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Inhibition of tumor necrosis factor-α induced neutrophil apoptosis by cyclic AMP: involvement of caspase cascade

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Abstract

Treatment of neutrophils with tumor necrosis factor-α (TNF-α) in the presence of cycloheximide induced apoptosis within 3 h, as evaluated by the occurrence of morphological nuclear changes characteristic of apoptosis. Pretreatment of neutrophils with dibutyryl cyclic AMP (dbcAMP) suppressed the TNF-α/cycloheximide-induced apoptosis in neutrophils in a concentration-dependent manner, while dbcAMP by itself did not induce any morphological changes. Forskolin, or a phosphodiesterase inhibitor, also produced a concentration-dependent inhibition on apoptosis. This inhibition by dbcAMP was completely reversed by pretreatment with the protein kinase A inhibitor, *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinoline sulphonamide (H-89). DbcAMP also inhibited the TNF-α/cycloheximide-induced activation of caspase-3, but it had no effect on the activation of caspase-8 in human neutrophils. Furthermore, dbcAMP did not directly inhibit activated caspase-3 activity. Inhibitor of protein kinase C, phosphatidylcholine-specific phospholipase C, tyrosine kinase, nitric oxide synthase, or granulocyte colony-stimulating factor or granulocyte monocyte colony-stimulating factor did not affect apoptosis. These results indicate that the elevation of levels of endogenous intracellular cyclic AMP and subsequent activation of protein kinase A play a crucial role in the prevention of apoptosis triggered by TNF-α/cycloheximide in human neutrophils, and that the possible target of cyclic AMP is a product in the metabolic pathway between caspase-8 and caspase-3.

Keywords: Apoptosis; Blood neutrophil; Human; cAMP; TNF-α (tumor necrosis factor-α); Cycloheximide; Caspase

1. Introduction

Neutrophils take part in host defense mechanisms against infection and in inflammatory and allergic reactions such as asthma. To fulfill this role, neutrophils migrate from blood to various tissues. The number of neutrophils in the circulation is maintained within a narrow range by a balance between the constant production of cells by bone marrow (Mauer et al., 1960) and their death following spontaneous apoptotic processes (Savill et al., 1989; Grigg et al., 1991). Apoptotic senescent neutrophils in tissue are recognized and phagocytosed by macrophages. This apoptotic process has been suggested to represent an in vivo mechanism to limit the tissue injury caused by neutrophils at sites of inflammation.

Tumor necrosis factor-α (TNF-α), a 17-kDa mammalian cell macrophage/monocyte-derived lymphokine originally defined for its anti-tumor activity, binds to specific receptors on most mammalian cells, having various effects on target cells (Tracey and Cerami, 1994). Recently, TNF-α has been shown to initiate apoptotic cell death and DNA fragmentation in several mammalian cell lines including human leukemia and murine fibrosarcoma cell lines (Obeid et al., 1993). TNF-α is also a potent neutrophil activator, stimulating functions such as adherence, phagocytosis, degranulation and oxidative metabolism (Klebanoff et al., 1986). It is reported that TNF-α induces apoptosis in neutrophils (Murray et al., 1997), and that this effect is greatly enhanced in the presence of cycloheximide (Takeda et al., 1993; Tsuchida

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et al., 1995; Niwa et al., 1997). Furthermore, it has been reported that with longer incubation periods cycloheximide (and actinomycin D) by itself accelerates spontaneous neutrophil apoptosis (Whyte et al., 1997). Although the mechanism of cycloheximide has been not clarified, endogenous survival proteins may be involved in neutrophil apoptosis (Whyte et al., 1997). These apoptotic processes may be a protective mechanism to limit neutrophil-induced tissue damage in both rats (Tsuchida et al., 1995) and humans (Niwa et al., 1997).

Cyclic AMP and its modulating drugs have been widely used in the clinics for the treatment of various disease states. They have been used in cases where superoxide or neutrophil dysfunction is believed to play a role in the pathogenesis of disease, e.g., asthma, ischemic diseases, and hypertension (McCord, 1992). Previous reports have established that the second messenger cAMP can regulate certain neutrophil functions, such as inhibition of chemotactic activity (Harvath et al., 1991), enzyme release (Ignarro and George, 1974), and superoxide production (Simchowitz et al., 1980). With regard to the relation between cyclic AMP and apoptosis, elevated levels of intracellular cAMP are known to induce of apoptotic cell death in rat leukemia cells (Gjertsen et al., 1994), primary granuloma cells (Aharoni et al., 1995), thymocytes (McConkey et al., 1990; Sakuta et al., 1996), and in human glomerular mesangial cells (Muhl et al., 1996), B lymphocytes (Lomo et al., 1995) and cells of the leukemic T cell line (Kiefer et al., 1995). In contrast, however, it is also reported that elevation of levels of intracellular cyclic AMP inhibits apoptotic cell death in murine macrophages (Messmer et al., 1995) and T lymphocyte hybridoma cells (Hoshi et al., 1994), in human eosinophils (Hallsworth et al., 1996) and in rat cerebellar granule cells (Chang et al., 1996). In the case of neutrophils, contradictory results have been reported: elevation of intracellular cyclic AMP levels shortened neutrophil survival by accelerating apoptosis (Aoshiba et al., 1995), but inhibited spontaneous apoptosis (Rossi et al., 1995). Furthermore, both effects of cyclic AMP on apoptotic process in neutrophils were antagonized by a specific protein kinase A inhibitor (Aoshiba et al., 1995; Rossi et al., 1995). It has been reported that prostaglandin E_2 also inhibits spontaneous neutrophil apoptosis, due to protein kinase A activation (Ottonello et al., 1998). More recently, Parvathenani et al. (1998) reported that dbcAMP inhibited caspase-3 activation in human neutrophils; however, this inhibition was not antagonized by treatment with a protein kinase A inhibitor. Thus, the role of endogenous cyclic AMP in neutrophil apoptosis remains unknown.

Recently, Kizaki et al. (1993) reported that TNF-α enhances cyclic AMP-induced programmed cell death in mouse thymocytes. We further investigated the relationship between TNF-α and cyclic AMP by testing whether cyclic AMP inhibits or accelerates neutrophil apoptosis triggered by TNF-α. Therefore, in the present study, we evaluated the effects of cell-permeable cyclic AMP (dibutyryl cAMP, dbcAMP) and endogenous cyclic AMP-elevating reagents, forskolin and type IV phosphodiesterase inhibitor, on TNF-α triggered neutrophil apoptosis. Furthermore, we also attempted to determine the site of action of protein kinase A in the TNF-α-induced apoptosis pathway.

2. Materials and methods

2.1. Materials

Cycloheximide, ribonuclease-A, proteinase-K, ethidium bromide, genistein, potassium tricyclo-(5,2,1,0)-decyl-[9(8)-xanthogenate] (D609), N^{ω}-nitro-L-arginine methyl ester (L-NAME) and histopaque were purchased from Sigma (USA). DbcAMP, dibutyryl cyclic GMP (dbcGMP) and granulocyte–macrophage colony-stimulating factor (GMCSF) were purchased from Funakoshi (Japan). *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulphonamide (H-89) was obtained from Calbiochem (Nottingham, UK). Triton X-100, Agarose DNA marker, and Hoechst 33258 were obtained from Kishida Kagaku (Japan), Nippon Gene (Japan), Life Technologies (USA) and Wako (Japan), respectively. Dextran (M.W. 208,000), HEPES, Ac-DEVD-AMC, and Z-IETD-AFC were purchased from Nacalai (Japan), DOJIN (Japan), Peptide Institute, (Japan) and BRL (Japan), respectively. Recombinant human TNF-α and granulocyte colony-stimulating factor (GCSF) were kind gifts from Dainippon Pharmaceutical (Japan) and Kirin Brewery (Japan), respectively. Radioimmunoassay systems for cyclic AMP was purchased from Yamasa (Japan).

2.2. Preparation of neutrophils

Human neutrophils were isolated as previously described (Boyum, 1968) with minor modifications (Niwa et al., 1995, 1996, 1997). Briefly, venous blood from healthy volunteers was collected on sodium citrate solution (3.8%), centrifuged ($110 \times g$, 10 min), and the platelet-rich plasma was discarded. The remaining part of the blood was mixed (1:1, v:v) with a solution of 3% dextran in 0.9% sodium chloride solution in a plastic syringe and fixed vertically for 20 min at 25°C. Neutrophil-rich plasma was collected from the upper layer of the suspension and centrifuged ($250 \times g$, 10 min). The pellet was subjected to hypotonic lysis to destroy the remaining erythrocytes, centrifuged and then suspended in HBSS (Hank's Balanced Salt Solution containing 10 mM HEPES, pH 7.4). The suspension was cushioned carefully on Histopaque solution ($d = 1.077$) and centrifuged ($420 \times g$, 30 min) at 20°C. The purified neutrophils of the bottom pellet were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum,

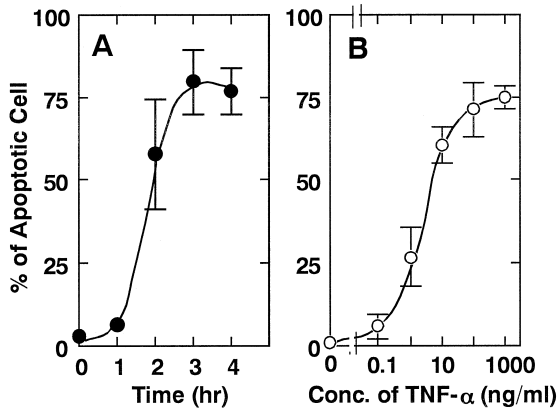

Fig. 1. TNF-α-induced apoptosis in human neutrophils. The time course (A) and dose dependence (B) of TNF-α-induced apoptosis in human neutrophils in the presence of cycloheximide are shown. (A) Human neutrophils were incubated with TNF-α in the presence of 1 μg/ml cycloheximide at 37°C for the indicated times (h). (B) Human blood neutrophils were incubated at 37°C for 3 h with TNF-α in the presence of 1 μg/ml cycloheximide. Then May–Grünwald–Giemsa staining was performed and apoptotic cells were counted as described in Section 2. Each value represents the mean ± S.D. of three separate experiments.

300 mg/ml L-glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin (RPMI 1640 medium). Purification of neutrophils was performed to minimize exposure of the cells to bacterial endotoxin.

The purity of neutrophils was greater than 95%. Cells were counted with a Coulter counter model ZM (Coulter, USA) and diluted in RPMI 1640 medium to the final concentrations and kept on ice until examined.

2.3. Evaluation of apoptosis

For morphological assessment, neutrophils were suspended at 2×10^6/ml in RPMI 1640 medium and then incubated with TNF-α and cycloheximide at 37°C for up to 3 h. Neutrophils incubated under specific conditions were spun down onto a glass slide in a cytocentrifuzer (CF-12SB, Sakurai-Seiki, Japan), dried in cool air, and stained with May–Giemsa solution (Merck, Germany) for light microscopic evaluation. The percentage of apoptotic cells was assessed by counting at least 500 cells/slide (Niwa et al., 1997). To confirm the appearance of nuclear chromatin condensation in apoptotic neutrophils, Hoechst 33258 staining was also performed.

DNA fragmentation in neutrophils was analyzed by using agarose gel electrophoresis. Neutrophils (2×10^6) were harvested and incubated in 100 μl of 10 mM Tris–HCl, pH 7.4, containing 10 mM EDTA and 0.5% Triton X-100 for 10 min at 4°C, and then centrifuged at 22,000 × *g* for 20 min. The supernatant was collected and incubated with 2 μl of 20 mg/ml ribonuclease-A at 37°C for 1 h. Two microliters of 20 mg/ml proteinase-K was then added and the incubation was continued for an additional 1 h. After the incubation, the mixture was kept at −20°C overnight with 120 μl of isopropyl alcohol and 20 μl of 5 M NaCl. Then the mixture was centrifuged at 22,000 × *g* for 15 min, the supernatant was discarded and the pellet was dissolved in 15-μl 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 0.25% bromophenol blue and 40% sucrose. Samples were loaded into each well of 2% agarose gels, and electrophoresis was carried out at 100 V for 1 h. The DNA in gels was visualized under ultraviolet light after staining with ethidium bromide (Niwa et al., 1997).

2.4. Measurement of caspase-3 and caspase-8 activity

Neutrophils were harvested after being exposed to TNF-α/cycloheximide for 3 h for caspase-3 or for 20 min for caspase-8 determination, in the presence or absence of dbcAMP, and resuspended in hypotonic lysis buffer (25 mM HEPES, pH 7.5, containing 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A and 10 μg/ml leupeptin). Then cells were lysed by subjecting them to four cycles of freezing and thawing. After centrifugation (15,000 × *g*, for 20 min at 4°C) of the cell lysates, the supernatant was used for the measurement of caspase activity. Caspase-3 and caspase-8 activities of the cell extracts were determined by using Ac-DEVD-AMC, a specific caspase-3 substrate, and Z-IETD-AFC, a specific caspase-8 substrate, as described previously (Nicholson et al., 1995). Caspase-3 and caspase-8 activities are expressed as the amount of AMC (7-amino-4-methylcoumarin) cleaved from Ac-DEVD-AMC and AFC (7-amino-4-trifluoromethyl coumarin) cleaved from Z-IETD-AFC, respectively, measured with a spectrofluorometer (Fluoroskan, Dainippon Pharmaceutical, Japan).

2.5. Cyclic AMP measurement

Cyclic AMP was measured by using the iodinated assay system of Yamasa (Japan). The experiment was performed

Table 1
Apoptosis in human neutrophils (expressed as a percentage) triggered by TNF-α and/or cycloheximide

Control	1.8 ± 1.0
TNF-α (100 ng/ml)	2.3 ± 2.0
cycloheximide (1 μg/ml)	3.6 ± 1.5
TNF-α (100 ng/ml) + cycloheximide (1 μg/ml)	75.0 ± 8.0[a]

Human neutrophils were incubated at 37°C for 3 h with 100 ng/ml TNF-α and/or 1 μg/ml cycloheximide. Then, May–Grünwald–Giemsa staining was performed and apoptotic cells were counted as described in Section 2. The values represent the mean ± S.D. of four separate experiments. Statistical significance ([a]$P < 0.05$) was determined by the Mann–Whitney-*U*-test comparing treatment vs. vehicle control.

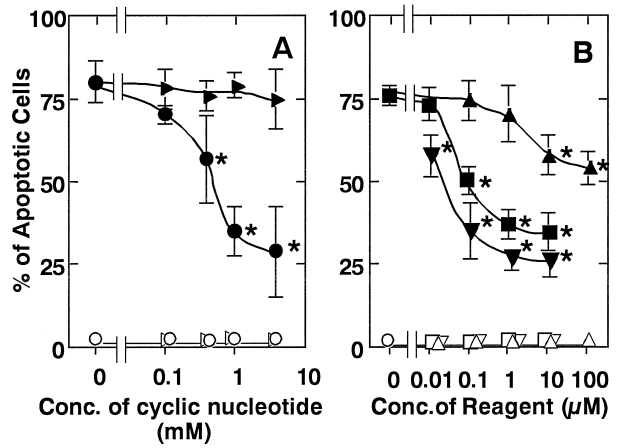

Fig. 2. Effect of cyclic nucleotides (A) and cyclic AMP-elevating agents (B) on TNF-α-induced neutrophil apoptosis. After treatment with db-cAMP (A, ● and ○), dibutyryl cyclic GMP (A, ▶ and ▷), forskolin (B, ▲ and △), Ro 2017–24 (B, ■ and □), or Ro 2017–24 plus 1 μM forskolin (B, ▼ and ▽) for 10 min, neutrophils were incubated for 3 h with 100 ng/ml TNF-α and 1 μg/ml cycloheximide (closed symbols) or vehicle (open symbols). Then, May–Grünwald–Giemsa staining was performed and apoptotic cells were counted as described in Section 2. Each value represents the mean ± S.D. of four separate experiments. Statistical significance (*$P < 0.05$) was determined by the Mann–Whitney-*U*-test comparing treatment vs. vehicle control.

by exposing 1.4×10^6 cells/ml to Ro-201724 and/or forskolin. The reaction was terminated after 3 min by addition of 0.1 volume of 20% ice-cold perchloric acid and incubation in an ice bath. The cyclic AMP content was determined in 100 μl aliquots of the supernatant by radioimmunoassay (Volker et al., 1985; Niwa et al., 1995).

3. Results

3.1. TNF-α triggered apoptosis in human circulating blood neutrophils

Following the addition of TNF-α (100 ng/ml) to the incubation medium, the proportion of neutrophils demonstrating morphological features of apoptosis, as revealed by nuclear pyknosis or chromatin condensation, progressively increased with time, reaching a maximum at approximately 3 h (75 ± 8%) in the presence of 1 μg/ml cycloheximide (Figs. 1A and 3). The addition of 0.1–1000 ng/ml of TNF-α to the incubation medium, which contained 1 μg/ml cycloheximide, resulted in a concentration-dependent increase in the proportion of apoptotic cells (Fig. 1B). No significant changes in the number of apoptotic cells was seen after treatment with either TNF-α or cycloheximide alone (Table 1).

3.2. Effect of dbcAMP, forskolin and Ro-201724 on TNF-α-induced apoptosis in neutrophils

Pretreatment of neutrophils with dbcAMP (0.1 to 4 mM), a stable and membrane-permeable analogue of cAMP, resulted in a concentration-dependent decrease in the number of apoptotic cells at 3 h with an IC50 of 0.62 mM. Apoptosis was triggered with 100 ng/ml TNF-α plus 1 μg/ml cycloheximide (Figs. 2A and 3). DbcGMP, a related cyclic nucleotide analogue, in the same concentration range did not affect neutrophil apoptosis. DbcAMP by itself did not cause morphological changes (Fig. 2A). We also evaluated the morphology of neutrophils by fluorescence microscopy after Hoechst 33258 staining. Use of

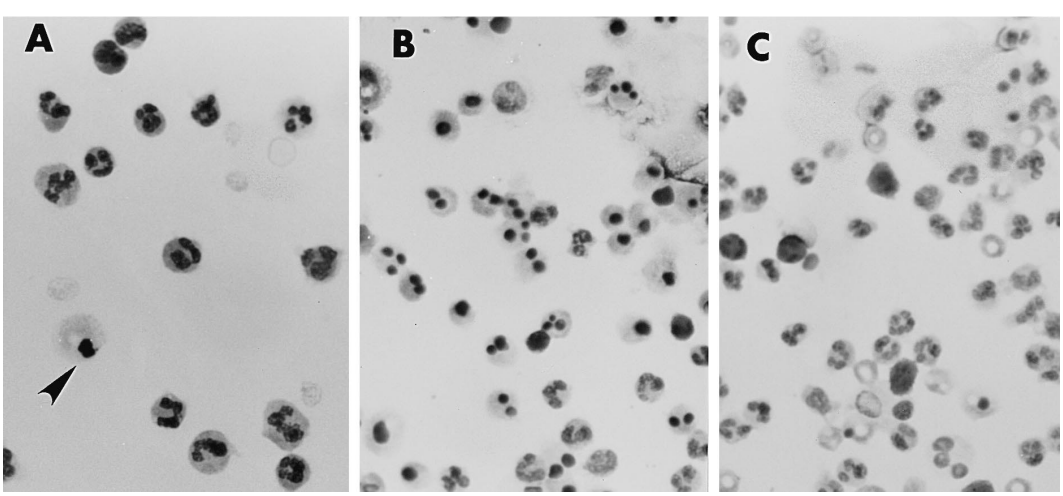

Fig. 3. Morphological features of apoptosis induced in neutrophils by treatment with TNF-α (100 ng/ml) in the presence of 1 μg/ml cycloheximide, and its inhibition by pretreatment with dbcAMP. (May–Grünwald–Giemsa staining, X600). Untreated peripheral blood neutrophils incubated at 37°C for 3 h with vehicle have multilobular nuclei (A). One neutrophil (arrowhead) shows morphological feature of spontaneous apoptosis. After treatment with vehicle (B) or dbcAMP (4 mM, C) for 10 min, neutrophils were incubated with TNF-α (100 ng/ml) and 1 μg/ml cycloheximide for 3 h. Nuclei show chromatin-condensed apoptotic bodies (B). This apoptosis was inhibited by pretreatment with dbcAMP (C).

this assay method confirmed the finding that dbcAMP protected against the apoptotic effect of TNF-α plus cycloheximide (data not shown). To investigate the step(s) by which the cyclic AMP-sensitive element affects the pathway leading to apoptosis induced by TNF-α/cycloheximide, dbcAMP was added 10 min before, or 30 or 60 min after TNF-α/cycloheximide addition. The addition of dbcAMP 10 min before treatment with TNF-α/cycloheximide significantly inhibited TNF-α/cycloheximide-induced apoptosis, whereas no significant effect was observed when it was added 30–90 min after treatment of neutrophils with TNF-α/cycloheximide (Fig. 4).

As with cyclic AMP, preincubation of neutrophils with reagents that elevate endogenous cyclic AMP, such as forskolin, a direct activator of adenylyl cyclase, and Ro-201724, a type IV phosphodiesterase inhibitor, caused a concentration-dependent inhibition of TNF-α/cycloheximide-induced apoptosis (Fig. 2B). The maximum inhibition seen with forskolin was relatively weaker than that produced by Ro-201724 and dbcAMP. Concomitant treatment of neutrophils with both agents enhanced the inhibition of TNF-α/cycloheximide-induced apoptosis (Fig. 2B). In separate experiments, we evaluated the effects of these agents on the intracellular cyclic AMP level in neutrophils. Treatment of neutrophils with either forskolin, Ro-201724, or both significantly increased the cyclic AMP level (Table 2). It appears that there was a correlation between the increased cyclic AMP level produced by these compounds and their ability to inhibit TNF-α/cycloheximide-induced neutrophil apoptosis. These observations indicate that the accumulation of cyclic AMP plays a key role in the inhibition of TNF-α/cycloheximide-induced apoptosis in neutrophils.

Table 2
Effect of forskolin or Ro-201724 on the cyclic AMP level in human neutrophils

	fmol/10^6 cells
Control	59.8 ± 6.7
Forskolin (10 μM)	78.5 ± 3.1[a]
Ro 201724 (10 μM)	83.5 ± 4.5[a]
Forskolin (1 μM) + Ro 201724 (10 μM)	102.2 ± 9.3[a]

Human neutrophils were incubated at 37°C for 3 min with forskolin and/or Ro-201724, and then the intracellular cyclic AMP level of neutrophils was determined as described in Section 2. The values represent the mean ± S.D. of four separate experiments. Statistical significance ([a]$P < 0.05$) was determined by the ANOVA with PLSD test.

3.3. Antagonism by protein kinase A inhibitor of the inhibition of apoptosis by cAMP-related agents

Preincubation of neutrophils with H-89 (1 μM), specific protein kinase A inhibitor (Nishizuka, 1986), completely suppressed the inhibition of TNF-α/cycloheximide-triggered neutrophil apoptosis by dbcAMP (Fig. 5). H-89 (1 μM) had no effect on the morphological changes in intact neutrophils or neutrophils treated with TNF-α alone (without cycloheximide) within 3 h of treatment (data not shown).

3.4. Effect of dbcAMP on TNF-α-triggered DNA fragmentation

To confirm that the above-mentioned inhibition of morphological changes in neutrophils was accompanied by inhibition of DNA fragmentation, which is regarded as

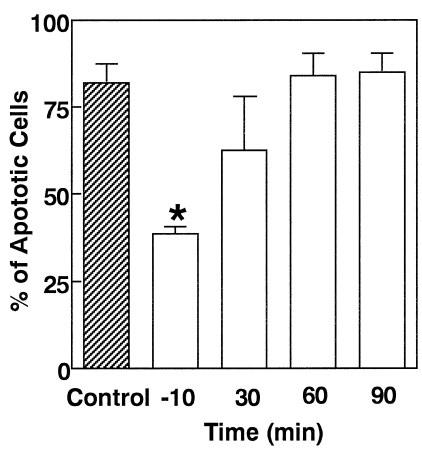

Fig. 4. Effect of time of addition of dbcAMP on the TNF-α-induced apoptosis of neutrophils. Neutrophils were incubated with dbcAMP 10 min before or 30, 60 or 90 min after addition of 100 ng/ml TNF-α and 1 μg/ml cycloheximide. Apoptosis of neutrophils was microscopically evaluated after May–Grünwald–Giemsa staining as described in Section 2. Each value represents the mean ± S.D. of three separate experiments. Statistical significance (*$P < 0.05$) was determined by the Mann–Whitney-*U*-test comparing treatment data with control data measured in the absence of dbcAMP.

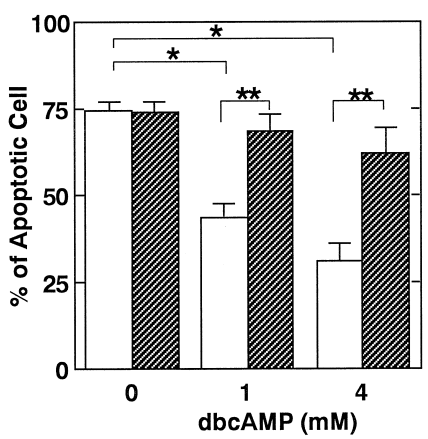

Fig. 5. antagonism by H-89 of inhibition of TNF-α-induced neutrophil apoptosis by dbcAMP. After pretreatment neutrophils with H-89 (rectangle with diagonal lines) or vehicle (rectangle), the inhibitory effect of dbcAMP on TNF-α-induced apoptosis in the presence of 1 μg/ml cycloheximide was assessed after May–Grünwald–Giemsa staining as described in Section 2. Each value represents the mean ± S.D. of four separate experiments. Statistical significance was determined by the Mann–Whitney-*U*-test. *$P < 0.05$ vs. without dbcAMP; **$P < 0.05$ vs. without H-89.

another indicator of apoptosis, DNA was extracted from neutrophils and electrophoresed on agarose gels (Fig. 6). Concomitant treatment of neutrophils with TNF-α and cycloheximide caused marked DNA fragmentation, showing a distinctive 'ladder pattern' of multiple approximately 200-base pair fragments. This indicates that there was activation of an endonuclease, which is characteristic of apoptosis (Fig. 6, lane 1). DbcAMP (1.0 mM, lane 2) inhibited this DNA fragmentation, an effect that was correlated with the inhibition of the morphological changes.

3.5. Activation of caspase-3 and caspase-8 by TNF-α/cycloheximide, and their inhibition by dbcAMP

The morphological and DNA fragmentation results strongly suggest that dbcAMP inhibits TNF-α/cycloheximide-induced neutrophil apoptosis. Members of the caspase family, especially caspase-3, are regarded as important regulators of apoptosis. Caspase-8, which acts upstream of caspase-3, is also thought to affects apoptosis pathways, especially TNF-α-mediated apoptosis (Boldin et al., 1996). However, the contribution of caspase-3 and caspase-8 to TNF-α-induced neutrophil apoptosis has not been clarified. Therefore, we evaluated the effects of cyclic AMP on caspase-3 and caspase-8 activation in human neutrophils, using their specific fluorogenic tetrapeptide substrates. In the presence of 1 μg/ml cycloheximide, 100 nM of TNF-α significantly increased caspase-3 and caspase-8 activities. This caspase-3 activation was reduced by pretreatment with dbcAMP in a concentration-dependent manner (Table 3). No significant inhibition of caspase-8 activity was observed following pretreatment with dbcAMP (Table 3). Furthermore, we also evaluated whether dbcAMP could directly inhibit activated caspase-3. DbcAMP did not affect the activity of caspase-3 in the caspase-rich fraction from TNF-α- and cycloheximide-treated neutrophils (data not shown).

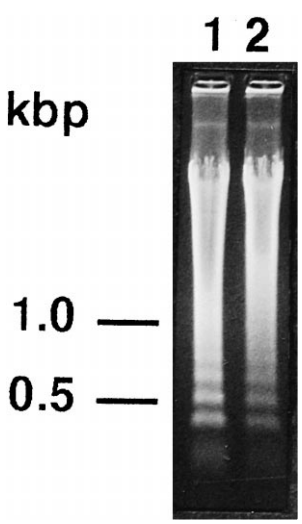

Fig. 6. Effect of dbcAMP on DNA fragmentation induced by treatment of neutrophils with TNF-α in the presence of 1 μg/ml cycloheximide. After treatment with vehicle (lane 1) or dbcAMP (1.0 mM, lane 2) for 10 min, human neutrophils were incubated with TNF-α (100 ng/ml) and 1 μg/ml cycloheximide for 2 h. DNA extracted from neutrophils was electrophoresed on a 1% agarose gel.

Table 3
Inhibition by dbcAMP of TNF-α/cycloheximide-induced caspase-3 and caspase-8 activation in human neutrophils

TNF-α + cycloheximide	dbcAMP (mM)	Caspase activity (pmol/mg/min)	
		Caspase-3	Caspase-8
–	–	15.3 ± 11.5	57.6 ± 14.5
+	–	340.8 ± 35.0[a]	128.0 ± 14.2[a]
+	0.1	288.9 ± 28.1[a]	120.9 ± 20.0[a]
+	0.4	255.6 ± 31.2[a,b]	120.5 ± 9.8[a]
+	1	207.1 ± 25.9[a,b]	119.3 ± 17.8[a]
+	4	194.5 ± 20.5[a,b]	118.5 ± 16.8[a]

Human neutrophils were incubated at 37°C for the times indicated in Section 2 with 100 ng/ml TNF-α and 1 μg/ml cycloheximide. Then, both caspase activities in neutrophils were determined as described in Section 2. DbcAMP was added 10 min before TNF-α and cycloheximide. The values represent the mean ± S.D. of four separate experiments. Statistical significance ($P < 0.05$) was determined by the ANOVA with PLSD test.
[a,b] Indicate significant difference from intact neutrophils and TNF-α + cycloheximide-treated control neutrophils, respectively.

3.6. Effect of calphostin C, D609, genistein, L-NAME and GCSF and GMCSF on TNF-α-induced apoptosis of neutrophils

Calphostin C (20–200 nM), a protein kinase C inhibitor, D609 (5–50 μg/ml), a phosphatidylcholine-specific phospholipase C inhibitor, genistein (5–50 μM), a tyrosine kinase inhibitor, or L-NAME (10–100 μM), a nitric oxide synthase inhibitor, did not affect TNF-α/cycloheximide-induced apoptosis in human neutrophils. Neither GCSF (100–2500 ng/ml) nor GMCSF (100–2500 ng/ml) had a significant effect on neutrophil apoptosis (data not shown).

4. Discussion

The present study addressed the question whether neutrophil apoptosis triggered by TNF-α in the presence of cycloheximide is inhibited by an elevation of intracellular levels of cyclic AMP ex vivo. Elevation of intracellular levels of cyclic AMP, achieved by adding the cell membrane-permeable analogue of cyclic AMP, dbcAMP, by activating adenylyl cyclase with forskolin, or by inhibiting the hydrolysis of cyclic AMP with Ro-201724, a type IV phosphodiesterase inhibitor, significantly inhibited neutrophil apoptosis triggered by TNF-α in a dose-dependent fashion in the presence of cycloheximide. In addition, H-89, a specific inhibitor of cyclic AMP-dependent protein kinase A, completely suppressed the inhibitory effect of dbcAMP. These findings suggest that the inhibitory effects

of these agents resulted from an increase in intracellular cyclic AMP levels in neutrophils. Furthermore, dbcAMP also inhibited TNF-α/cycloheximide-induced caspase-3 activation in human neutrophils, while it had no effect on TNF-α/cycloheximide-induced caspase-8 activation. DbcAMP did not appear to produce its inhibition by a direct action on caspase-3. Since it has been reported that caspase-8 is upstream of caspase-3 in TNF-α mediated apoptosis (Boldin et al., 1996), the results of the studies with caspase substrates suggest that a possible target site cyclic AMP is a product in the metabolic pathway between caspase-8 and caspase-3.

It has been reported that cycloheximide enhances the capacity of TNF-α to induce apoptosis in neutrophils (Takeda et al., 1995; Niwa et al., 1997), suggesting that certain protective protein(s), such as manganous superoxide dismutase (Wong and Goeddel, 1988), counteract the cytotoxicity of TNF-α on neutrophils and that protein synthesis is not required for the stimulation of apoptosis by TNF-α even though it has been shown that protein synthesis is a prerequisite for the induction of apoptosis in other systems (Shi et al., 1989). Additionally, treatment with cycloheximide alone resulted in an acceleration of apoptosis (Takeda et al., 1993; Whyte et al., 1997). These results suggest that spontaneous apoptosis in neutrophils is usually inhibited by synthesized protein(s). In the present study, because cyclic AMP inhibited TNF-α/cycloheximide-triggered apoptosis in neutrophils, the target of cyclic AMP in its inhibition of apoptosis might not be protein synthesis.

Cyclic AMP has previously shown to have an inhibitory effect on neutrophil function. An elevation of cyclic AMP levels is associated with the inhibition of several physiological functions in neutrophils including chemotaxis (Harvath et al., 1991), enzyme release (Ignarro and George, 1974) and oxygen burst (Simchowitz et al., 1980). The present observation that cyclic AMP inhibited neutrophil apoptosis induced by TNF-α suggests a critical role of this intracellular second messenger in controlling the turnover of human neutrophils. Inhibition of cyclic AMP hydrolysis with selective inhibitors of phosphodiesterase has also been used in various studies to elevate cyclic AMP levels. In neutrophils collected from several species, cyclic AMP is degraded by type IV phosphodiesterase (Nielson et al., 1990). Consistent with this finding, we reported in our previous paper that treatment of human or rabbit neutrophils with a selective inhibitor of type IV phosphodiesterase, Ro-201724, led to an increase in cAMP levels, resulting in the inhibition of formyl-methionyl leucyl–phenylalanine (fMLP)-induced superoxide generation (Al-Essa et al., 1995). Forskolin is another agent that elevates intracellular cyclic AMP levels by directly activating adenylyl cyclase in neutrophils (Tyagi et al., 1991). We also confirmed in this study that both forskolin and Ro-201724 elevated intracellular cyclic AMP levels in neutrophils. Similar to dbcAMP, both forskolin and Ro-201724 significantly inhibited TNF-α/cycloheximide-induced neutrophil apoptosis, although the maximal inhibition achieved with forskolin was smaller than that achieved with Ro-201724. This may suggest the existence of a threshold level of intracellular cyclic AMP for protecting against TNF-α/cycloheximide-induced neutrophil apoptosis. The cause of the inconsistency between cAMP levels and apoptosis is not known but future experiments are being designed to address this apparent paradox. The ability of these agents, which modulate intracellular cyclic AMP levels, to inhibit TNF-α/cycloheximide-induced apoptosis correlated with those for suppressing superoxide generation stimulated by fMLP as previously reported (Al-Essa et al., 1995). Apoptotic neutrophils display a reduced ability to respond to stimulation with the receptor-dependent stimulus, fMLP, by undergoing respiratory burst (Whyte et al., 1993). Although the underlying mechanism still remains to be clarified, inhibition of neutrophil function by intracellular cyclic AMP accumulation leads to the prevention of apoptosis of neutrophils triggered by TNF-α/cycloheximide.

Although the activation of protein kinase A is the main pathway subsequent to intracellular cyclic AMP accumulation, there is evidence that cyclic AMP can also activate protein kinase G (Lincoln et al., 1990). In our experiments, however, a membrane-permeable cyclic GMP analogue did not affect neutrophil survival, indicating that protein kinase G is not involved in the control of apoptotic death in human neutrophils.

Nathan and Sanchez (1990) reported that an increase in intracellular cyclic AMP levels blocked the TNF-α-induced respiratory burst in neutrophils when it occurred any time before, but not after, the onset of superoxide generation. This finding indicates that a fall in intracellular cyclic AMP levels is a prerequisite for the TNF-α-induced respiratory burst. Once the respiratory burst has started, however, the level of intracellular cyclic AMP is no longer important. We found similar results in our experiment: TNF-α/cycloheximide-induced apoptosis of neutrophils could be suppressed only if the cyclic AMP analogue or cyclic AMP-elevating agents were added *before* treatment with TNF-α. This indicates that the apoptotic process in neutrophils starts immediately after the addition of TNF-α and that cyclic AMP is no longer a determinant after initiation of the apoptotic process.

Nitric oxide also facilitates the apoptotic process in macrophages (Albina et al., 1993; Messmer et al., 1994; Shimaoka et al., 1995) and thymocytes (Fehsel et al., 1995), whereas it is reported to inhibit apoptosis in ovarian follicles (Chun et al., 1995), human eosinophils (Beauvais et al., 1995) and B lymphocytes (Genaro et al., 1995). In addition, there is a report that nitric oxide protects D-galactosamine-sensitized mouse liver from TNF-α/cycloheximide-induced apoptosis (Bohlinger et al., 1995). The present results showed that pretreatment of neutrophils with the nitric oxide synthetase inhibitor, L-

NAME, as well as with the cell permeable analogue, dbcGMP, had no effect on TNF-α/cycloheximide-induced apoptosis. These results suggest that the nitric oxide signaling pathway, at least the cyclic GMP-dependent component, is unlikely to be involved in neutrophil apoptosis triggered by TNF-α/cycloheximide.

Recent evidence indicates that phosphatidylcholine-specific phospholipase C is involved in the cytotoxic action of TNF-α in L929 and Wehi164 cells (Machleidt et al., 1996) and TNF-α-mediated NF-kappa B activation (Rensing-Ehl et al., 1995). To determine whether phosphatidylcholine-specific phospholipase C contributes to the inhibitory action of cyclic AMP on TNF-α-triggered apoptosis in neutrophils, we assessed the effect of D609, a specific phosphatidylcholine-specific phospholipase C inhibitor. The result indicated that phosphatidylcholine-specific phospholipase C does not contribute to apoptosis.

Although the functional activation of neutrophils in response to various external stimuli has been widely investigated, the modulation of neutrophil survival is still unclear. GCSF is reported to protect mature neutrophils from apoptosis (Lopez et al., 1986; Begley et al., 1986). Contrary to this, GCSF and GMCSF failed to inhibit TNF-α/cycloheximide triggered apoptosis of neutrophils in our experiment. This discrepancy may indicate that CSF and cyclix AMP inhibit apoptosis by different mechanisms. Interestingly, Hallsworth et al. (Hallsworth et al., 1996) reported that cyclic AMP inhibited the GMCSF-induced survival of human eosinophils, whereas, in the absence of GMCSF, cyclic AMP acted as inhibitor of spontaneous apoptosis in the same cells. Furthermore, they also reported that the inhibition by cyclic AMP of the GMCSF-induced prolongation of survival, but not the inhibition by cyclic AMP of spontaneous apoptosis, was dependent on the protein kinase A pathway. These data, taken together with ours, indicate that in neutrophils TNF-α/cycloheximide triggered apoptosis is mechanistically different from spontaneous apoptosis.

In conclusion, elevation of the level of endogenous intracellular cyclic AMP plays a crucial role in preventing apoptosis triggered by TNF-α/cycloheximide in human neutrophils. Studies with the caspase substrate suggest that cyclic AMP acts upstream of caspase-3, and downstream of caspase-8, although the exact target sites have still to be elucidated. It is suggested that this second messenger may be an important factor in the regulation of neutrophil number in the circulation.

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