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ADENOSINE AS AN ENDOGENOUS MEDIATOR OF HYPOXIA FOR INDUCTION OF VASCULAR ENDOTHELIAL GROWTH FACTOR mRNA IN U-937 CELLS

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Summary Adenosine induced by hypoxia exerts various effects via different types of receptors. Recently, hypoxia was shown to be a strong inducer of vascular endothelial growth factor, a secreted endothelial cell specific mitogen. In this report, we studied on effects of adenosine on inducibility of VEGF and possible mediation of hypoxia for its induction in U-937 cells. Hypoxia induced expression of VEGF mRNA with an early peak at 1 hour. 5'-N-ethylcarboxamidoadenosine, an adenosine analog, strongly induced VEGF mRNA, which was inhibited by 3,7-dimethyl-1-propargylxanthine (DMPX), an A_2-antagonist. The hypoxic induction was inhibited by adenosine deaminase, 7-(β-hydroxyethyl)theophyline, a non-selective adenosine receptor antagonist and DMPX. These results suggest that the hypoxic induction of VEGF mRNA is mediated by adenosine via A_2-receptor in U-937 cells.

Adenosine, an endogenous nucleoside produced in many cells and tissues acts locally to yield a number of specific effects (1). Adenosine production is influenced by a variety of factors, but levels are primarily increased by oxygen deprivation or cellular ATP depletion (2). Primary effects of adenosine depend on the different types of receptors present within the tissues and cells(3). Adenosine receptors can be subdivided based on inhibitory (via A_1 subtype) or stimulatory (via A_2 subtype) effects on adenylyl cyclase activity (4). Mononuclear cells in the blood expresses both subtypes (5).

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Vascular endothelial growth factor (VEGF) is a secreted, endothelial cell specific growth factor (6) produced by a number of normal and tumor tissues and cultured cells (7,8). Recent reports provide evidences for the role of VEGF on tumor neovascularization in vivo (9). In addition, hypoxia is suggested to be a strong inducer of VEGF mRNA in tumor and normal tissues (10,11).

To elucidate mechanism of hypoxia sensing, we studied on involvement of adenosine in the induction of VEGF mRNA using U-937 cells, a lymphocytic cell line with VEGF expression(6).

Materials and Methods

Reagents. 5'-N-ethylcarboxamidoadenosine (NECA), 7-(β-hydroxyethyl)theophyline and 3,7-dimethyl-1-propargylxanthine (DMPX) were from Research Biochemicals Inc. Adenosine deaminase (ADA) was from Worthington Biochemical Corp. Reagents for cell culture were from Sigma.

Cell culture. U-937 DE-4 cells were obtained from RIKEN Cell Bank and grown as described (12) in tissue culture dishes with RPMI-1640 containing 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C under 5% CO_2. For hypoxic and drug treatments, cells were resuspended in the medium at a concentration of 1 x 10^6 cells/ml.

Hypoxic treatment. U-937 cells were resuspended in the medium preequilibrated by anoxic gas (95 % N_2 - 5% CO_2) at a concentration of 1 x 10^6 cells/ml and incubated at 37°C in a semi-sealed spinner flask (Hario) with the anoxic gas bubbled into the medium. In this system, PO_2 of the medium was maintained at around 40 mmHg throughout the experiment. PO_2 of 90mmHg was obtained by bubbling of the appropriate mixture of the anoxic gas and gas of 95% O_2-5%CO_2.

RNA isolation and first-strand DNA synthesis. Total RNA was prepared from the cells by the acid guanidinium method (13). First strand cDNA was prepared from 2 μg of polyadenylated RNA by incubation in 40 μl of aliquots containing 40 U of avian myeloblastosis virus reverse transcriptase(Life Sciences) and 100 pmol of oligo-dT primer for 1 h at 37°C.

Polymerase chain reaction(PCR) cloning, and sequencing. Complementary DNA corresponding to that of VEGF was obtained from first strand cDNA by PCR using an automated thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT). The primers for VEGF (14) were 5'-CAGGAATTCCGAAACCATGAAC (5' oligonucleotide) and 5'-CCGGGCAGGAGGAAGCTTATC (3' oligonucleotide). The PCR products were analyzed by agarose gel electrophoresis. Following digestion with restriction endonucleases, corresponding fragments were purified and cloned into pBluescript II (Stratagene). Sequencing was performed in both directions on double-stranded templates by the dideoxynucleotide chain-termination method using Sequenase kit (United States Biochemical).

Northern blot analysis. Fifteen μg of total RNA was denatured, electrophoresed in 1.3% agarose gels containing 17% formaldehyde, and transferred onto nylon sheets (Hybond N; Amersham Corp.). RNA was fixed by baking for 2 h at 80°C, followed by prehybridization for 2 h at 45°C in 50% formamide, 5X standard saline citrate (SSC; 1X is 150 mM sodium chloride, 15 mM sodium citrate), 5X Denhardt's solution (1X is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 1% SDS, and 100 μg/ml salmon sperm DNA. Hybridization was then performed with the addition of 25 ng random-primed [^{32}P]DNA probes prepared from corresponding cDNAs (0.6kb) using a commercial kit at the same temperature for 16 h. Washes were twice in 1X SSC, 0.1% SDS at 45°C and twice in 0.1X SSC, 0.1% SDS at 65°C. The filters were scanned and

analyzed by Bio-Imaging analyzer BAS2000 (Fujix, Japan). Quantitation of expressed mRNA was performed with compensation by the corresponding G3PD signals.

Results and Discussion

Time course of hypoxic induction of VEGF mRNA

Effect of hypoxia on VEGF mRNA induction in U-937 cells was analyzed by Northern blot analysis as shown in Fig.1. The major band of VEGF mRNA was detected at 3.9 kb. This band is identical to the one predicted by the major form of VEGF peptide produced by U-937 cells(6). As to the weaker band, seen at around 18S when the signal of the major band is strong, additional experiments are required to descriminate among alternatively spliced spieces(15) since the hybridization probes in our experiments were designed specifically to detect all VEGF mRNAs. Peak induction was obtained rapidly at 1 hour of hypoxic culture. The induction began to decline at 2 hour and returned to the basal level by 4 to 6 hours. It became even lower than the control level after prolonged hypoxia of 9 and 24 hours. Control cells were incubated in normoxic medium for 1 hour. Control incubation for up to 24 hours did not cause induction of the mRNA at any time point shown for hypoxic culture in fig.1(data not shown). Viability of the cells counted by trypan blue exclusion showed no significant changes throughout the hypoxic culture up to 24 hours. Peak induction at 1 hour was 3 to 4 folds of the control. These results suggest that hypoxia is a strong inducer of VEGF mRNA also in U-937 cells as has been shown in tumor and normal tissues(10,11). Time course of the hypoxic induction in U-937 cells rather resembles that in cardiac tissues(11). Although adenosine concentrations in the culture media were measured by enzyme immunoassay, the values were at very low range and their changes in the time course of hypoxic culture were not statistically significant.

Induction of VEGF mRNA by NECA in U-937 cells

Effects of NECA, an adenosine analog(16), and DMPX, an A_2-antagonist(17), on VEGF mRNA expression in U-937 cells were studied (Fig. 2). NECA was used because of its stability in the culture medium as compared with adenosine. NECA after 1 hour incubation induced VEGF mRNA in U-937 cells in a dose-dependent manner (Fig.2 A). Peak induction by 10^{-6} M NECA was 3 fold of the control, comparable to that of hypoxia. The increase of VEGF mRNA by 10^{-7} M NECA was inhibited by 10^{-7} and 10^{-6} M DMPX (Fig.2 B). Basal control level of VEGF mRNA was also reduced by 30% and 70% by 10^{-7} M and 10^{-6} M DMPX, respectively. Dibutyril cAMP (10^{-6} M) increased expression of the mRNA (Fig.2 C). These findings suggest that basal level of endogenous adenosine acts on U-937 cells to maintain basal expression of VEGF mRNA and exogenous adenosine upregulate VEGF mRNA via A_2-receptor mediated pathway. The increase of the mRNA by cAMP, consistent with the report by Claffey et al(18), support A_2-receptor mediation in that activation of A_2-receptor increases of cAMP as an intracellular messenger. Increase of the mRNA by 10^{-8} M A_2-antagonist might be from original nature of the drug. In addition, decay of VEGF mRNA was also estimated to be

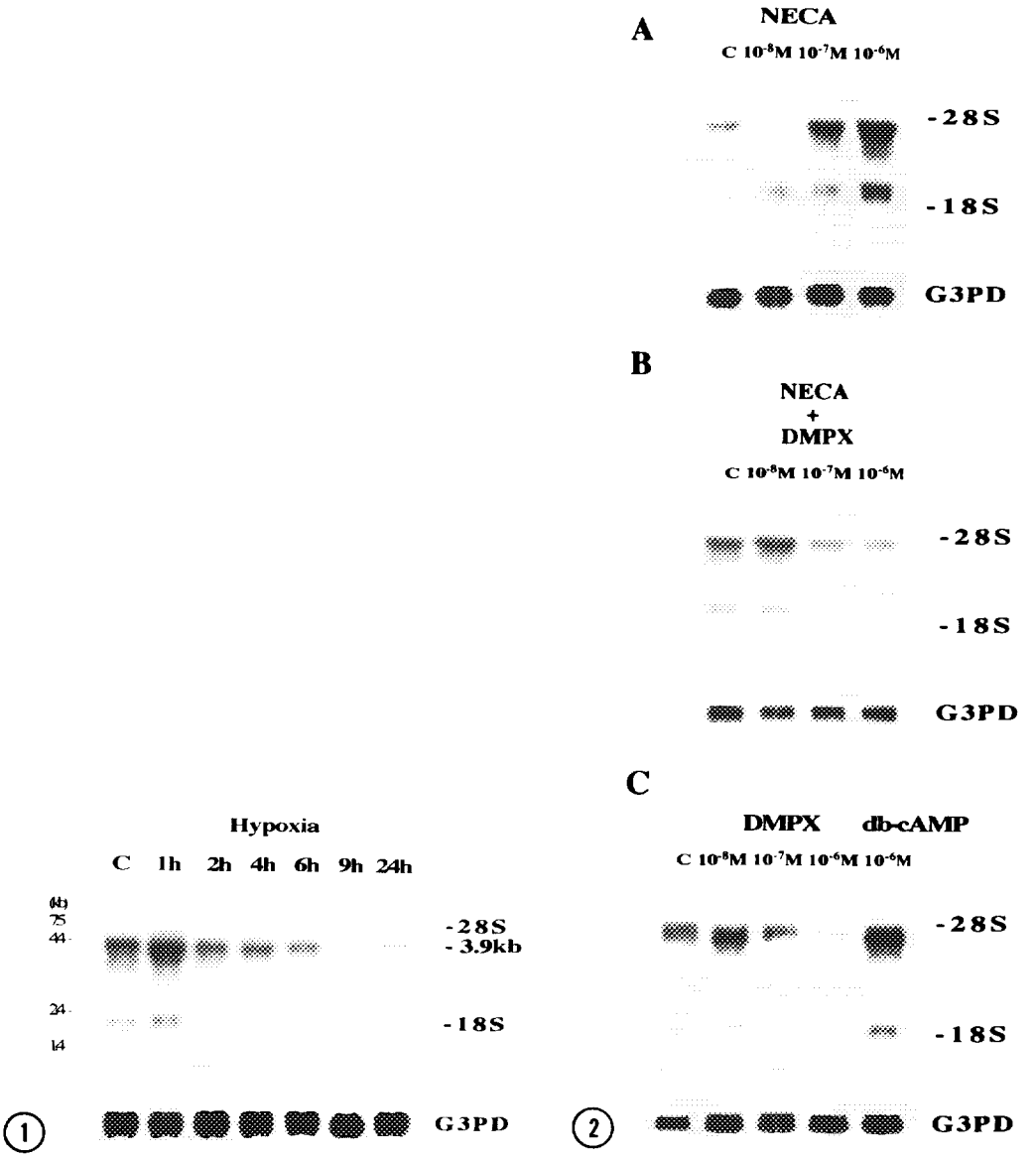

Fig. 1.Time course of hypoxic induction of VEGF mRNA in U-937 cells.
Cells at a concentration of $1x10^6$ cells/ml were incubated in hypoxic condition (PO_2 of the medium kept at 40 mmHg) as descibed in the Materials and Methods. Control cells were incubated in normoxic condition (pO_2 at 140 mmHg) for 1 hour. Cells were collected and reduced to Northern blot analysis.

Fig. 2. Induction of VEGF mRNA by NECA in U-937 cells.
A:Dose-dependent induction of VEGF mRNA by NECA in U-937 cells.
Cells at a concentration of $1x10^{-6}$ cells/ml were incubated for 1 hour in normoxic condition in the presense of NECA (10^{-8}, 10^{-7} and 10^{-6} M).
B:Inhibition of NECA-induced VEGF mRNA by DMPX.
Cells were incubated for 1 hour in the presense of 10^{-8}, 10^{-7} and 10^{-6} M DMPX in addition to NECA 10^{-7} M.
C:Decrease of basal level of VEGF mRNA by DMPX and induction by dibutyryl cAMP.
Cells were incubated for 1 hour in the presense of 10^{-8}, 10^{-7} and 10^{-6} M DMPX or 10^{-6} M dibutyryl cAMP.

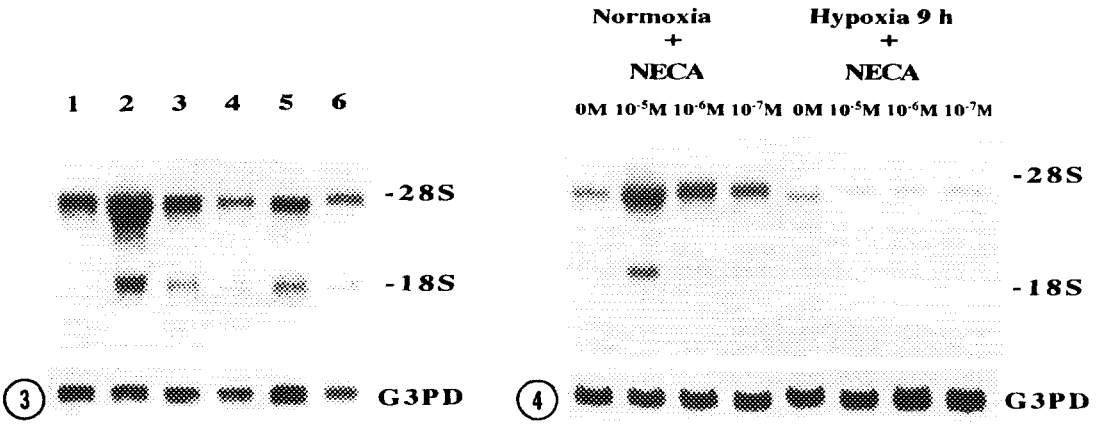

Fig. 3. Inhibition of hypoxia-induced VEGF mRNA by ADA and adenosine antagonists.
Cells were incubated for 1 hour in hypoxic condition (PO_2 at 40 mmHg) in the presence of ADA(25 U/ml), 7-(β-hydroxyethyl)theophyline(10^{-6} M) and DMPX(10^{-6} M). For mild hypoxic condition, medium with PO_2 at 90 mmHg were used as described in the Matetials and Methods. Lane 1: control, 2:hypoxia (pO_2=40mmHg), 3: hypoxia (pO_2=90mmHg), 4, 5 and 6: hypoxia (pO_2=40mmHg) in the presense of ADA (25U/ml), 7-(β- hydroxyethyl)theophyline (10^{-6}M) and DMPX (10^{-6}M), respectively.

Fig. 4. Desensitization of adenosine receptor mediated induction of VEGF mRNA in prolonged hypoxia.
After incubating in hypoxic condition for 9 hours, cells were collected and resuspended in normoxic medium containing 10^{-5}, 10^{-6}, 10^{-7} and 0 M NECA and incubated for 1 hour. Control cells were incubated in normoxic medium containing 10^{-5}, 10^{-6}, 10^{-7} and 0 M NECA for 1 hour.

rapid because the control level of the mRNA was reduced by 70 % in 1 hour by A_2-antagonist and the hypoxia-induced mRNA returned to the basal level within 3 hours.

Inhibition of hypoxic induction of VEGF mRNA by adenosine receptor antagonists

To investigate if adenosine produced by hypoxia acts directly on VEGF mRNA induction, ADA, 7-(β-hydroxyethyl)theophyline, a non-selective adenosine receptor antagonist(19) and DMPX were used in the hypoxic culture(fig.3). All of the drugs inhibited the induction by 1 hour of hypoxia below the control level. There was no significant difference among the VEGF mRNA levels inhibited by the three drugs(fig.3, lane 4-6). These results suggest that endogenous adenosine produced by hypoxia directly induces VEGF mRNA in U-937 cells. Threshold of extent of hypoxia for VEGF mRNA induction may be between 90 to 40 mmHg because hypoxia of PO_2 90 mmHg did not induce the mRNA(lane 3). Time for peak induction in our hypoxic culture in Fig.1 may be shorter than those reported by others(10). Although they have not confirmed time course of the induction, mediation by adenosine may be a rapid responsive system to hypoxia for induction of VEGF.

Desensitization of adenosine receptor mediated induction of VEGF mRNA in prolonged hypoxia

As to the decreased level of VEGF mRNA after prolonged exposure to hypoxia (Fig.1), possibility of adenosine receptor desensitization was studied (Fig.4). After incubation in hypoxic condition for 9 hours, subsequent incubation for 1 hour with NECA of 10^{-7}, 10^{-6}, 10^{-5} M in normoxic condition did not cause a dose-dependent induction of VEGF mRNA, in contrast to the control. Prolonged exposure to increased level of adenosine as produced by hypoxia may cause receptor desensitization.

Although the hypothesis of adenosine mediation may be true only to cells or tissues with A_2-receptor expression, we have also confirmed the induction of VEGF mRNA by NECA in perfused normal rat hearts (data not shown).

As to the induction of erythropoietin, another hypoxia-inducible peptide, only additional increase by NECA to hypoxic induction was confirmed and adenosine antagonists do not inhibit the hypoxic induction in producing cells (20). In this sense, our data are the first to reveal that hypoxia can be put into biochemical mediator as adenosine to act directly on the cells to induce growth factors. VEGF gene induction by hypoxia in relation to adenosine-A_2 receptor mediation can be a task to be solved.

In that anti-VEGF antibody suppresses tumor growth in vivo(9), use of adenosine inhibitor could be another target point for tumor therapy at least for tumors with A_2-receptor expression. In addition, adenosine and its analogs can be a therapeutic for ischemic disorders in hope not only of its direct protective effects on ischemic tissues(21), but of effect on angiogenesis possibly induced by VEGF(22).

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