and Dziak reported that high concentration of TNFα (10^{-8} M) significantly increased the cyclooxygenase pathway, with PGE_2 being a major product.[18]

In this study, we found out the antiproliferative effect of PGE_2 on AGS, human gastric cancer cell line via apoptosis and TNFα can augment the growth inhibitory effect even on gastric cancer cell through enhancing cyclooxygenase pathway.

MATERIALS AND METHODS

1) Chemicals

PGE_2 was purchased from Sigma Chemical Co. (St. Louis, Mo) and dissolved in 100% ethanol and diluted to the appropriate concentrations at the time of use. Recombinant human TNFα was purchased from R & D system Inc. (Minneapolis, MN) and all other reagents were of analytical grade. [^{14}C]-Arachidonic acid (40~60 mCi/mmol) was purchased from NEN (Life science Products, Inc., Boston, MA).

2) Cell culture

AGS, human gastric adenocarcinama was purchased from America Type Culture Collection (Rockvill, MD). The cells were maintained in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal bovin serum (Gibco BRL), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Gibco BRL) at 37°C and in humidified air with CO_2.

3) Assay of antiproliferative activity

To examine the effect of PGE_2 on the AGS cell growth, colorimetric MTT assay was performed as described before.[19] AGS cells were seeded at 10^4 cells/well into 96-well plates (Corning, Cambridge, MA) and allowed to adhere overnight and then PGE_2 was added. After incubated for the indicated times, removed the medium and added 200 μl of fresh medium per well. 50 μl MTT in PBS (5 mg/ml) was added immediately, the plate was incubated for 4h at 37°C, wrapped in foil. Medium containing MTT was removed and cell were lysed with 200 μl of dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO) per well.

The absorbency was read at 570 nm on an ELISA reader (SPECTRA MAX250, Molecular Devices Corp. Sunnyvale, CA). To compare the effects of PGE_2 with that of co-treatment with TNFα or that of TNFα-treatment only, cells were plated at a density of 10^4 cells/well in 96-well culture plates and incubated with PGE_2 and/or TNFα. The number of viable cells was counted daily by trypan blue exclusion tests.

4) DNA fragmentation analysis by agarose gel

After treatment with PGE_2, cells ($\geq 5\times10^5$) were centrifuged, washed with PBS two times, and resuspended in 0.5 ml of the lysis buffer (10mM Tris-HCl pH 8.0, 100 mM NaCl, 25 mM EDTA and 0.5% SDS containing 20 μg RNase A). After incubation at 50°C for 1 h, 5 μl of 20 mg/ml proteinase K was added and incubated overnight at 50°C. Samples were extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (v/v/ v, 25 : 24 : 1). DNA was precipitated overnightin 2 volume of ethanol, 1/10 volume of sodium acetate and centrifuged at 12,000 rpm for 20 min. A DNA pellet was washed with 70% ethanol and resuspended with TE buffer. DNA samples were separated on 1.5% agarose gel and visualized under UV using ethidium bromide.

5) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted according to the method of Chomczynski and Sacchi using TRI Reagent (Molecular Research Center, Inc., Cincinnati. OH).[20] cDNA was prepared by reverse transcription at 37°C, 60 min in a 25 μl reaction mixture containing 2 μg total RNA, 5 μg oligo $(dT)_{15}$, 1 mM dNTPs, 5 μl 5× reverse-transcriptase (RT) buffer, 40 U RNasin (Promega, Madison, WI) and 25 U MMLV-RT (Promega). 2 μl of cDNA was amplified by PCR in the presence of 4 μM of each primer, 10×buffer, 0.2 mM dNTPs and 0.5 U Taq polymerase. The mixture was amplified in a Perkin-Elmer thermal cycler (GeneAmp PCR System 9600) with PCR cycle. 5 μl of PCR product was electrophoresed in a 1.5% agarose gel in Tris/boric acid/EDTA buffer. To identify the mRNA for *c-myc* and GAPDH in AGS cells, exact primers were synthesized. The *c-myc* primers were left: 5'-GGTCAGCGTCTGG-ATCACCTTC-3' and right: 5'AGGATGTATGCT-GTGGCTTTTT-3', giving rise to a 406 bp PCR product. The GAPDH primers were left: 5'-CCTC-TGACTTCAACAGCGAC-3' and right: 5'-ACA-TGACAAGGTGCGGCTCC-3', giving rise to a 500 bp PCR product. PCR was performed for 25 cycles, using a cycling program of 94°C for 30 sec, 60°C for 1 min and 72°C for 2 min.

6) [^{14}C]-Arachidonic acid (AA) release

In order to incorporate [^{14}C]-AA release into cellular lipids, AGS confluent monolayers were trypsinized and 7×10^4 cells/well were placed in 24-well tissue culture plates. [^{14}C]-AA (0.1 μ Ci/7×10^4 cells)was added to the well, and the cells were incubated overnight. The cells were then washed 3 times with media containing 0.1% fatty acid-free bovine serum albumin and treated with RPMI 1640 containing 10% FBS alone or with RPMI 1640 containing 10% FBS and TNFα (50 ng/ml) for the experimental time period. After incubation, the medium was decanted into tubes and cells were dissolved in 0.5 M NaCl. Both fractions were assayed for radioactivity in a beta counter after the addition of Cocktail solution.

RESULTS

To determine whether PGE_2 can affect gastric cancer cell growth, AGS cell were incubated with various concentrations of PGE_2 (up to 50 μg/ml) for 72 h. The results shown in Fig. 1. indicated that cell growth was inhibited by PGE_2 in a time- and dose-dependent manner as determined by the MTT assay. When AGS cells were incubated with ≥ 12.5 μg/ml of PGE_2, the cells began to lose their viability after 36 h, and this growth inhibitory effect was persisted up to 72 h. In the examination of the various dose of PGE_2, dramatic changes of morphology were observed. With the increase of the dose of PGE_2, loss of cell

Fig. 1. The effect of PGE_2 on proliferation and the viability of AGS cells. Exponentially growing cells in cultures containing 10% FBS were untreated (◆) or treated with 5 μg/ml (■), 12.5 μg/ml (▲), 25 μg/ml (□) and 50 μg/ ml (○) PGE_2. The OD value was measured in a triplicate culture.

adhesive ability was increased and membrane blebbing was observed (data not shown).

We then examined DNA integrity of AGS cells by agarose gel electrophresis (Fig. 2). Even though a relatively low dose of PGE_2 (10 μg/ml) was used, cellular DNA was cleaved into multiples of 180 bp. DNA ladder, the hallmark of apoptosis, began to appear after PGE_2 treatment for 48 h and this phenomenon was much more marked after 72 h incubation.

As growth inhibition by PGE_2 was due to apoptosis, an apoptosis-relative gene, *c-myc* expression in the AGS cells following PGE_2 treatment was examined. RT-PCR analysis demonstrated that *c- myc* level began to increase at 24 h and remained high for up to 48 h after treatment (Fig. 3). As the expression of GAPDH gene, the internal control, was not changed with PGE_2 treatment, we confirmed that *c-myc* mRNA was induced by PGE_2 treatment.

To investigate the effects of TNFα on the PGE_2 actions to AGS cells, TNFα was added at various times with/without PGE_2 on AGS cells (Fig. 4). In our previous experiments, AGS cells showed a cytotoxic response to TNFα at a concentration of 50 ng/ml. Although the single treatment of PGE_2 or TNFα did not affect cell growth at an early time

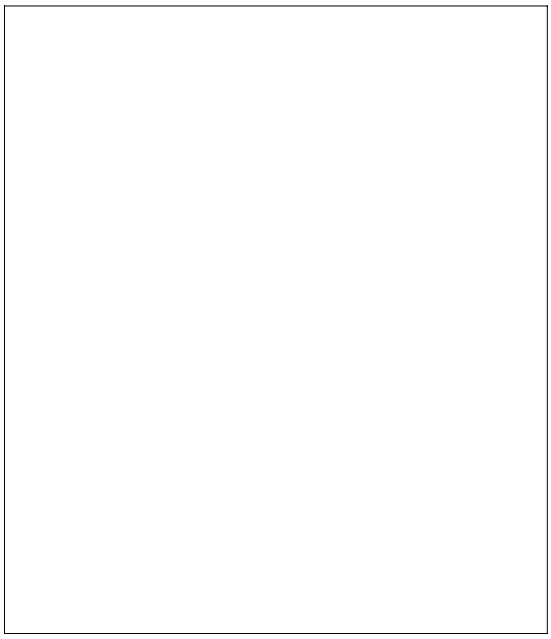

Fig. 2. Electrophoretic analysis of DNA from PGE_2 (10 μg/ml) treated AGS cells. DNA was analyzed on a 1.5% agarose gel and stained with ethidium bromide. Cells were incubated without (lane 1) or with PGE_2 for 6, 12, 24, 48, 72 h (lane 2-6, respectively). Lane M represents the 123 bp DNA size marker.

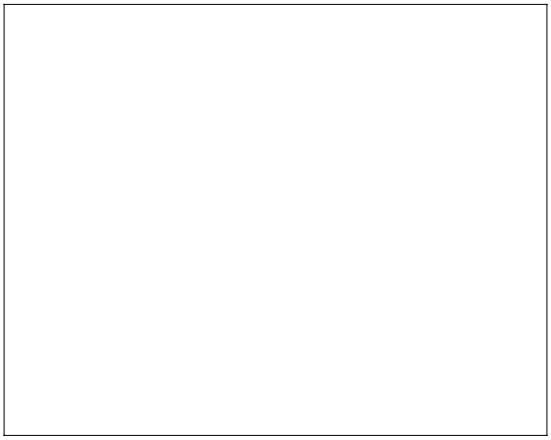

Fig. 3. RT-PCR analysis of *c-myc* gene expression in AGS cells. PCR products loaded in the lanes were as follows: M, DNA size marker; 1, untreated control cells; 2, 6 h treatment; 3, 12 h treatment; 4, 24 h treatment; 5, 36h treatment; 6, 48 h treatment of 10 μg/ml PGE_2.

Table 1. Effect of TNFα on the release of [^{14}C]-arachidonic acid from AGS cells

Treatment	[^{14}C]-Arachidonic acid release (% of total [^{14}C])*			
	12 h	24 h	36 h	48 h
TNFα treated cells	25.23±2.7	21.89±1.20	31.42±1.7	49.03±4.40
Control cells	19.55±6.16	18.03±1.63	25.64±3.48	49.61±2.15

*Values are the mean±SEM of three replicates.

Fig. 4. The effect of co-treatment of PGE_2 and TNFα on AGS cell growth. Exponentially growing cells in cultures containing 10% FBS were untreated or treated with 10 μg/ml PGE_2 only and/or 50 ng/ml TNFα. Single treatment of TNFα (50 ng/ml) was also performed. AGS cells were tested in a 96-well microplate in triplicate for the indicated times and the number of viable cell was counted after trypan blue staining.

(up to 24 h), combination treatment began toinhibit cell growth significantly at this time, and after 48 h, the growth inhibitory effects of the combination treatment overwhelmed control and single treatments. One of the possible hypotheses responsible for this phenomenon is the regulation of arachidonic acid release and PGE_2 production by TNFα. We then measured the quantities of [^{14}C]-AA metabolites released from [^{14}C]-pre-labled AGS cells stimulated by TNFα (Table 1). Stimulation of AGS cells with TNFα (50 ng/ml) increased the rate of [^{14}C]-AA release over that of unstiulated control cells. The effect of TNFα was detectable within 12 h and continued during the 36 h of the assay period. The slow decline in [^{14}C]-AA after 36 h in our study is probably due to a re-uptake of fatty acid by the cells.

DISCUSSION

During the last decade, significant advances have been made in understanding the role of eicosanoids as physiological modulators and therapeutic agents in the gastrointestinal tract. However, substantial inconsistencies exist between studies evaluating the physiological role of eicosnoids in the gastrointestinal tract. Also, the regulatory control of arachidonic acid metabolism, including eicosanoid receptor biology and coupled intracellular signal transduction pathway, has not been fully characterized. Since the initial observation that some PGs inhibit the growth of certain tumor cells, their antiproliferative effects have been confirmed in a variety of cultured cells.[21,22] Among the various PGs, we demonstrated that AGS cells incubated with a cytotoxic dose of PGE_2 showed signs of apoptosis, indicating that cytotoxicity induced by PGE_2 is mediated via an endogenous apoptotic pathway. This phenomenon was confirmed by observing DNA fragmentation in agarose gel and *c-myc* mRNA expression from RT-PCR, which are typical features of apoptosis. Possible intracellular signaling mechanisms in the initiation of apoptosis include the influx of calcium ions[23] and the altered expression of oncogenes *c-fos* and *c-myc*.[24] Little is known, however, of the intra-cellular effector mechanisms in apoptosis and, in

particular, of the regulation of DNA cleavage. In this study, a relatively low dose of PGE_2 can cleave cellular DNA into a 180 bp-sized ladder, and along the incubation time, *c-myc* expression was increased. Other investigators using tumor cell lines reported similar responses to exogenous PGE_2.[25,26] It has been proposed that the antitumor activities of PGE_2 are exclusively due to its dehydration product, PGA_2, which shares a cyclopentenone ring structure.[21] Santoro *et al.*[5] reported that a 74 kDa protein was induced by PGA_1, or PGJ_2 in human K562 erythroleukemia cells; the protein has been identified as a heat-shock protein, and its appearance was associated with changes in cell proliferation.

Consideration that TNFα inhibited the AGS cell growth in our previous report,[27] co-treatment may show a different effect compared with the single treatment of TNFα or PGE_2. The result of the co-treatment of PGE_2 and TNFα on AGS cells showed a synergistic effect on cell growth. Pelus and Brockman[28] reported that macrophages from tumor-bearing animals have a markedly augmented capacity to metabilize arachidonic acid. Mori *et al.*[29] reported that a addictive antiproliferative effect of TNFα and Δ^{12}-PGJ_2 appeared in human gynecologic tumor cell lines, but there was no synergistic interaction between TNFα and PGA_2 in the same cell lines. An especially relatively high concentration of TNFα (10^{-8} M) significantly increased the cyclooxygenase pathway, with PGE_2 being a major product. Our result also demonstrated that 50 ng/ml TNFα stimulates AGS cells to release arachidonic acid metabolites over that of unstimulated control cells. These released arachidonic acid metabolites could act as autocrine factors that inhibit the growth of AGS cells. These results suggest that a combined treatment with TNFα and PGE_2 could provide a new approach to obtaining increased responses in clinical trials.

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