Endogenous Hydrogen Sulfide Enhances Cell Proliferation of Human Gastric Cancer AGS Cells

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Hydrogen sulfide (H$_2$S), the third gasotransmitter, is endogenously generated by certain H$_2$S synthesizing enzymes, including cystathionine-$\gamma$-lyase (CSE) and cystathionine-$\beta$-synthase (CBS) from l-cysteine in the mammalian body. Several studies have shown that endogenous and exogenous H$_2$S affects the proliferation of cancer cells, although the effects of H$_2$S appear to vary with cell type, being either promotive or suppressive. In the present study, we determined whether endogenously formed H$_2$S regulates proliferation in human gastric cancer AGS cells. CSE, but not CBS, was expressed in AGS cells. CSE inhibitors, n-propargylglycine (PPG) and $\beta$-cyano-l-alanine (BCA), significantly suppressed the proliferation of AGS cells in a concentration-dependent manner. CSE inhibitors did not increase lactate dehydrogenase (LDH) release in the same concentration range. The inhibitory effects of PPG and BCA on cell proliferation were reversed by repetitive application of NaHS, a donor of H$_2$S. Interestingly, nuclear condensation and fragmentation were detected in AGS cells treated with PPG or BCA. These results suggest that endogenous H$_2$S produced by CSE may contribute to the proliferation of gastric cancer AGS cells, most probably through anti-apoptotic actions.

Key words hydrogen sulfide; cystathionine-$\gamma$-lyase (CSE); cell proliferation; gastric cancer AGS cell; anti-apoptotic effect

Materials and Methods

Chemicals DL-Propargylglycine (PPG) and $\beta$-cyano-l-alanine (BCA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), and NaHS was from Kishida Chemical (Osaka, Japan).

Cell Culture Human gastric adenocarcinoma AGS cells were purchased from Sumitomo Dainippon Pharma (Osaka, Japan) and cultured in glucose-containing RPMI-1640 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal calf serum (FCS; Thermo Electron, Melbourne, Australia), 100 U/mL penicillin and 100 $\mu$g/mL streptomycin (Gibco, Carlsbad, CA, U.S.A.) in a 5% CO$_2$ incubator at 37°C.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay AGS cells (2×10$^3$ cells/well) were seeded in 96-well plates in the culture medium mentioned above and cultured for one day. Then, CSE inhibitors were added and further cultured for 48 h. The cell number was determined by using a MTT cell proliferation kit (Cayman Chem., Ann Arbor, MI, U.S.A.). NaHS, an H$_2$S donor, at 1.5 mM was repetitively applied at 0, 12, 24 and 36 h after the addition of CSE inhibitors. The data are represented as the percentage of the cell numbers just before the addition of CSE inhibitors.

Lactate Dehydrogenase (LDH) Release Assay Forty eight hours after the addition of CSE inhibitors, the activity of LDH released from the cells into the culture medium was measured with an LDH cytotoxicity detection kit (TaKaRa Bio, Otsu, Japan). The activity of released LDH was standardized by the total amount of LDH in the cells, which was obtained by determining the LDH activity from the cells lysed with 1% Triton X-100 (Kishida Chemical). The data are represented as the percentage of the levels of LDH activity just

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Hoechst 33258 Staining for Detection of Apoptosis  AGS cells (1.5 × 10^5 cells) were seeded in 6-well plates and cultured for one day. After 48-h incubation with CSE inhibitors, cells were harvested with a cell scraper, fixed in phosphate buffered saline (PBS) containing 1% glutaraldehyde for 30 min at room temperature, and then, stained with Hoechst 33258 (Wako Pure Chemical Industries, Ltd.). A drop of the cell suspension was placed on a slide glass and covered with a coverslip. The nuclear condensation and fragmentation of the cells were morphologically evaluated under UV excitation light with an inverted fluorescence microscope (BX50; Olympus, Tokyo, Japan). The cells with nuclear condensation and fragmentation were counted, and the results are expressed as the percentage of total cells (the proportion of apoptotic cells).

Western Blotting  AGS cells (1.2 × 10^6 cells/dish) were seeded in 100 mm culture dishes and cultured for one day. The cells were lysed in sodium dodecyl sulfate (SDS) buffer (2% SDS, 62.5 mM Tris–HCl and 10% glycerol, pH 6.8). The protein sample was separated by electrophoresis on 12.5% SDS-polyacrylamide gels (Wako Pure Chemical Industries, Ltd.), and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.). Primary antibodies used in the present study were: rabbit anti-CSE polyclonal antibody (Sigma-Genosys/Sigma-Aldrich) against a peptide corresponding to the amino acid sequence, (C)80GGTNRYFRR89V, in rat CSE,14 mouse CBS monoclonal antibody (clone 3E1; Abnova Co., Taipei, Taiwan) and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (sc-25778; Santa Cruz Biotechnol., Santa Cruz, CA, U.S.A.). We used the polyclonal antibody against rat CSE in the present study, because we have accumulated evidence that this antibody specifically recognizes not only rat and mouse CSE,15,16 but also human CSE.17 On the other hand, we used the monoclonal antibody against human CBS, the specificity of which was confirmed in our recent study.17 After washing the primary antibodies, the membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies (Cell Signaling Technol., Beverly, MA, U.S.A.). Immunolabelled proteins were visualized by Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan). The protein sample of mouse liver homogenate was used as positive control for CSE and CBS.

Statistics  Data are represented as mean ± standard error...
of the mean (S.E.M.). Statistical significance was evaluated by ANOVA followed by Tukey’s test. Significance was set at a level of $p<0.05$.

RESULTS AND DISCUSSION

Protein expression of CSE, but not CBS, was detected in AGS cells (Fig. 1A). Cell proliferation of AGS cells for 48 h was suppressed by CSE inhibitors, DL-propargylglycine (PPG) and β-cyano-L-alanine (BCA), in a concentration-dependent manner (Figs. 1B, C). The suppressive effects of PPG and BCA were significant at 0.5–5 and 0.95–5 mM, respectively. CSE inhibitors in the same range of concentrations did not increase LDH release from the cells (Figs. 1D, E), indicating that these CSE inhibitors suppressed the cell proliferation without causing cell death. To determine whether endogenous H$_2$S synthesized by CSE actually contributes to the proliferation of AGS cells, effects of NaHS, an H$_2$S donor, on the inhibitory effects of PPG and BCA on proliferation were examined. Repetitive application of NaHS significantly reversed the suppression of proliferation by PPG and BCA (Fig. 2). Both PPG and BCA significantly increased proportion of apoptotic cells with nuclear condensation and fragmentation, as assessed by the Hoechst 33258 staining (Fig. 3).

Our data suggest that the proliferation of human gastric cancer AGS cells is enhanced by the endogenous H$_2$S synthesized by CSE, which exhibits anti-apoptotic activity. CBS is not considered responsible for H$_2$S production in AGS cells, since the protein expression of CBS was hardly detected in AGS cells (Fig. 1A). It has been reported that the increased H$_2$S production plays a crucial role in cell proliferation and the related angiogenesis in several types of cancer cell lines, such as colonic and ovarian cancers. In addition, there are many papers showing that endogenous or exogenous H$_2$S modulates cell proliferation and migration in gastric, oral, breast, pancreas, lung or prostate cancer and also leukemia, whereas, H$_2$S promotes and suppresses cancer growth in distinct cells. Thus, the effects of H$_2$S on cancers might vary with the tissues, conditions surrounding cancer, the amount of synthesized H$_2$S, and so on.

Our data suggest the involvement of anti-apoptotic effect of H$_2$S in the enhancement of cell proliferation via the CSE/H$_2$S pathway (Fig. 3). It has been reported that H$_2$S enhances the activity of nuclear factor kappaB (NF-κB) through the sulfhydration of the p65 subunit, leading to anti-apoptotic transcriptional activity. In addition, an independent group has reported the contribution of Ca$_3$2.2 T-type calcium channels in the H$_2$S-mediated proliferation of AGS cells, because we could not detect the T-type calcium channel-dependent currents in AGS cells by the whole cell patch clamp technique (data not shown). Further studies are needed to clarify the target molecules of H$_2$S for the H$_2$S-mediated proliferation in future.

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Conflict of Interest The authors declare no conflict of interest.

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