J Clin Exp Hematop
Vol. 53, No. 2, Aug 2013

Original Article

Characterisation of Apoptosis in myb-Transformed Hematopoietic Cell (MTHC-A) Lines : TNF-α-Induced Apoptosis and Prevention by cAMP#

Kenji Sorimachi,[1] Paul Waring,[2] Andrew J Hapel,[3] Ichio Fukasawa,[1] Yuko Kaneko,[4] and Nobuhide Masawa[4]

Myb-transformed haematopoietic cell (MTHC) lines have been developed and clones selected based on their ability to respond to tumor necrosis factor (TNF)-α. MTHC-A cells underwent apoptosis in response to TNF-α (MTHC-A). The apoptotic effect of TNF-α in MTHC-A was mimicked by a specific inhibitor of protein kinase A (PKI 5-24) and by the tyrosine kinase inhibitor genistein, suggesting that phosphorylation of tyrosine and PKA activity were important in protecting MTHC from apoptosis. Agents that elevate intracellular levels of cAMP, e.g. cholera toxin and dibuteryl cAMP, protected MTHC-A from the apoptotic effects of TNF-α, and also reduced the apoptotic effects of PKI and genistein. MTHC-A thus provides a useful model for investigating the role of TNF-mediated apoptosis in regulation of the myeloid lineage of cells. 〔*J Clin Exp Hematop 53(2) : 115-120, 2013*〕

Keywords: myb-transformed haematopoietic cell, TNF-α, apoptosis, cAMP, protein kinase A

INTRODUCTION

The use of a truncated form of Myb in the generation of myeloid cell lines that can be used as homogeneous populations for *in vitro* studies of differentiation.[1] The transfected cells grow continuously, resembling other factor-dependent myeloid cell lines such as FDC-P1.[2] Myb is normally expressed during proliferation of blood cells, but its expression is auto-regulated via the carboxy-terminal region[1] that is deleted in our vector. Continuous expression of Myb confers immortality on the transformed cells so long as they are maintained using an appropriate mitogenic cytokine [granulocyte macrophage colony-stimulating factor (GM-CSF), or interleukin-3 for instance]. Importantly, myb-transformed hematopoietic cells (MTHC) retain the ability to respond to a wide variety of stimuli and so appear to retain most of the properties of progenitor cells.

Cell lines were cloned by dilution of MTHC cultures to a density of one cell per ten wells. Replicates were then made of each positive well. Screening of a variety of cytokines by addition to the replicate wells revealed that some clones responded in quite different ways to the same stimulus. The majority of clones were induced to differentiate (MTHC-D) in the presence of tumor necrosis factor (TNF)-α but other clones died (MTHC-A ; MTHC underwent apoptosis by TNF-α) in the presence of TNF-α.

Apoptosis is a regulated form of cell death, which can be induced by a variety of extracellular stimuli.[3] During haematopoiesis, apoptosis is believed to play a regulatory role in maintaining homeostasis among the various mature populations of blood cells.[4] Apoptosis may also be a way of eliminating cells that are damaged or pre-leukaemic.[3] Progenitor cells from bone marrow undergo apoptosis if those growth factors required for survival are withdrawn.[5] This effect is mimicked by the protein synthesis inhibitor cycloheximide.[6] However apoptosis can also be actively triggered by active engagement of cell surface receptors. Notable among these are the TNF-α receptor family whose activation can lead to a variety of outcomes ranging from cell differentiation, activation or apoptosis.[7, 8]

Engagement of the TNF-α receptor Rp55 commonly results in elevated levels of intracellular cAMP.[9] The effect of

Received : December 25, 2012
Revised : February 1, 2013
Accepted : February 11, 2013
[1]Department of Obstetrics and Gynecology, Dokkyo Medical University, Mibu, Tochigi, Japan
[2]Faculty of Chemistry, Australian National University, Canberra, ATC, Australia
[3]Department of Haematology, Division of Science and Design, University of Canberra, Canberra, ACT, Australia
[4]Department of Anatomic Diagnostic Pathology, Dokkyo Medical University, Mibu, Tochigi, Japan
Corresponding author : Dr. Kenji Sorimachi, Department of Obstetric and Gynecology, Dokkyo Medical University, Mibu, Tochigi 321-0293, Japan
E-mail : kenjis@jcom.home.ne.jp

elevated intracellular cAMP is clearly cell type specific. In haematopoietic progenitor cells elevation of cAMP seems to be linked to differentiation,[9] while other studies have shown that elevated cAMP levels can trigger apoptosis.[10] In differentiated cells such as thymocytes elevation of cAMP coupled to histone H3 phosphorylation is linked to apoptosis.[11] On the other hand, in haematopoietic progenitor cells and in neurones, cAMP and protein kinase A (PKA) can have protective effects.[12] Dibuteryl cAMP has been shown to protect liver cells *in vivo* from TNF-α-induced apoptosis,[13] and BAD, a pro-apoptotic member of the Bcl-2 family, has been shown to be phosphorylated and inactivated by a cAMP-dependent kinase associated with the mitochondrial membrane.[14]

This manuscript describes some of the properties of the MTHC-A cells and investigates the mechanism of cell death induced by TNF-α.

MATERIALS AND METHODS

Growth factors and cytokines

Recombinant GM-CSF provided by Drs. N. Gough and T. Wilson (Walter and Eliza Hall Institute) was made in a yeast expression system and used as a crude culture supernatant with activities of 8×10^4 U/mL. No endotoxin was detected in the sample.

Recombinant mouse TNF-α was supplied by Ernst-Boehringer-Institute Fur Arzeneimittel-Forschung (Vienna) and Prof. W. Fiers (University of Gent, Belgium) with activities of 7×10^7 and 1.2×10^7 U/mL and endotoxin concentrations of 2.0 ng/mL and 51 pg/mL respectively. Human TNF-α was a gift from Dr. J. Tavernier (Roche Research, Gent, Belgium) and had a specific activity of 1.1×10^9 U/mL and an endotoxin content of 3.2 ng/mL.

The endotoxin concentration of each cytokine was determined using the Pyrogent Plus Advantage kit (Whittaker Bioproducts Inc., Walkersville, MD, USA) exactly as described in the instructions.

Cell lines

The JCS subclone[8] of the WEHI-3B cell line was grown in Iscove's modified Eagle's medium (IMEM) containing antibiotics and 20% foetal calf serum.

Transformation of foetal liver cells with myb, and maintenance of MTHC

Liver cells were prepared from 14 day foetuses and infected with recombinant *myb* retro virus RED (CT3myb) as previously described.[1,15,16] Foetal liver cells (10^6) were cultured on a feeder layer of 10^6 irradiated (25 Gy) virus-producing Y2 cells in the presence of 400 units/mL of GM-CSF. Following infection, foetal liver cells were removed from the feeder layer and washed twice using phosphate buffered saline (PBS), then suspended in IMEM containing antibiotics, 20% foetal calf serum supplemented with GM-CSF (400 units/mL). Cultures were then split twice weekly. Cloned cell lines were derived by limiting dilution of MTHC in the presence of GM-CSF.[1] In order to maintain their phenotypic properties, all cell lines derived in this manner were recloned by limiting dilution at intervals of roughly 30 passages. The cell lines described in this manuscript were derived from A/J and C3H/HeJ mice from the specific pathogen free animal facility at JCSMR.

DNA fragmentation

Detection of intra nucleosomal DNA fragmentation was done by electrophoresis in agarose gels according to the method of Eckhardt as modified by Barry and Eastman.[17] A 2% agarose gel in Tris-boric acid EDTA electrophoresis buffer was allowed to set with wells approximately 3 cm from the top of the gel. The top part of the gel was removed and a 1% agarose gel containing 25 SDS and 64 μg/mL of proteinase K in Tris-borate buffer was used to replace the top of the gel. Treated cells (10^6), were washed once in cold PBS, pelleted, then loading buffer (5 mg/mL RNA'se A, 10% glycerol, 10 mM Tris pH 8.0, 0.1% w/v bromophenol blue) (15 μL) was gently added and the sample added to the gel for electrophoresis at 50 V for 16 hrs at room temperature. Gels were stained with ethidium bromide (2 μg/mL in distilled water) for 1 hr and de-stained in distilled water for 4 hrs with several changes of water. The DNA bands were visualised using an ultraviolet illuminator and photographed using Polaroid high-speed film (Polaroid 57, Cambridge MA).

Cell cycle analysis

MTHC were washed once in IMEM and resuspended at 10^6/mL in IMEM plus supplements. Cytokines were added at appropriate concentrations and replicate cultures were maintained for different times. Cells were collected at designated times by centrifugation at 800 G for 10 min at room temperature then resuspended in 1 mL of PBS and 3 mL of 95% ethanol. Cells were fixed at 4℃ overnight, washed twice with cold PBS, and resuspended in propidium iodide staining solution (PBS plus 20 μg/mL PI, 200 μg/mL RNA'se A) and incubated for 30 min at room temperature in the dark. Samples were then analysed using a Becton-Dickinson FACScan with a flow rate of 100 cells/second. The FL-2 profile was adjusted so that the G0/G1 mean was at channel 200 on a 1,024 channel scale and 10,000 events were collected per sample. Data was analysed using Modfit LT, 1.0 (Verity Software House Inc, Maine) for Macintosh. The percentage of apoptotic cells was estimated by data modelling

using MODFIT, with model ranges set to encompass the populations of interest.

RESULTS

MTHC could be divided into two groups on the basis of their response to TNF-α. MTHC-A responded by dying within 4-6 hrs while MTHC-D responded by differentiating as described in detail elsewhere.[1] In the present study, MTHC-A was used to investigate apoptosis.

The specific protein kinase A inhibitor PKI [5-24], has previously been shown to inhibit apoptosis induced by gliotoxin in thymocytes and P815 cells, implicating a PKA-dependent mechanism in the apoptotic event.[12] TNF-α is known to induce an increase in intracellular cAMP in haematopoietic progenitor cells[10] and does so transiently in MTHC-D (data not shown). This suggested that PKA activity might be differentially involved in the regulation of apoptosis in these cell lines.

To determine the mechanism of cell death we examined the DNA extracted from cultures of PKI [5-24]-treated MTHC. Analysis of MTHC-A (C3H-A2) was undertaken by transmission electron microscopy. Fig. 1 shows chromatin condensation typical of apoptotic MTHC.

To examine the role of PKA in TNF-α-induced apoptosis in MTHC-A cells we treated MHTC-A cells with PKI [5-24] to examine whether this would inhibit apoptosis. During apoptosis specific endonucleases cleave genomic DNA at linker regions between nucleosomes to give fragments that resolve by agarose gel electrophoresis as multiples of 180-200 bp.[18] DNA fragmentation was assessed at 0, 0.5 and 1 mM PKI [5-24] after 4 hrs PKI [5-24] addition, and at 0, 2 and 4 hrs after the addition of 1 mM PKI [5-24]. DNA fragmentation was found in all cultures of MTHC-A treated with PKI [5-24] (Fig. 1).

PKI (5-24) did not inhibit apoptosis in TNF-α-treated MTHC-A cells, and in fact increased the apoptotic effect significantly (Fig. 2). Furthermore, control MTHC-A cells treated with PKI alone, died with the typical biochemical and morphological features of apoptosis (Fig. 1). This suggested that PKA [5-24] may actively protect MTHC-A cells from apoptosis and that inhibition of endogenous PKA activity may trigger apoptosis in these cells. The cell-permeable analogue dibutyryl cAMP inhibited TNF-α-induced apoptosis in MTHC-A cells (Fig. 3) and had a small inhibitory effect on apoptosis in PKI [5-24] treated cells (data not shown).

Genistein, nominally a protein tyrosine kinase inhibitor, has also been shown to inhibit the effects of cAMP dependent kinase, a serine kinase.[11] Consistent with this we also found that genistein induces apoptosis in MTHC-A and this effect was inhibited by db-cAMP and cholera toxin (Fig. 4) again indicating that cAMP dependent phosphorylation exerts an important protective effect in these cells.

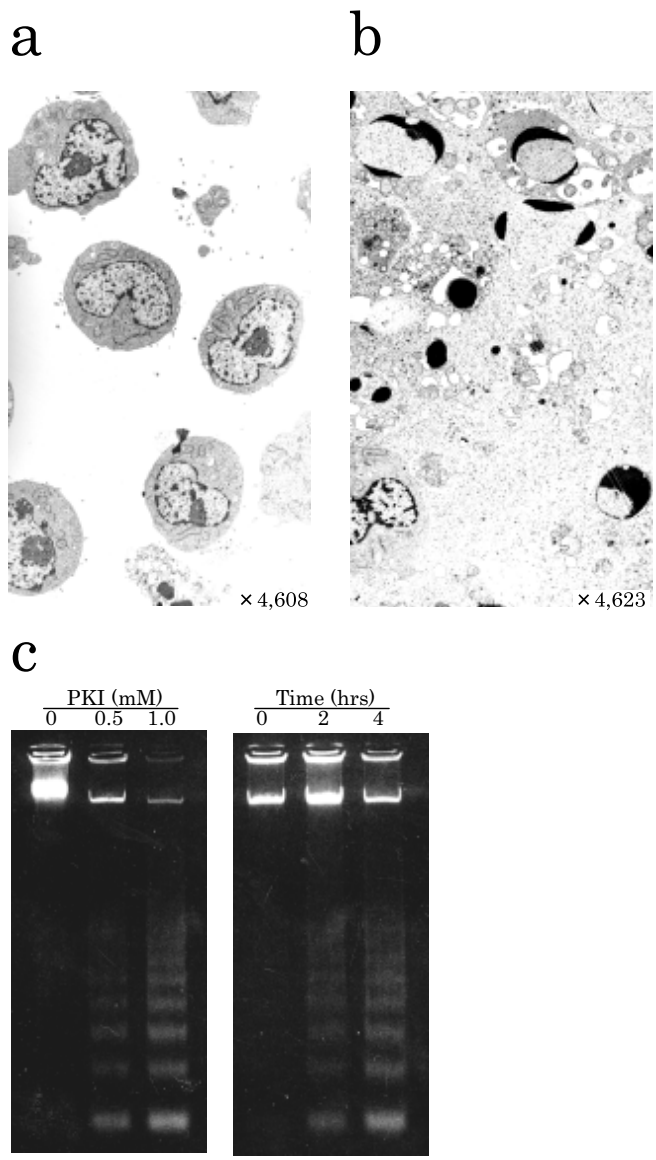

Fig. 1. Electron micrographs of MTHC-A undergoing PKI [5-24]-induced apoptosis and DNA fragmentation on gel electrophoresis. (*1a*) control cells, (*1b*) treated with PKI [5-24] at 1 mM for 4 hrs, (*1c*) treated with PKI [5-24] at different concentrations and for different times. MTHC-A, myb-transformed haematopoietic cell underwent apoptosis by tumor necrosis factor-α ; PKI, specific inhibitor of protein kinase A

Cycloheximide is a general inhibitor of protein synthesis and induces apoptosis in many cell types. Cycloheximide induces apoptosis in MTHC-A cells and this apoptotic effect is reversed by db-cAMP. Thus apoptosis induced by a non-specific agent in MTHC is also abrogated by activation of PKA (Fig. 5).

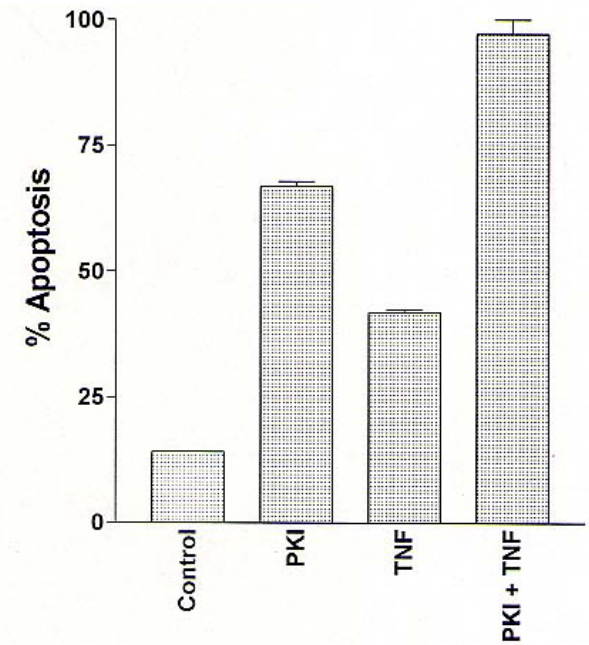

Fig. 2. The effect of PKI [5-24] on apoptosis in MTHC-A. Cells were treated for 4 hrs with 0.5 mM PKI in the presence of 250 U/mL tumor necrosis factor (TNF)-*α*. PKI, specific inhibitor of protein kinase A ; MTHC-A, myb-transformed haematopoietic cell underwent apoptosis by TNF-*α*

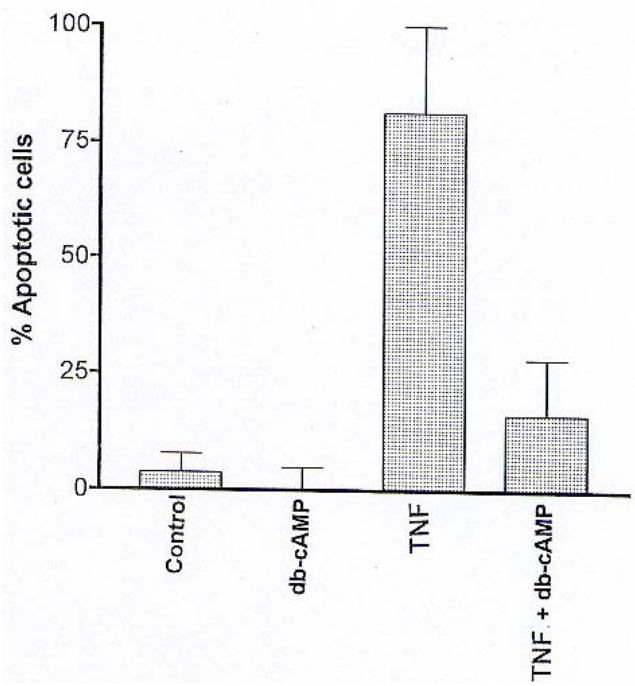

Fig. 3. Dibutyryl cAMP inhibits tumor necrosis factor (TNF)-*α*-induced apoptosis in MTHC-A. Dibutyryl cAMP was added to TNF-*α*-treated cells at 0.5 mM. MTHC-A, myb-transformed haematopoietic cell underwent apoptosis by TNF-*α*

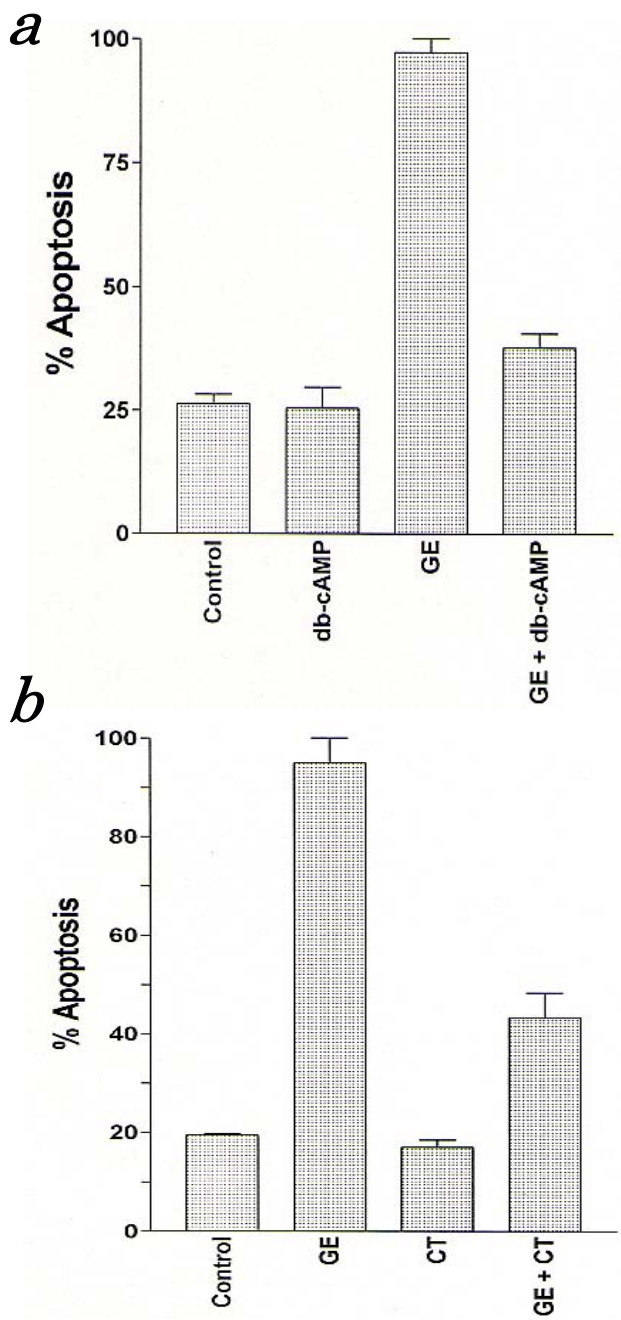

Fig. 4. Inhibitory effect of dibutyryl cAMP on apoptosis induced in MTHC-A by genistein or cholera toxin. Cells were treated with genistein (*GE*) at 100 μM (*4a*) or cholera toxin (*CT*) at 100 ng/mL (*4b*) for 4 hrs with and without db-cAMP at 0.5 mM. MTHC-A, myb-transformed haematopoietic cell underwent apoptosis by tumor necrosis factor-*α*

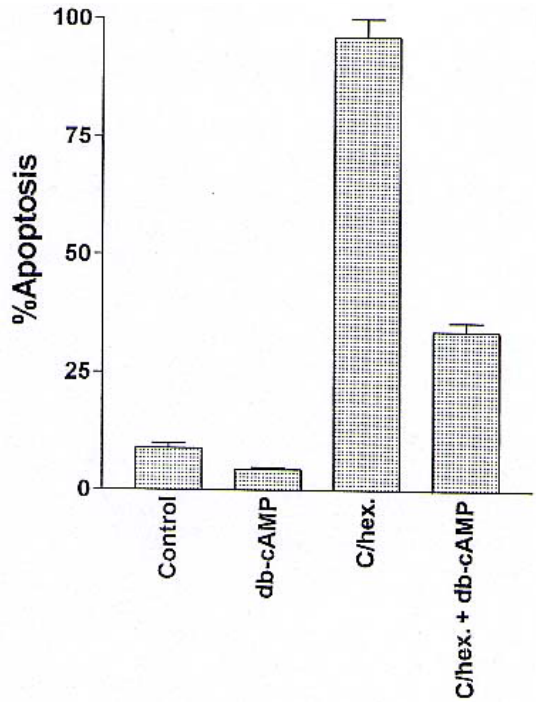

Fig. 5. Inhibitory effect of dibuteryl cAMP on apoptosis induced in MTHC-A by cycloheximide. Cells were treated with cycloheximide (*C/hex.*) at 10 μM with and without db-cAMP at 0.5 mM for 4 hrs. MTHC-A, myb-transformed haematopoietic cell underwent apoptosis by tumor necrosis factor-α

DISCUSSION

Here we describe myeloid cell lines that are susceptible to the apoptotic effects of TNF-α. These cell lines were cloned from cultures of myb-transformed fetal liver cells that grew in the presence of GM-CSF. Because of their apoptotic response to TNF-α we have designated the cell lines MTHC-A (myb-transformed haematopoietic cell-apoptotic) to distinguish them from the differentiating form of MTHC (MTHC-D) that we have described previously.[1] Cloned MTHC-A were obtained from A/J and C3H. HeJ mice and the clones had similar properties.

MTHC-A requires GM-CSF for continued growth. In the presence of low doses of TNF-α (10% of the amount required to induce differentiation in MTHC-D) MTHC-A are induced to pass more rapidly through the cell cycle then to undergo growth arrest and cell death. By all the criteria used (fluorescence activated cell sorting analysis, DNA laddering and electron microscopy) the cells die as a result of apoptosis. This is consistent with previous reports describing the effects of TNF-α on the cell cycle. Growth arrest in G1 has been shown to correlate with increased resistance to TNF-induced cell death in fibroblasts,[19] while a transient elevation in the rate of DNA synthesis, and hence transition from G1 to G2/M, has been associated with increased sensitivity to TNF-α-induced apoptosis.[20]

Many hormones, via coupling to signal transducing G proteins, act through the second messenger cAMP to stimulate metabolism and proliferation.[21] The target for cyclic AMP is cyclic AMP dependent protein kinase or PKA and the pleiotropic effects of this kinase presumably results from phosphorylation of different substrates, either cytosolic or nuclear, by the kinase. Many of these targets remain to be identified. PKA-dependent phosphorylation of the transcription factor CREB results in its activation and gene transcription.[22] A number of studies have shown that elevated cAMP levels induce apoptosis in immature thymocytes[11,23,24] and that this effect can be abrogated by the specific inhibitor of PKA, PKI [5-24].[11] PKI [5-24] is a truncated form of PKI, an endogenous inhibitor of PKA.[25] One possible nuclear target for active PKA during apoptosis is histone H3 which becomes phosphorylated on ser10 during gliotoxin induced apoptosis. In general however, cAMP and its analogues such as the cell permeant db-cAMP inhibit the effects of pro-inflammatory cytokines such as TNF. This protective effect has been ascribed to the production of the anti-inflammatory cytokine interleukin-10.[26,27] Our data showing that db-cAMP and cholera toxin which is known to raise cAMP levels, both protect against TNF induced apoptosis in MTHC-A. Experiments with PKI [5-24], a specific inhibitor of PKA, imply that basal PKA activity is required for viable MTCH-A cells suggesting that PKA dependent phosphorylation of unknown substrates is important for maintaining viability in these cells. The fact that genistein, a relatively non-specific inhibitor of protein phosphorylation, also induces apoptosis in these cells which is again abrogated by agents which raise cAMP levels supports this idea.

Interestingly apoptosis induced by TNF in MTHC-A cells in culture does not require the simultaneous presence of cycloheximide. The latter is often required to suppress synthesis of anti-apoptotic molecules, which abrogate the effects of TNF-α. MTHC-A cells may lack the ability to produce those anti-apoptotic molecules following engagement of the TNF-α receptor. In fact cycloheximide induces extensive apoptosis in the MTHC and this effect is also overcome by cAMP.

ACKNOWLEDGEMENTS/FUNDING

The assistance of Gail DeCello and Allan Sjaarda is acknowledged.

This work was funded by a grant from Dokkyo University School of Medicine to K. S. and a grant from Glaxo Smith Kline to A. J. H. The main part of this work was carried out at the John Curtin School of Medical Research, the Australian National University, Canberra, ACT, Australia.

REFERENCES

1 Gonda TJ, Macmillan EM, Townsend PV, Hapel AJ: Differentiation state and responses to hematopoietic growth factors of murine myeloid cells transformed by myb. Blood 82:2813-2822, 1993

2 Hapel AJ, Warren HS, Hume DA: Different colony-stimulating factors are detected by the "interleukin-3"-dependent cell lines FDC-Pl and 32D cl-23. Blood 64:786-790, 1984

3 Hale AJ, Smith CA, Sutherland LC, Stoneman VE, Longthorne VL, *et al*.: Apoptosis : molecular regulation of cell death. Eur J Biochem 236:1-26, 1996

4 Vaux DL, Haeker G, Strasser A: An evolutionary perspective on apoptosis. Cell 76:777-779, 1994

5 Cohen JJ, Duke RC, Fadok VA, Sellins KS: Apoptosis and programmed cell death in immunity. Annu Rev Immunol 10:267-293, 1992

6 Martin SJ, Lennon SV, Bonham AM, Cotter TG: Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. J Immunol 145:1859-1867, 1990

7 Mak N-K, Fung M-C, Leung KN, Hapel AJ: Monocytic differentiation of a myelomonocytic leukemic cell (WEHI 3B JCS) is induced by tumour necrosis factor-α (TNF-α). Cell Immunol 150:1-14, 1993

8 Heller RK, Song K, Fan N, Chang DJ: The p70 tumor necrosis factor receptor mediates cytotoxicity. Cell 70:47-56, 1992

9 Vairo G, Argyriou S, Bordun AM, Whitty G, Hamilton JA: Inhibition of the signaling pathways for macrophage proliferation by cyclic AMP. Lack of effect on early responses to colony stimulating factor-1. J Biol Chem 265:2692-2701, 1990

10 Chen TC, Hinton DR, Zidovetzki R, Hofman FM: Up-regulation of the cAMP/PKA pathway inhibits proliferation, induces differentiation, and leads to apoptosis in malignant gliomas. Lab Invest 78:165-174, 1998

11 Waring P, Khan T, Sjaarda A: Apoptosis induced by gliotoxin is preceded by phosphorylation of histone H3 and enhanced sensitivity of chromatin to nuclease digestion. J Biol Chem 272:17929-17936, 1997

12 Goswami R, Dawson SA, Dawson G: Cyclic AMP protects against staurosporine and wortmannin-induced apoptosis and opioid-enhanced apoptosis in both embryonic and immortalized (F-11$\times$7) neurons. J Neurochem 70:1376-1382, 1998

13 Takano M, Arai T, Mokuno Y, Nishimura H, Nimura Y, *et al*.: Dibutyryl cyclic adenosine monophosphate protects mice against tumor necrosis factor-α-induced hepatocyte apoptosis accompanied by increased heat shock protein 70 expression. Cell Stress Chaperones 3:109-117, 1998

14 Harada H, Becknell B, Wilm M, Mann M, Huang LJ, *et al*.: Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol Cell 3:413-422, 1999

15 Gonda TJ, Buckmaster C, Ramsay RG: Activation of c-myb by carboxy-terminal truncation : relationship to transformation of murine haemopoietic cells *in vitro*. EMBO J 8:1777-1783, 1989

16 Gonda T, Ramsay RG, Johnson GR: Murine myeloid cell lines derived by *in vitro* infection with recombinant c-myb retroviruses express myb from rearranged vector proviruses. EMBO J 8:1767-1775, 1989

17 Barry MA, Eastman A: Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. Arch Biochem Biophys 300:440-450, 1993

18 Wyllie AH: Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284:555-556, 1980

19 Shankey TV, Rabinovitch PS, Bagwell B, Bauer KD, Duque RE, *et al*.: Guidelines for implementation of clinical DNA cytometry. International Society for Analytical Cytology. Cytometry 14:472-477, 1993

20 Darzynkiewicz Z, Williamson B, Carswell EA, Old LJ: Cell cycle-specific effects of tumor necrosis factor. Cancer Res 44:83-90, 1984

21 Birnbaumer L, Abramowitz J, Brown AM: Receptor-effector coupling by G proteins. Biochem Biophys Acta 1031:163-224, 1990

22 Andrisani OM: CREB-mediated transcriptional control. Crit Rev Eukaryot Gene Expr 9:19-32, 1999

23 Anderson KL, Anderson G, Michell RH, Jenkinson EJ, Owen JJ: Intracellular signalling pathways involved in the induction of apoptosis in immature thymic T lymphocytes. J Immunol 156:4083-4091, 1996

24 McConkey DJ, Orrenius S, Jondal M: Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. J Immunol 145:1227-1230, 1990

25 Walsh DA, Glass DB: Utilization of the inhibitor protein of adenosine cyclic monophosphate-dependent protein kinase, and peptides derived from it, as tools to study adenosine cyclic monophosphate-mediated cellular processes. Methods Enzymol 201:304-316, 1991

26 Arai T, Hiromatsu K, Kobayashi N, Takano M, Ishida H, *et al*.: IL-10 is involved in the protective effect of dibutyryl cyclic adenosine monophosphate on endotoxin-induced inflammatory liver injury. J Immunol 155:5743-5749, 1995

27 Uotila P: The role of cyclic AMP and oxygen intermediates in the inhibition of cellular immunity in cancer. Cancer Immunol Immunother 43:1-9, 1996