Role of Tumor Necrosis Factor α in Hyperthermia-induced Apoptosis of Human Leukemia Cells

Dörthe M. Katschinski,1 H. Ian Robins, Martina Schad, Stilla Frede, and Joachim Fandrey

Institute of Physiology, Medical University of Lübeck, d-23538 Luebeck, Germany [D. M. K., M. S., J. F.]; Comprehensive Cancer Center, University of Wisconsin, Madison, Wisconsin 53792 [H. I. R.]; and Institute of Physiology, University of Bonn, 53115 Bonn, Germany [S. F.]

ABSTRACT

We used the human myelomonoblastic leukemia cell line PLB-985 to study the effects of temperatures ranging from 37°C to 43°C for 1 h on the induction of apoptosis and cell cycle distribution in leukemia cells. The threshold temperature for the onset of apoptosis was 42°C. Whereas hyperthermia exerted no effect on the expression of Bcl-2 and Bax, heat induced a >30-fold increase of tumor necrosis factor (TNF) α mRNA expression and a significant increase in TNF-α protein secretion. This endogenous production of TNF-α correlated directly with the temperature-induced apoptotic effect. Blocking TNF-α expression via treatment with pyrrolidinedithiocarbamate or blocking TNF-α activity with neutralizing antibodies abrogated heat-induced apoptosis. In addition, exposure of cell culture supernatant of heat-treated PLB-985 cells to untreated cells induced an apoptotic effect. These data indicate a TNF-α-mediated self-eradication of the leukemia cells after heat exposure. Inducing apoptosis with wild-type TNF-α or p55 and p75 protein muteins demonstrated that this effect was mediated by the p55 receptor. Interestingly, the autocrine suicidal loop found in immature leukemia cells was lost after granulocytic differentiation with 0.5% N,N-dimethylformamide. These data should be of critical importance for the understanding of the biological impact of fever as well as for developing therapeutic approaches to malignant diseases.

INTRODUCTION

Apoptosis is a suicidal mode of cell death, which is of critical importance for proper ontogeny and tissue homeostasis as well as in the pathogenesis of a variety of diseases including cancer. Apoptotic cell death requires the active participation of the affected cell in its self-destruction. Activation of a preprogrammed cascade of molecular events will result in DNA degradation and nuclear disintegration, leading to cell death (1). Several protein families such as the Bcl-2 family (2) or the caspases, which are engaged in distinct apoptotic pathways, have been discovered in the past.

Tumor cell apoptosis can be induced by DNA-damaging treatments like chemotherapy or radiotherapy. Equally effective are physiological stress conditions, i.e., growth factor starvation, hypoxia, or heat (3, 4). Abnormalities of these environmental factors are common features in tumor biology and may affect tumor cell proliferation and response to antitumor therapy (5).

Pyrexia associated with immune and inflammatory responses has been implicated in host defenses against infection and neoplasia. In addition, heat is used in conjunction with radiotherapy or chemotherapy, locally or systemically, for antitumor therapy (6). Although fever is generally not directly cytotoxic for malignant cells, a distinct thermal sensitivity of leukemias and lymphomas has been demonstrated in vitro and clinically (7–9). This characteristic property has been related to heat-induced apoptosis (4, 10, 11). However, the threshold temperature and underlying mechanism are not well defined. Without this information, the potential clinical relevance of temperature-induced apoptotic cell death for tumor biology or antitumor strategies remains uncertain. In addition, conceptual approaches of heat-induced cytotoxic effects against tumor cells must address factors affecting therapeutic index, i.e., the relative toxicity of a modality for neoplastic cells versus normal tissues.

To broaden the knowledge about these pending questions, we have determined the effects of short-term exposure to elevated temperatures, i.e., ranging from 37°C to 43°C for 1 h, on the induction of apoptosis, the cell cycle, and Bcl-2 and Bax protein expression in the human promyelocytic leukemia cell line PLB-985. The results show the induction of apoptotic cell death starting at a temperature of 42°C; however, apoptosis was not correlated to changes in Bcl-2 and Bax protein expression. Treatment at 43°C caused a strong induction of TNF-α2 (1) mRNA expression and protein secretion. Additional mechanistic studies using neutralizing TNF antibodies or PDTC revealed a mechanistic link between heat-induced apoptosis and endogenous TNF-α production. Apoptotic cell death and TNF-α induction were prevented when PLB-985 cells were differentiated by treatment with 0.5% DMF for 6 days before heat exposure.

MATERIALS AND METHODS

Chemicals and Antibodies. Wild-type TNF-α (recombinant human; specific activity, 6.6 × 106 units/mg by L929 bioassay) was a gift from BASF/Knoll (Ludwigshafen, Germany). Human TNF-α mutants, which bind specifically to the p55 TNF-α receptor (TNFR-p55/Trp32Thr86TNF-α) or p75 TNF-α receptor (TNFR-p75/Asn143Arg145TNF-α), were provided by Hoffmann La Roche AG (Basel, Switzerland). The TNFR-p55 and TNFR-p75 bind with high affinity to TNF p55 and p75 receptors, respectively, but not vice versa (12, 13). All other chemicals used for flow cytometry and cell culture studies were purchased from Sigma Chemical Co. (St. Louis, MO).

The mouse anti-Bcl-2, rabbit anti-Bax, and goat anti-actin antibodies as well as the appropriate horseradish peroxidase-labeled secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Jose, CA) and used according to the manufacturer’s recommendations. Neutralizing rabbit anti-human TNF-α antibodies were purchased from Genzyme (Cambridge, MA; neutralizing activity, 100 units/μl by L929 bioassay). The nonspecific IgG control antibodies were purchased from Sigma Chemical Co.

Cell Lines and Culture Conditions. The human myelomonoblastic leukemia cell line PLB-985 was a kind gift from M. Dinauer (Herman B. Wells Center for Pediatric Research, Indiana University Medical Center, Indianapolis, IN). The cells were grown in RPMI 1640 with 10% fetal bovine serum in a humidified 5% CO2–95% air atmosphere at 37°C. For a heat experiment, PLB-985 cells were seeded in 25-cm2 tissue culture flasks, allowed to grow for 24 h, and then exposed to the indicated temperature for 1 h. After heat treatment, cells were grown at 37°C, and samples were collected at various times for whole-cell protein extracts, cell cycle distribution/quantitation of apoptotic cells (via flow cytometry), cell counts, fluorescent microscopy, and DNA extraction. Cell viability was determined using the trypan blue exclusion test. Before each experiment, cells were scrutinized to have a viability of >90%. Time 0 was considered the point at which the cells were removed from the water bath after heat treatment. All experiments were repeated at least three times for statistical analysis.

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1 To whom requests for reprints should be addressed, at Institut für Physiologie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany. Phone: 0049-451-500-4152; Fax: 0049-451-500-4151; E-mail: katschinski@physio.muelbeck.de.

2 The abbreviations used are: TNF, tumor necrosis factor; PDTC, pyrrolidinedithiocarbamate; DMF, N,N-dimethylformamide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NBT, nitroblue tetrazolium; ROS, reactive oxygen species.

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times. Data shown in the graphs for various parameters represent the mean ± SE, and data sets were compared using the Student’s t test.

**Induction of Differentiation.** PLB-985 cells were induced to differentiate along a granulocytic pathway for each experiment by treatment with 0.5% (v/v) DMF for 6 days. Every third day, the medium was exchanged, and fresh DMF was added. Functional differentiation was verified by determining respiratory burst activity after stimulation with 1 μM porphol myristate acetate and 800 nM formyl-methionyl-leucin phenylalanine. Under these conditions, burst activity was observed in DMF-PLB cells but not in PLB-985 cells. In addition, the ability to produce ROS was detected by NBT staining (14).

**Temperature Treatment.** The 25-cm² tissue culture flasks were placed into plastic freezer bags and filled with 5% CO²/air before sealing. The flasks were removed from the freezer bags and returned to the 37°C incubator. At frequent time intervals thereafter, cells were assayed for apoptosis by propidium iodide staining and flow cytometry. Data points represent three independent experiments; bars, SE. Typical DNA histograms of PLB-985 cells before heat exposure (b) and 18 h after exposure to 43°C for 1 h (c) are shown.

**Flow Cytometric Analysis of Apoptosis and Cell Cycle Distribution.** At various intervals after the 1-h heat exposure, PLB-985 cells were fixed in 400 μl of 4% ethanol-PBS, resuspended in 500 μl of PBS, and lysed with 50 m M Tris-HCl, 120 m M NaCl, 100 m M NaF, 1 m M EDTA (pH 8), and 0.2% Triton X-100 and treated with 60 μg of proteinase K for 1 h at 50°C. Then 100 μl of 5 m NaCl solution and 500 μl of isopropanol were added, and this solution was incubated at −20°C overnight. The precipitate was resuspended in TE buffer [10 m M Tris and 1 m M EDTA (pH 7.5)], and DNA was digested with 4 units of RNase for 1 h at 50°C. DNA (10 μg) was run on a 1.5% agarose gel and stained with 0.5 μg/ml ethidium bromide.

**Bioassay.** TNF-α-sensitive mouse fibrosarcoma LM cells (ATCC CCL 1.2) were cultured in RPMI 1640 with the addition of 100 units/ml penicillin and 100 μg/ml streptomycin in 25-cm² culture flasks. For the bioassay, LM cells were seeded on 96-well microtiter plates. LM cells were either incubated with a dilution series of TNF-α standards or with PLB-985 culture supernatants for 24 h. The viability of the LM cells was assessed by the tetrazolium salt dye method as described previously (15).

**Western Immunoblot Analyses.** Cells were spun down, washed with PBS, and lysed with 50 μl Tris buffer (pH 8), 120 μl NaCl, 100 μl NaF, 0.5% NP40, 200 μl sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml phenylmethylsulfon fluoride and stored at −80°C. Protein (50 μg) was run on a 15% polyacrylamide gel and blotted onto nitrocellulose membranes by semi-dry electroblotting (Bio-Rad, München, Germany); membranes were stained with Ponceau S to verify equal protein loading per lane. After overnight blocking (5% nonfat milk powder and 0.05% Tween 20 in PBS), blots were probed for 1 h with antibodies against Bcl-2, Bax, or actin (diluted 1:200, 1:200, and 1:500, respectively) and detected with 1:2000 diluted horseradish peroxidase-conjugated antibodies and the enhanced chemiluminescence system (Amersham, Buckinghamshire, England).

**RNA Extraction, Reverse Transcription-PCR, and TNF mRNA Quantitation.** Extraction of total RNA, reverse transcription, and competitive TNF-α mRNA PCR were performed exactly as described previously (16, 17). PCR for the p55 receptor was performed with upstream primer 5'-TCTACTC-CATTG-3' and downstream primer 5'-AGACGCTAGACA-3' for 30 cycles (temperature profile, 94°C for 1 min, 50°C for 1.5 min, and 72°C for 3 min). For GAPDH mRNA PCR, upstream primer 5'-ATCATCCCTGCTCC-TACTGG-3' and downstream primer 5'-TGGGTGTCGCTGTTAAGTC-3'.
were used (temperature profile, 94°C for 1 min, 55°C for 1.5 min, and 72°C for 3 min; 28 cycles). Expression of GAPDH mRNA was used as an internal standard for RNA integrity and equal gel loading.

RESULTS

Hyperthermia-induced Apoptosis. A 1-h exposure of PLB-985 leukemia cells to 42°C or 43°C led to the appearance of preapoptotic sub-G₀-G¹ (<2 N DNA) cells as determined by flow cytometry. No significant numbers of apoptotic cells were observed after treatment with temperatures below 42°C (Fig. 1a). Hyperthermia-induced apoptosis peaked with about 30% apoptotic cells in the population at 18–24 h and disappeared by 48 h after exposure to 43°C. Apoptosis after treatment with 43°C for 1 h was confirmed by nuclear disintegration and condensation as visualized by acridine orange/propidium iodide staining (Fig. 2a). DNA laddering was observed 18 h after exposure to 42°C and 43°C for 1 h, whereas the DNA extracted from cells treated with temperatures below 42°C was found to be intact (Fig. 2b). Thus, the threshold temperature for the induction of apoptosis in undifferentiated PLB-985 cells appears to be 42°C.

In addition to apoptosis, temperature-dependent alterations in cell cycle distribution were also detected. However, in contrast to the induction of apoptosis at 42°C, effects on the cell cycle were already elicited after exposure to 40.5°C and 41°C (Fig. 3). After heat treatment, the number of cells in G₀-G¹ phase decreased continuously with increasing temperatures. Nadir cell counts were observed 18 h after treatment (Fig. 3a). Coincidently, a significant G₂-M-phase cell cycle checkpoint accumulation was observed, which disappeared 24 and 48 h after heat treatment (Fig. 3c). Moreover, 4–8 h after treatment with 40.5°C to 43°C for 1 h, the number of cells in S phase increased significantly (Fig. 3b). The temperature profile and kinetics of cell cycle changes as well as induction of apoptosis correlated with cell survival after hyperthermia treatment: a significant increase in cell number was seen after treatment with 40.5°C for 1 h compared to treatment with 37°C whereas temperatures above 42°C were clearly cytotoxic (Fig. 3d).

Hyperthermia-induced Apoptosis Is No Longer Present after Differentiation with DMF. Most interestingly, when PLB-985 cells were DMF differentiated along the granulocytic pathway (DMF-PLB...
cells), exposure to 42°C or 43°C for 1 h did not result in the induction of apoptotic cell death (Fig. 2b). The DMF-PLB cells presented with an average population of 73.2 ± 1.5% G₀-G₁ phase cells, 18 ± 1.3% S-phase cells, and 8.7 ± 0.3% G₂-M-phase cells after the induction of differentiation compared to 41.2 ± 2.4% G₀-G₁-phase cells, 37 ± 1.1% S-phase cells, and 20.9 ± 1.3% G₂-M-phase cells in non-differentiated PLB-985 cells.

Induction of Apoptosis Does Not Correlate to Bcl-2 or Bax Protein Expression. To gain insight into the hyperthermia-induced apoptotic pathway in PLB-985 and DMF-PLB cells, Bcl-2 and Bax protein expression was determined. Differential sensitivity to hyperthermia-induced apoptosis of PLB-985 versus DMF-PLB cells did not correlate with steady-state protein levels of Bcl-2 or Bax (Fig. 4a). In addition, induction of apoptotic cell death in PLB-985 cells after exposure to 43°C was not associated with changes in Bcl-2 or Bax protein expression (Fig. 4b).

Endogenous TNF-α Production Correlates with Hyperthermia-induced Apoptotic Effects in PLB-985 and DMF-PLB Cells. Subjecting PLB-985 cells grown at 37°C for 4 h (acceptor cells) to supernatant of PLB-985 cells treated for 1 h with 43°C 18 h earlier (donor cells) induced apoptotic cell death in the acceptor cells (Fig. 5). In search of the proapoptotic factor(s) in the culture supernatant of heat-treated donor cells, a dramatic increase of TNF-α mRNA expression and TNF-α protein secretion by PLB-985 cells exposed to 43°C for 1 h was observed. After heat exposure, TNF-α mRNA levels increased over 30-fold at 12 h after treatment, whereas they remained unchanged in the 37°C-treated control cells for up to 48 h (Fig. 6, a and b). Consistent with the lack of hyperthermia-induced apoptosis in DMF-PLB cells, no effect on TNF-α mRNA expression was observed in differentiated cells exposed to 43°C for 1 h (Fig. 6c). In addition to the effects on TNF-α mRNA levels, TNF-α protein concentration was significantly increased after treatment with 43°C compared to 37°C control cells (Fig. 7).

Treatment of PLB-985 cells with recombinant human TNF-α confirmed the sensitivity of PLB-985 cells to undergo TNF-α-induced apoptotic cell death (Fig. 8). Of the two TNF-α mutants, TNFR-p55 and TNFR-p75 [which stimulate only either of the two TNF receptors (12, 13)], only TNFR-p55 provoked apoptosis in PLB-985 cells (Fig. 8). The constitutive presence of the p55 TNF-α receptor mRNA was confirmed in PLB-985 and DMF-PLB cells (Fig. 6). However, levels
of p55 TNF-α receptor mRNA were not affected by temperature treatment with 37°C or 43°C in PLB-985 cells (Fig. 6, a–c).

Hyperthermia-induced Apoptosis Is Mediated by an Autocrine TNF-α-stimulated Pathway. The above findings suggest a role for an autocrine TNF-α-mediated program of apoptosis as a consequence of hyperthermia treatment. In accordance with this notion, hyperthermia-induced apoptosis was significantly inhibited after the addition of neutralizing TNF-α antibodies (Fig. 9a). DNA fragmentation was almost completely abolished after cotreatment of PLB-985 cells with 43°C for 1 h and increasing amounts of neutralizing TNF-α antibodies. Equal amounts of nonspecific IgG antibodies did not abrogate the inhibition of anti-TNF-α antibodies toward hyperthermia-induced apoptosis, demonstrating the specificity of the neutralizing antibodies (Fig. 9b).

PDTC is known to inhibit the formation of ROS, for which an induction of TNF-α expression via activation of nuclear factor κB has been demonstrated (18). Cotreatment of PLB-985 cells with 43°C for 1 h and increasing amounts of PDTC abolished heat-induced apoptosis (Fig. 10a). Substantiating the hypothesis of an autocrine TNF-α-
mediated apoptotic program, the induction of TNF-α mRNA expression by heat treatment was prevented by PDTC (Fig. 10b).

DISCUSSION

In this study, we provide evidence for an autocrine effect of endogenously produced TNF-α that is responsible for heat-induced apoptosis in nondifferentiated PLB-985 cells. The ability to undergo heat-induced apoptosis was lost in neutrophilic differentiated PLB-985 cells, which appears to be related to the lack of heat-inducible TNF-α expression in differentiated cells. TNF-α-dependent apoptosis in nondifferentiated PLB-985 cells is mediated via the p55 TNF-α receptor. As demonstrated in this study, the protein expression of Bcl-2 and Bax did not change after heat exposure, which implies that the heat-induced apoptotic process is independent of Bcl-2 or Bax. This observation is in agreement with the work of Takasu et al. (4).

Previous studies have shown that similar TNF-α-mediated self-inflicted apoptotic pathways play a significant role as negative feedback mechanisms to regulate the natural killer cell immune response after maximal stimulation with interleukin 12 and interleukin 15 (19, 20). Autocrine suicidal effects have also been demonstrated for other apoptotic death factors such as the FAS ligand (21). With regard to the meaning of endogenous TNF-α production in leukemia cells, Giri and Aggarwal (22) have recently demonstrated the importance of an autocrine TNF-α loop for sensitivity toward TNF-α-induced apoptosis and cell survival in leukemia cells. In general, TNF-α-induced effects are mediated by the p55 and p75 TNF receptors. Only the p55 receptor contains the intracellular death domain, which is critically important for recruiting several proteins involved in the TNF-α-mediated apoptotic program (23, 24). Of the two TNF-α mutants used in this study, i.e., TNFR-p55 and TNFR-p75, only TNFR-p55 provoked apoptosis in PLB-985 cells. Therefore, our finding that TNF-α-induced apoptosis is most likely mediated by the p55 TNF receptor is in line with reports from studies on other leukemia cell lines (25, 26).

It has been shown previously that HL-60 cells undergo apoptosis when treated with temperatures above 42°C (4). However, no studies on the possible mechanism of apoptosis induction or the state of differentiation of leukemia cells are reported. We propose that heat-induced apoptosis in undifferentiated PLB-985 cells, a myelomonocytic cell line, depends on the capability to increase TNF-α expression upon heat treatment. This ability is lost when PLB-985 are driven into neutrophilic differentiation. Heat-induced TNF-α mRNA expression and apoptosis were prevented by the addition of PDTC, which is able to block nuclear factor κB activity by inhibition of ROS formation. It is tempting to speculate that reactive oxygen intermediates are involved in the induction of TNF-α expression by heat. A similar mechanism was demonstrated for the induction of CD95 ligand mRNA expression by cytostatic drugs (27). While this work...
PLB-985 cells were treated with 43°C (Lane 2) to clinical application, our data demonstrate a protection with respect to
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was in progress, Hiroaka et al. (28) have demonstrated the role of oxygen
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topoisomerase I inhibitor, in PLB-985 cells. In their study, as in our
work, PLB-985 cells did not lose their susceptibility to undergo apoptosis
upon differentiation. The differential induction of heat-induced apoptosis
in granulocytic differentiated cells versus nondonorated differentiated cells may be
explained by the observation that differentiated PLB-985 cells express higher levels of enzymes involved in the degradation of ROS-like
glutathione peroxidase (29). Higher levels of antioxidant enzymes that lower
cellular ROS levels could be the reason for the lack of heat-induced
TNF-α expression in the DMF-PLB cells.

The physiological role of the heat-stimulated cell cycle changes and eventual apoptosis is not entirely clear. It is interesting to note that
hyperthermia-induced apoptosis has a threshold temperature of 42°C, which is the upper temperature that can be safely tolerated systemically
(6) or is achieved in high-grade fever situations. For further understanding of the immunological mechanisms of fever, additional studies with different heating durations should be performed. Relative to clinical application, our data demonstrate a protection with respect to
heat-induced apoptosis as a function of cell differentiation. Taken collectively, this apparent therapeutic window may be exploited to clinical advantage, e.g., as a purging modality in the setting of autologous bone marrow transplantation (9).

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