



Detection of inducible nitric oxide synthase (iNOS) mRNA by RT-PCR in ATL patients and HTLV-I infected cell lines: clinical features and apoptosis by NOS inhibitor

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Various tumors have been reported to express an inducible form of nitric oxide synthase (iNOS), and nitric oxide (NO) may affect the clinicopathological features of these tumors. Previously, Burkitt's lymphoma and Epstein-Barr virus (EBV)-infected cells were shown to express iNOS constitutively at a low level. We analyzed iNOS expression by the reverse transcriptase-polymerase reaction method (RT-PCR) in eight HTLV-I-infected cell lines (five were ATL-derived lines and there were *in vitro* transformed lines), nine ATL patients (three were chronic, two were acute, and four were lymphoma type), and an HTLV-I-negative T cell line (CEM). In four ATL derived and in all three *in vitro* transformed cell lines, iNOS was expressed constitutively, but it was not expressed in CEM cells. Four out of nine ATL patients also showed iNOS expression. The expression of iNOS was found in all subtypes of ATL. Three of four iNOS-positive patients had infiltration of ATL cells to organs such as skin, lung, or liver. In NOS inhibitor (N^G-monomethyl-L-arginine: L-NMMA)-containing medium, an iNOS-positive ATL cell line (K3T) showed growth inhibition and DNA ladder. Although only a limited number of patients was analyzed, our results suggest that NO may be involved in the invasive character of ATL cells. The NOS inhibitor can induce apoptosis in an ATL cell line, as it does in EBV-infected cell lines.

Keywords: HTLV-I; ATL; iNOS; nitric oxide; apoptosis

Introduction

Nitric oxide (NO) plays an important role in regulating blood vessel dilatation and the immune response, and functions as a neurotransmission signal in the brain and peripheral nervous system.^{1–3} NO is synthesized from arginine by nitric oxide synthase (NOS) *in vivo*. At present, the genes for three isoforms of NOS have been cloned: brain NOS (bNOS or type I), endothelial NOS (eNOS or type III), and inducible NOS (iNOS or type II). bNOS and eNOS are expressed constitutively in the neurosystem and endothelium, respectively. iNOS expression is induced by immune stimulations such as lipopolysaccharide (LPS), interferon γ (INF γ), or tumor necrosis factor α (TNF α), in rodent macrophages. Recent studies have shown that NO may be involved in cell proliferation, differentiation,⁴ and apoptosis.^{5–7} Some tumors are reported to express the iNOS gene and NO is thought to affect the clinicopathological features of the tumors expressing iNOS.^{8,9} Regarding human lymphocytes, constitutive, low-level iNOS expression was detected in Epstein-Barr virus (EBV)-infected B cells, and NO inhibited apoptosis and EBV reactivation in these cells.⁵ In splenic B cells, NO was also reported to play a role in regulating bcl-2 expression and cell survival.⁶

Adult T cell leukemia/lymphoma (ATL) is a human T lym-

photrophic virus type I (HTLV-I)-infected T cell malignancy.¹⁰ ATL is classified into four clinical subtypes: smoldering, chronic, acute and lymphoma types. The latter two types are highly aggressive and have a poor prognosis. Clinically, ATL cells often infiltrate to organs such as skin, lung, and liver. For proliferation in such organs, sufficient blood supply to infiltrated ATL cells may be required. NO has been reported to play a role in vascularization or permeability of the vascular wall;^{11–15} thus, we thought that NO might be associated with the invasive character of ATL cells.

In this study, we analyzed iNOS expression by reverse transcriptase-polymerase chain reaction (RT-PCR) in ATL patients and HTLV-I-infected cell lines, and compared the results with clinical features. We also showed that a NOS inhibitor caused cell proliferation retardation and apoptosis in an ATL-derived cell line (K3T).

Materials and methods

Patients and cell lines

Primary ATL cells from nine patients, including three chronic, two acute and four lymphoma types, were obtained. All patients were admitted to Kumamoto University Hospital between 1985 and 1994. The lymphocytes and ATL cells in peripheral blood were counted after May-Giemsa staining. The subjected cells from patients were harvested after centrifugation on a Ficoll-Hypaque gradient. After centrifugation, the cells were washed with PBS and provided for the flowcytometric analyses and the cell pellets were stocked in nitrogen liquid for further analyses. Postmortem studies were performed in cases 4, 6 and 8. Histological examinations were performed on skin biopsy specimens of case 1 and on autopsy specimens. Case 1 presented systemic eruption, and the skin biopsy confirmed infiltration of ATL cells. Histological examinations of cases 4 and 6 showed infiltration of ATL cells into lung, spleen, kidney, liver and intestine. In case 8, swelling of numerous intra-abdominal lymph nodes was observed, but invasion of ATL cells into other organs was not seen. SKT1B,¹⁶ ED,¹⁷ HUT102¹⁸ and K3T¹⁹ cell lines were derived from ATL patients. MT2,²⁰ YT-1²¹ and YY cell lines were established by *in vitro* HTLV-I infection to umbilical cord blood cells, T cells from a paroxysmal nocturnal hemoglobinuria patient, and T cells from a normal healthy donor, respectively. CEM is an HTLV-I-negative T cell line. A172 is a human glioblastoma cell line²² which expresses iNOS when induced by LPS, TNF α , or IFN γ treatment. The total RNA from LPS-, TNF α -, IFN γ -treated A172 cells was provided by Dr T Akaike of the Department of Microbiology, Kumamoto University School of Medicine, and used as a positive control for iNOS expression.

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Received 20 August 1998; accepted 15 January 1999

Immunophenotypic analysis

Cell surface marker analysis was performed as described previously.^{23,24} The cells were reacted with fluorescein isothiocyanate (FITC)-conjugated OKT4 (Ortho Pharmaceutical, Raritan, NJ, USA). The stained cells were analyzed by Spectrum III (Ortho Diagnostic Systems, Westwood, MA, USA) or FAC-Scan (Becton Dickinson Monoclonal Center, Mountain View, CA, USA).

RT-PCR

The total RNAs were isolated from the frozen cell pellets of patients and packed cells of the cell lines by acid-guanidine phenol-chloroform extraction. First-strand DNA was made from 1 μ g of total RNA using Superscript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD, USA) and random hexamers (25 pmol) as primers (Perkin-Elmer Cetus, Foster City, CA, USA), following the manufacturer's instructions. Out of a total of 20 μ l, 3 μ l of the resulting cDNA was PCR amplified in 25- μ l reactions using *Pfu* DNA polymerase (Stratagene, San Diego, CA, USA) and primers specific for human hepatic iNOS.²⁵ The reaction mixture included 2 U of *Pfu* polymerase and 50 pmol of each primer. The primer pairs were designed by referring to a previous report:⁵ (5'-CTGTCCTGG-AAATTTCTGTT-3' and 5'-TGGCCAGATGTTCTCTATT-3', nucleotide sequences 212–232 and 699–680 of hepatic iNOS cDNA, respectively). The PCR conditions were: denaturation once at 95°C for 5 min, and then 35 cycles of 1 min of denaturation at 95°C, 1 min of annealing of 58°C, and 2 min of extension at 72°C, using a Robo-Cycler system (Stratagene). Ten microliters of each PCR product was separated in a 1.2% agarose gel and visualized by 0.5 μ g/ml ethidium bromide staining.

Sequence analysis

The PCR product from A172 cDNA was ligated into the *HincII* site of plasmid pCDNAII (Stratagene) and then cloned. The sequence was determined by an auto sequencer system.

Southern blot analysis

The PCR products were transferred on to nylon membranes (Hybond+, Amersham, Japan). The blots were prehybridized at 65°C for 2 h, and hybridized overnight to a ³²P-labeled iNOS cDNA-specific fragment (398 bp) at 65°C. To avoid cross-hybridization to the primers, we generated a 398-bp fragment by PCR using internal iNOS primers (5'-AGTATGCAATGAATGGGGAA-3' and 5'-ATTCGATAGCTTGAGGTAGA-3', nucleotide sequences 250–269 and 642–623 of hepatic iNOS cDNA, respectively) (Ref. 25 and GenBank database, accession No. L09210) and A172 cDNA as template. The hybridized blots were washed twice in 2 \times SSC/0.1% SDS at room temperature for 10 min, and twice in 0.1 \times SSC/0.1% SDS at 65°C for 1 h. The blots were exposed to X-ray film at -80°C.

Cell culture and assays for cell growth and apoptosis

K3T and CEM were cultured in RPMI 1640 medium containing 10% fetal calf serum in the presence or absence of a NOS

inhibitor (NG-monomethyl-L-arginine; L-NMMA purchased from Sigma Chemical, St Louis, MO, USA). The RPMI 1640 medium contained 1.15 mM of L-arginine. Before determination of the initial cell density, we examined the survival curve of both cell lines for 72 h. To avoid cell death due to over-growth during the experiments, we started the cell culture at initial cell density 12×10^5 /ml. The dose of L-NMMA utilized in this study was determined according to a previous report.⁵ The viable cell numbers were estimated by conventional trypan-blue exclusion. DNA contents were determined as described elsewhere.²⁴ Briefly, the cells were fixed in 70% ethanol on ice for 1 h, then stained with 50 μ g/ml propidium iodine. The cells were washed twice with 1 \times PBS and analyzed by flow cytometry (FACScan; Becton Dickinson). The fragmented DNA was extracted as previously described,²⁶ and electrophoresed in 1.5% agarose gels containing 0.5 μ g/ml ethidium bromide.

Results

Detection of iNOS mRNA by RT-PCR in HTLV-I infected cells and ATL cells

Using RT-PCR, the expected 491-bp RT-PCR product was detected in ethidium bromide-stained gels (Figure 1a). The 491-bp fragment derived from A172 cDNA was shown to be identical to human hepatic iNOS cDNA by sequence analysis (data not shown). To confirm these products as the iNOS cDNA fragment, we performed Southern blot analysis using the 398-bp fragment of iNOS cDNA as a probe. As shown in Figure 1b, the expected hybridized signal was detected in the RT-PCR products, indicating that these bands were iNOS cDNA fragments. No RT-PCR product was obtained from normal peripheral blood mononuclear cell samples using iNOS-specific primers. Three ATL-derived cell lines (SKT1B, ED and K3T) and three *in vitro* transformed cell lines (MT2, YY and YT-1) were positive for iNOS expression. On the other hand, the HTLV-I-negative T cell line CEM was negative for iNOS. We could detect the iNOS mRNA by Northern analysis only in the RNA sample from A172 (data not shown).

Clinical features and iNOS in ATL patients

Among nine primary ATL samples, four were positive for expression of iNOS gene (Figure 1 and Table 1). The iNOS-positive patients included two of three chronic, one of two acute, and one of four lymphoma types. Four of nine patients had involvement of organs and three out of four patients expressed iNOS gene (Table 1).

Cell growth retardation and apoptosis caused by a NOS inhibitor (L-NMMA)

In iNOS-positive EBV-infected B cells, the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) caused apoptosis.⁵ Thus, we examined the effect of L-NMMA on cell growth of ATL cells. The K3T cell line showed the strongest expression of iNOS when analyzed by RT-PCR; therefore, we compared K3T and an iNOS-negative T cell line, CEM. As shown in Figure 2, L-NMMA inhibited cell growth in a dose-dependent manner. Time course experiments showed that 5 mM L-NMMA caused growth retardation of K3T cell (Figure 3). To

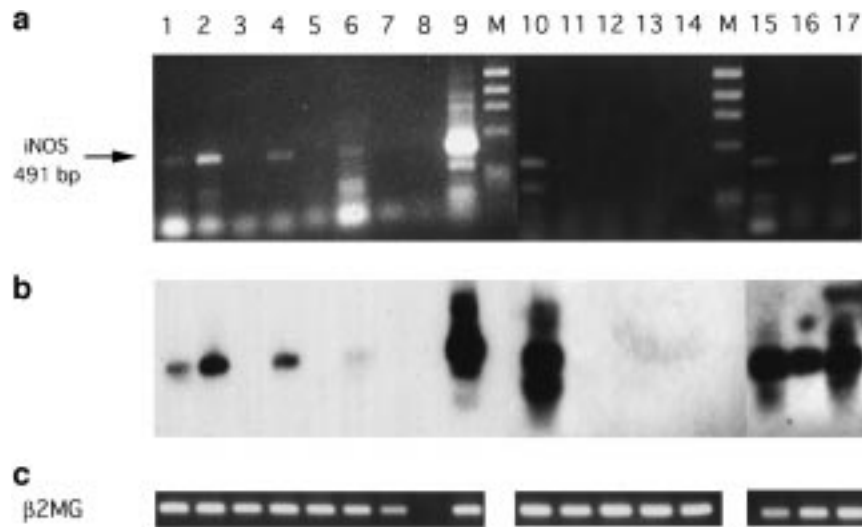


Figure 1 Representative results of RT-PCR for iNOS mRNA and Southern analysis. (a) The RT-PCR products were visualized by ethidium bromide staining. Lane 1, SKT1B; lane 2, K3T; lane 3, HUT102; lane 4, ED; lane 5, CEM; lane 6, case 3; lane 7, case 8; lane 8, H₂O; lane 9, A172; lane 10, case 4; lane 11, case 2; lane 12, case 5; lane 13, case 7; lane 14, normal peripheral blood mononuclear cells; lane 15, MT2; lane 16, YT-1; lane 17, YY; and M, size marker ($\phi \times 174/HaeIII$). (b) Southern blot analysis using an internal fragment of iNOS cDNA. (c) The β -2 microglobulin (β 2MG) fragment was amplified using the same first-strand cDNA as the DNA template. The 491-bp RT-PCR product of A172 was confirmed to be identical to that of the iNOS cDNA fragment by sequence analysis (data not shown).

Table 1 Patient profiles and results of RT-PCR for iNOS mRNA

Case	Type	iNOS	Organ infiltrations	Leukocytes (/ μ l)	Lymphocytes (L) and ATL cells (A) in peripheral blood (%) fl	CD4-positive cells in peripheral blood (PB) or lymph node (LN) (%)	GOT and LDH (U/l) ^a	RNA source
Case 1	Chronic	(+)	Sk	65 500	L 70, A 29	89.5 (PB)	GOT 7, LDH 615	PBMC
Case 3	Chronic	(+)	(-)	28 300	L 89	95.0 (PB)	GOT 24, LDH 218	PBMC
Case 4 ^b	Acute	(+)	Lu, Li, Sp, Ki, Int, Co	169 800	L 90	Not tested	GOT 42, LDH 1594	PBMC
Case 6 ^b	Lymphoma	(+)	Lu, Li, Sp, Ki, Int	7500	L 45, A 1	Not tested	GOT 33, LDH 694	LNC
Case 2	Chronic	(-)	(-)	20 600	L 79	75.4 (PB)	GOT 16, LDH, 181	PBMC
Case 5	Acute	(-)	Li	142 000	A 91	Not tested	GOT 831, LDH 27 960	PBMC
Case 7	Lymphoma	(-)	(-)	6000	L 1, A 2	72.7 (LN)	GOT 16, LDH 699	LNC
Case 8 ^b	Lymphoma	(-)	(-)	5900	L 19	81.7 (LN)	GOT 56, LDH 944	LNC
Case 9	Lymphoma	(-)	(-)	3400	L 12, A 3	44.2 (LN)	GOT 25, LDH 997	LNC

Sk, skin; Lu, lung; Li, liver; Sp, spleen; Ki, kidney; Int, intestine; co, colon PBMC, peripheral blood mononuclear cells; LNC, lymph node cells. fl The lymphocytes and ATL cells were counted after May-Giemsa staining.

^aNormal values of GOT (glutamine-ornithine transaminase) and LDH (lactate dehydrogenase) are 6–45 U/l and 150–320 U/l, respectively.

^bPostmortem studies were performed in these patients.

further examine the effect of L-NMMA on cell growth, we analyzed the cellular DNA content by flow cytometry. Figure 4a shows the dosage effect of L-NMMA on DNA content. In K3T cells, the proportion of cells with normal chromosome DNA content (2n or 4n) was decreased, while the proportion of cells having hypodiploid DNA content (below 2n) was increased. DNA ladder formation was observed in L-NMMA-treated K3T cells, indicating that apoptosis had occurred (Figure 4b). As seen in Figure 5, increasingly longer treatment with 5 mM L-NMMA caused an increasingly greater number of cells with lower DNA content. No such effect was observed in CEM cells (Figures 4 and 5). We examined the effect of L-NMMA on cell growth of ED cell line. ED cell showed cell growth inhibition by addition of L-NMMA, but we could not confirm the evidence of apoptosis in the cell line.

Discussion

Various human tumor cells have been reported to express iNOS, and NO appears to affect clinical features and pathological aspects of the tumors. A previous study revealed that Burkitt's lymphoma and EBV-infected B cells show low-level, constitutive expression of iNOS.⁵ ATL is also a virus-associated lymphoid malignancy; thus, we asked whether iNOS was expressed in ATL cells. Three out of four ATL-derived cell lines and three out of three *in vitro* HTLV-I-infected cell lines expressed iNOS. In normal PBMC, iNOS was not detected. These results suggested that HTLV-I infection caused iNOS expression in T lymphocytes. We also found iNOS expression in freshly isolated cells from four patients. ATL cells are known to produce various kinds of cytokine; therefore, we

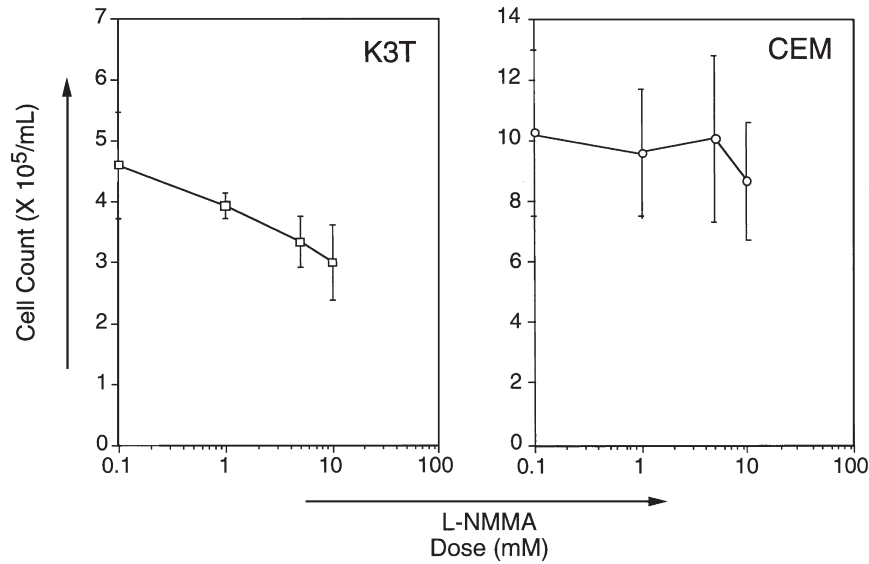


Figure 2 Growth inhibition of iNOS positive-ATL cell line (K3T) caused by a NOS inhibitor (L-NMMA). K3T and CEM cells growing in early log phase were cultured in various concentrations of L-NMMA for 48 h. The viable cells were counted using trypan-blue exclusion. To avoid cell death of over-growth during the experiments, we started the cell culture at initial cell density $1.2 \times 10^5/\text{ml}$. After 48 h culture, the cell count of the two cell lines was different due to its own cell growth rate.

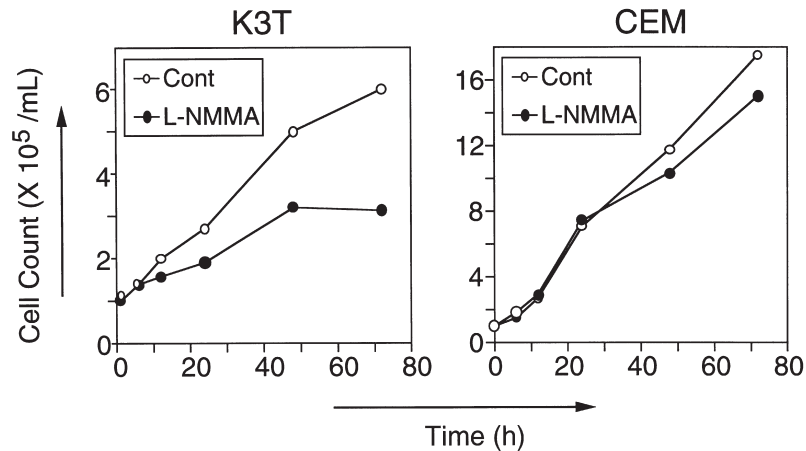


Figure 3 Retardation of K3T cell growth by L-NMMA. The cells were cultured in 5 mM L-NMMA-containing medium. The viable cells were counted using trypan-blue exclusion.

could not rule out the possible iNOS expression from other activated cells such as monocytes than from ATL cells in patient's samples. Molecular mechanism of the iNOS expression in ATL is not known, but previous studies showed that the viral Tax protein activates the transcriptional regulatory potential of nuclear factor-kappa B (NF- κ B) in ATL cells.^{27,28} NF- κ B binding DNA elements exist in the 5' region of the iNOS gene, which is responsible for regulation of iNOS gene expression.^{29,30} Moreover, HTLV-I-Tax protein elevate iNOS expression in human monocytoid cell line (U937).³¹ Thus, the viral Tax protein or activation of NF- κ B may play a partial role in constitutive iNOS expression in ATL cells. Recently, iNOS expression was also demonstrated in a hairy cell leukemia cell line³² and B chronic lymphocytic leukemia cells.³³ Our results showed that HTLV-I-infected T cells also expressed iNOS mRNA.

ATL patients show various prognoses and clinical features. In central nervous system tumors, pathological grade has been

shown to be correlated with NOS activity, and iNOS was expressed in highly anaplastic astrocytoma or glioblastoma.⁹ ATL is classified into four subtypes on the basis of clinical features. Our results suggested that iNOS expression and clinical subtype seemed not to be correlated. Next, we examined the relationship between iNOS expression and organ involvement. Three of four iNOS-positive patients had infiltration of ATL cells into skin, lung or liver, whereas only one of five iNOS-negative patients showed hepatomegaly and an elevated serum transaminase value. On the basis of these results, we speculated that iNOS-positive ATL cells might have a tendency to invasively proliferate in organs. Only a limited number of patients was analyzed; thus, further studies are required for understanding iNOS expression and clinicopathological aspects of ATL.

A previous study revealed that NOS inhibitors induced apoptosis in EBV-infected B cells.⁵ Thus, we examined the effect of a NOS inhibitor on cell growth of an iNOS-positive

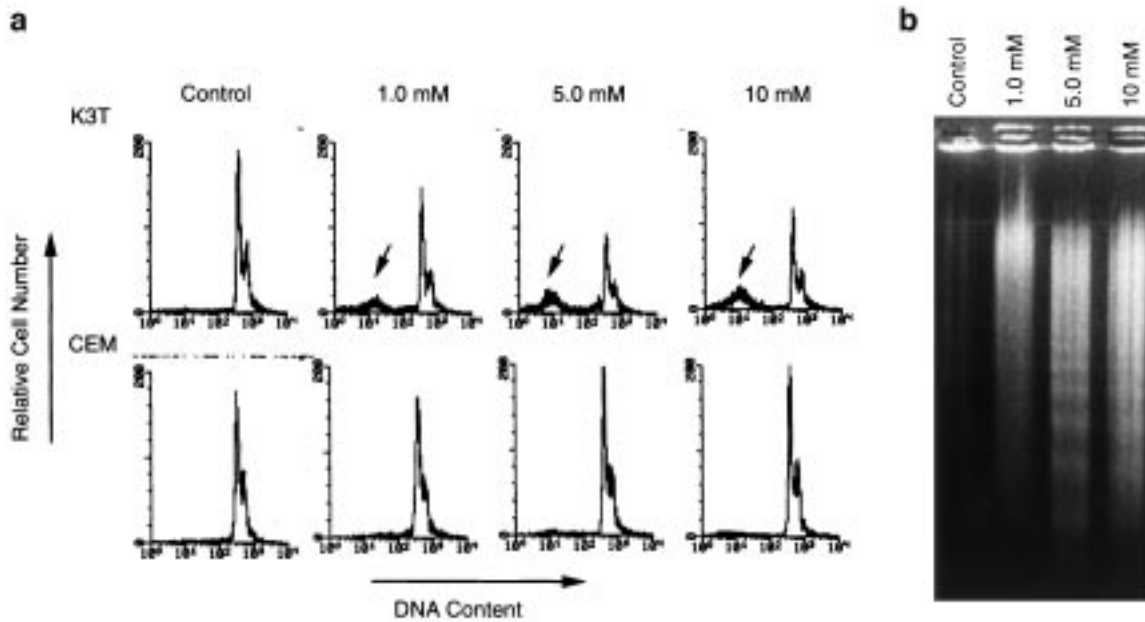


Figure 4 (a) DNA content analysis of L-NMMA-treated K3T and CEM cells. The cells were cultured in various concentrations of L-NMMA-containing medium for 48 h. The DNA was stained with propidium iodide, and analyzed by flow cytometry. Cells with hypodiploid DNA content (arrow) appeared in a dose-dependent manner. (b) DNA ladder formation in the L-NMMA-treated K3T cells. K3T cells were cultured in the presence of various concentration of L-NMMA for 48 h. DNA ladder formation was induced by L-NMMA.

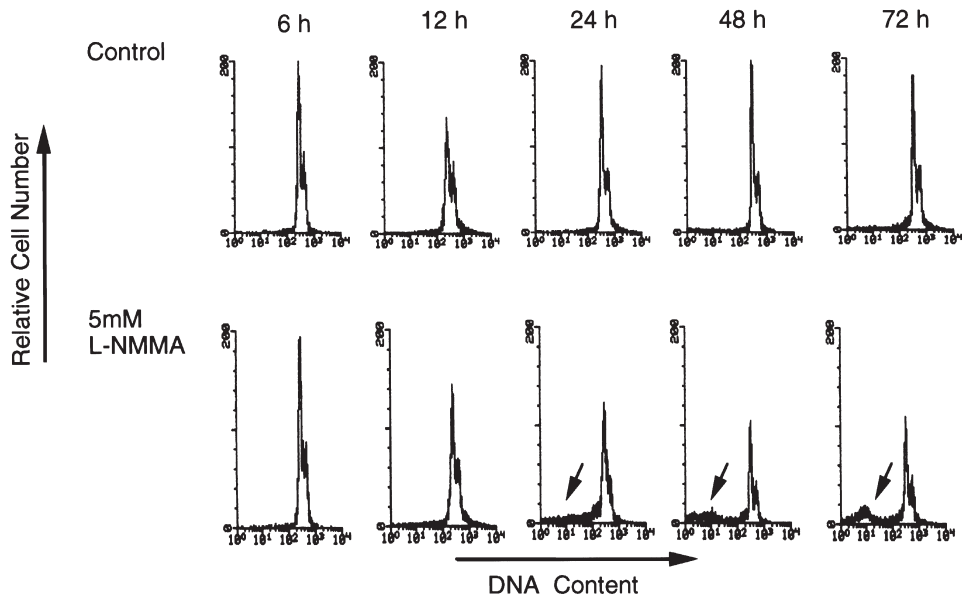


Figure 5 Time course of effects of L-NMMA on growth of K3T cells. The cells were cultured in 5 mM L-NMMA-containing medium. In L-NMMA-containing medium, the number of cells with hypodiploid DNA content (arrow) was increased.

ATL cell line, K3T. In K3T cells, growth inhibition and apoptosis were induced by a NOS inhibitor, L-NMMA. These results were similar to those in EBV-positive B cells. NO plays a role in the control of apoptosis as both inducer and suppressor.^{34,35} At present, many physiological effects of NO have been reported, and NO has been implicated in redox regulation in cells.³⁶ Regarding ATL, the ATL-derived factor (ADF), which augments interleukin 2 receptor expression on ATL cells, has been shown to be a redox regulator.³⁷ The NO produced by iNOS in ATL cells may be involved in cellular redox regulation and may play a role in cell survival.

Acknowledgements

We thank Dr S Tamiya in our laboratory and Dr T Akaike (Department of Microbiology, Kumamoto University School of Medicine) for providing the total RNA of ATL patients and A172, respectively. We also thank Dr N Arima (Kagoshima University, Kagoshima, Japan), Dr RC Gallo (National Cancer Institute, Bethesda, MD), Dr M Maeda (Chest Disease Research Institute, Kyoto University, Kyoto, Japan), Dr I Miyoshi (Kochi Medical University, Kochi, Japan), Dr S Nagakura and Dr H Nakakuma in our laboratory for providing

their cell lines. We are grateful to Dr T Gotoh and Dr M Takiguchi for their technical advice. This work was supported in part by a Grant-in Aid for Cancer Research (85-1) from the Ministry of Health and Welfare, grants (09254251 and 08770852) from the Ministry of Education, Science, Sports and Culture of Japan, and a grant from Haraguchi Memorial Fund for Cancer Research.

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