Inhibition by Sphingosine of Leukemic Cell Killing by Human Monocytes Activated with Interleukin-2: A Possible Role of Protein Kinase C

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Sphingosine and its analogs, which inhibit protein kinase C (PKC), are known to be potent inducers of apoptosis in tumor cells. However, we were concerned that sphingosine might also interfere with anti-tumor cells of the immune system. Therefore, we evaluated the effect of sphingosine on activation of human monocytes by interleukin-2 (IL-2) for killing of leukemic cells. Monocytes, purified by elutriation and adherence, were activated with IL-2 or interferon-gamma (IFN-γ) in the presence or absence of sphingosine or another inhibitor for 18 h. Then the monocytes were washed and the culture medium was replaced with fresh medium to remove the sphingosine. HL-60 and K562 leukemic cells were added to the monocyte cultures. Over the next 48 h, the cytotoxic activity of the monocytes towards the leukemic cells was assessed by means of an ^{111}indium-releasing assay. IL-2-activated monocytes lysed 48±3% of HL-60 cells and 44±3% of K562 cells. Sphingosine, dihydrosphingosine, N,N-dimethylsphingosine, and the PKC inhibitor H7 inhibited the activation of monocytes by IL-2, blocking cytotoxic activity against the leukemic cells by approximately 75%. These inhibitors were not toxic to monocytes at the concentrations used. In a PKC assay, sphingosine and H7 inhibited PKC activity in IL-2-treated monocytes. Thus, sphingosines, by inhibiting PKC activity, inhibited activation of monocytes by IL-2, which inhibited the killing of leukemic cells.

Key words: Interleukin-2 — Leukemic cell — Monocyte — Protein kinase C — Sphingosine

Macrophages play important roles in a number of processes, including antigen presentation, lymphocyte activation, tissue repair, and host defense against infections.[1] Besides these functions, macrophages can destroy tumor cells effectively while leaving normal untransformed cells unharmed.[2] To exhibit such activity, macrophages must be activated by lymphokines such as IFN-γ, or bacterial products such as LPS, or both.[2, 3] The biochemical mechanism of macrophage activation with these agents has been investigated. The activation process involves Ca^{2+} mobilization,[4] alteration of lipid mediators,[5] activation of PKC,[3, 6] protein tyrosine phosphorylation,[7–11] and activation of mitogen-activated protein kinase.[10, 11] We recently reported that a serine protease inhibitor, 4-(2-aminoethyl)benzenesulfonyl fluoride, inhibited the killing of leukemic cells by human macrophages treated with IFN-γ and LPS.[12] This result suggests that a serine protease is also involved in activation of human macrophages. Thus,

the activation mechanism of macrophages is complex and not yet fully elucidated.

IL-2 was originally described as a T cell growth factor.[13] Later, it was reported that monocytes and macrophages also possess IL-2 receptors on their surface.[14–16] In response to IL-2, monocytes and macrophages secrete several cytokines, including IL-1β,[17] TNF-α,[18] IL-6,[19] and IL-8.[20] IL-2 also primes monocytes for enhanced release of oxygen radicals.[21] Another prominent effect of IL-2 on monocytes and macrophages is the induction of cytocidal activity directed towards tumor cells. We[22, 23] and other investigators[16, 24–26] have reported that IL-2 or IFN-γ activates human monocytes and macrophages to be cytocidal towards leukemic cells or other tumor cells.

However, the biochemical mechanism by which IL-2 activates monocytes and macrophages is not completely understood.[27] Recently sphingosine and its analogs have been shown to inhibit isolated PKC, and to inhibit PKC activity within cells.[28] It is also known that sphingosine, or other PKC inhibitors such as H7 or staurosporine, induce apoptosis in leukemic cells.[29] Thus, sphingosine has a potential role as a therapeutic agent to induce apoptosis in tumor cells.[30] However, it is also important to examine the effect of sphingosine on the immune system, including its effects on the anti-tumor functions of monocytes and macrophages. Therefore, in this study, we investigated the effect of sphingosine, a potent PKC inhibitor,

on the activation of monocytes by IL-2 for the killing of leukemic cells.

MATERIALS AND METHODS

Reagents Recombinant human IFN-γ (1.0×10^7 U/mg) was purchased from Becton Dickinson (Bedford, MA), and recombinant human IL-2 (6.9×10^6 U/mg) from Genzyme (Cambridge, MA). D-*erythro*-Sphingosine, dihydrosphingosine, N,N-dimethylsphingosine, and H7 were purchased from Calbiochem (La Jolla, CA). Stock solutions of sphingosines were made in DMSO (Sigma Chemical, St. Louis, MO) at a concentration of 30 mM. Brief sonication was needed to dissolve dihydrosphingosine in DMSO. The stock solutions were stored at $-20°C$. When added to culture medium, the final concentration of DMSO did not exceed 0.1%. PMA was purchased from Sigma Chemical, dissolved in DMSO at 1 mM, and stored at $-20°C$. All reagents were LPS-free, as assessed by means of the *Limulus* amebocyte lysate assay.

Leukemic cells HL-60 and K562 cell lines were provided by the American Type Culture Collection, Rockville, MD. These leukemic cells were maintained in complete medium (RPMI 1640 (Life Technologies, Grand Island, NY), containing 5% heat-inactivated human AB serum, 50 U/ml penicillin, and 50 μg/ml streptomycin), and passaged three times weekly. Mycoplasma contamination was checked and no contamination was found.

Monocyte culture Monocytes were separated from buffy coats (Life Blood Mid-South Regional Center, Memphis, TN) as described previously.[12] Briefly, mononuclear cells were isolated from buffy coats by means of dextran (Calbiochem) sedimentation and Histopaque (Sigma Chemical) gradient centrifugation. The harvested mononuclear cells were then subjected to counterflow centrifugal elutriation. The purity of monocytes after this step was more than 80% as judged from nonspecific esterase staining. The monocytes were suspended in complete medium at a density of 2×10^6/ml and plated into 96-well microplates (Falcon 3072, Falcon, Franklin Lakes, NJ) (2×10^5/well) for the cytotoxicity assay. For the PKC assay, 5×10^6 monocytes were plated on 35 mm culture dishes (Falcon 3001). After 2 h incubation in a 5% CO_2 incubator, the plates or dishes were washed to remove non-adherent cells. At this step, more than 98% of the adherent cells were monocytes, as determined by nonspecific esterase staining and phagocytosis of *Candida albicans*.

Viability assay To examine the toxicity of the inhibitors to monocytes, monocyte viability in the microplates was assessed by MTT assay as previously reported.[12] Briefly, after treatment of the monocytes with IL-2 or IFN-γ, with or without sphingosine or another agent, 10 μl of MTT (Sigma Chemical, 5 mg/ml) was added to each well for 4 h. After formation of formazan crystals, the culture super-

natants were aspirated from the wells. The formazan crystals were dissolved in DMSO (150 μl/well) with the addition of 0.1 M glycine (25 μl/well). The absorbance was measured at 550 nm using a microplate spectrophotometer. Only live cells produce formazan.

Cytotoxicity assay After treatment of monocytes with IL-2 or IFN-γ in the presence or absence of sphingosine for 18 h, monocyte-mediated cytotoxicity was assayed as previously described.[12] Briefly, 3×10^6 target cells were labeled with [111]indium oxine (Amersham Healthcare, Medi-physics, Inc., Memphis, TN) for 15 min at room temperature. Labeled target cells (5×10^3) were added to the monocytes in the microplates, after the monocyte medium had been replaced with fresh complete medium. The total volume was 200 μl. The effector-to-target ratio was 40:1. After 48 h incubation in a 5% CO_2 incubator, the microplates were centrifuged at 250g for 5 min. Supernatants (50 μl) were collected to measure their radioactivity. The specific cytotoxic activity was calculated as follows: [(experimental cpm−spontaneous cpm)/ (maximum cpm−spontaneous cpm)]$\times100$%. The maximum cpm release was that obtained from target cells exposed to 1% sodium dodecyl sulfate (Sigma Chemical). The spontaneous cpm release was that obtained from target cells cultured in monocyte-free medium. The spontaneous release was less than 15% of the maximum release. The cytotoxicity assay was performed in triplicate.

PKC assay PKC activity of monocytes was assessed by using a Protein Kinase Assay Kit (Calbiochem). The assay kit is based on an enzyme-linked immunosorbent assay that utilizes a synthetic pseudo-peptide as the substrate for PKC and a monoclonal antibody that recognizes the phosphorylated form of the substrate. For the PKC assay, the monocytes were incubated overnight in 35 mm dishes, then treated for 30 min with IL-2 (1000 U/ml) or IFN-γ (100 U/ml), in the presence or absence of sphingosine. After the 30 min incubation with the agents being tested, the monocytes were washed with phosphate-buffered saline, then collected by scraping with a rubber policeman. The cells (5.0×10^6) were suspended in 0.5 ml of lysing buffer (20 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 0.3% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine, pH 7.5) and sonicated for 30 s on ice. The supernatants were obtained after centrifugation for 10 min at 150g. Samples of supernatants (12 μl) were assayed directly. Purified PKC of rat brain (Calbiochem) was used as a standard. One unit of kinase activity is defined as one nanomole of phosphate transferred to the substrate per min per ml. Results were calculated as units per mg protein, after the protein concentrations of the samples had been determined by the Lowry method.

Statistics Probabilities (P values) were determined by ANOVA, using Scheffe's F-test for post-hoc comparisons

among groups, except that in Fig. 5, showing PKC activity, *P* values were determined by ANOVA, using Fisher's protected least significant difference test. Single-factor ANOVA was always performed, and two-factor or three-factor ANOVA was also performed, when appropriate for the experimental design.

RESULTS

Leukemic cells were lysed by cytokine-activated monocytes. Although unactivated monocytes exhibited low cytolytic activity towards HL-60 and K562 cells, monocytes activated with IFN-γ (100 U/ml) or IL-2 (1000 U/ml) showed enhanced cytolytic activity towards these leukemic cells. Higher cytolytic activity was found in monocytes treated with IL-2, compared with monocytes activated with IFN-γ, as assessed by the ^{111}In-releasing assay (Table I).

In preliminary experiments, we determined the concentrations of IFN-γ and IL-2 that gave the optimal activation response, but with minimal toxicity to the monocytes, as measured by the MTT assay of monocyte viability (data not shown). IL-2 showed no significant toxicity at concentrations up to 1000 U/ml, the highest concentration used. IFN-γ showed slight but significant toxicity at 100 U/ml. Despite the slight toxicity, 100 U/ml of IFN-γ gave maximum activation of monocytes for killing of leukemic cells.

In earlier experiments using this system, we showed that activated monocytes lysed leukemic cells, but did not lyse normal lymphocytes.[23] Thus, the cytotoxicity of the IL-2-activated or IFN-γ-activated monocytes was directed specifically towards leukemic cells.

Sphingosine inhibited the killing of leukemic cells by monocytes. Sphingosine, which inhibits PKC, or another

Table I. The Killing Activity of Activated Monocytes towards Leukemic Cells

Target cells	Activation of monocytes[a]		
	None	IFN-γ	IL-2
	% Cytotoxicity[b]		
HL-60 cells	2.8±0.9	28.6±3.5	47.5±2.6
K562 cells	7.0±0.7	27.2±2.1	44.3±2.8

a) Monocytes were either unactivated (None) or activated with IFN-γ (100 U/ml) or IL-2 (1000 U/ml) for 18 h.
b) The cytotoxic activity of the monocytes towards HL-60 or K562 cells was assessed by means of an ^{111}In-releasing assay after co-cultivation for 48 h. Results are means±SE from five separate experiments, each with triplicate cultures. With both target cell lines, the killing after activation with IFN-γ was significantly greater than killing with no activation, and the killing after activation with IL-2 was significantly greater than the killing after activation with IFN-γ, by ANOVA with *P*<0.01.

PKC inhibitor H7, was added to monocyte monolayers during the activation phase with IFN-γ or IL-2. After the monocytes had been washed and the monocyte medium
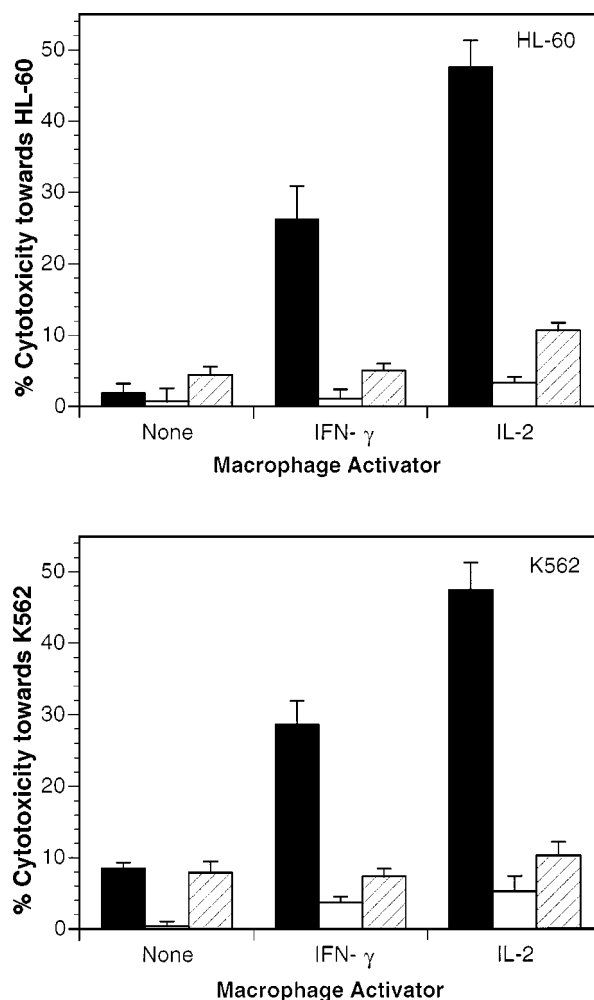

Fig. 1. Effect of sphingosine or H7 on activation of monocytes for killing of leukemic cells. Monocytes were either unactivated or activated with IFN-γ (100 U/ml) or IL-2 (1000 U/ml) for 18 h. At the beginning of this activation phase, in some cultures, sphingosine (25 *μM*) or H7 (25 *μM*) was added to the monocytes. After 18 h incubation, monocyte medium was replaced with fresh complete medium, and the target HL-60 or K562 leukemic cells were added. Then monocyte-mediated cytotoxicity towards the leukemic cells was assessed by use of the ^{111}In-releasing assay. Results are means±SE from three independent experiments, each with triplicate sets of monocyte cultures. Only cells activated by IFN-γ or IL-2 in the absence of inhibitor were significantly different from control cells that had been exposed to neither activator nor inhibitor. Both sphingosine and H7 significantly inhibited killing by IFN-γ-activated or IL-2-activated monocytes (*P*<0.01 by two-factor ANOVA). ■ no inhibitor, □ H7, ▨ sphingosine.

replaced with fresh complete medium to remove the inhibitor, leukemic cells were added to the monocyte culture, and the cytolytic activity of the monocytes towards the leukemic cells was assessed. Sphingosine (25 μM) or H7 (25 μM) inhibited the killing of HL-60 or K562 cells by monocytes activated with IFN-γ or IL-2 (Fig. 1). The vehicle for sphingosine, DMSO (0.1%), had no effect on the killing of leukemic cells by IFN-γ-activated or IL-2-activated monocytes (data not shown).

The inhibitory effect of sphingosine on the activation of monocytes by IL-2 was concentration-dependent (Fig. 2). A high concentration of sphingosine (24 μM) inhibited the killing of HL-60 by 74% and K562 by 81%. Because the monocytes were washed before addition of the leukemic cells, the leukemic cells were not directly exposed to significant concentrations of sphingosine in this protocol. Therefore, the inhibition of killing by sphingosine was due to an effect on the monocytes, not an effect on the target cells.

The inhibition by sphingosine might have been due to non-specific toxicity of sphingosine to monocytes. So we checked the viability of monocytes after treatment with inhibitors, with or without activators, by use of the MTT assay. No significant reduction of cell viability was seen after treatment of monocytes with sphingosine (25 μM), dihydrosphingosine (20 μM), N,N-dimethylsphingosine
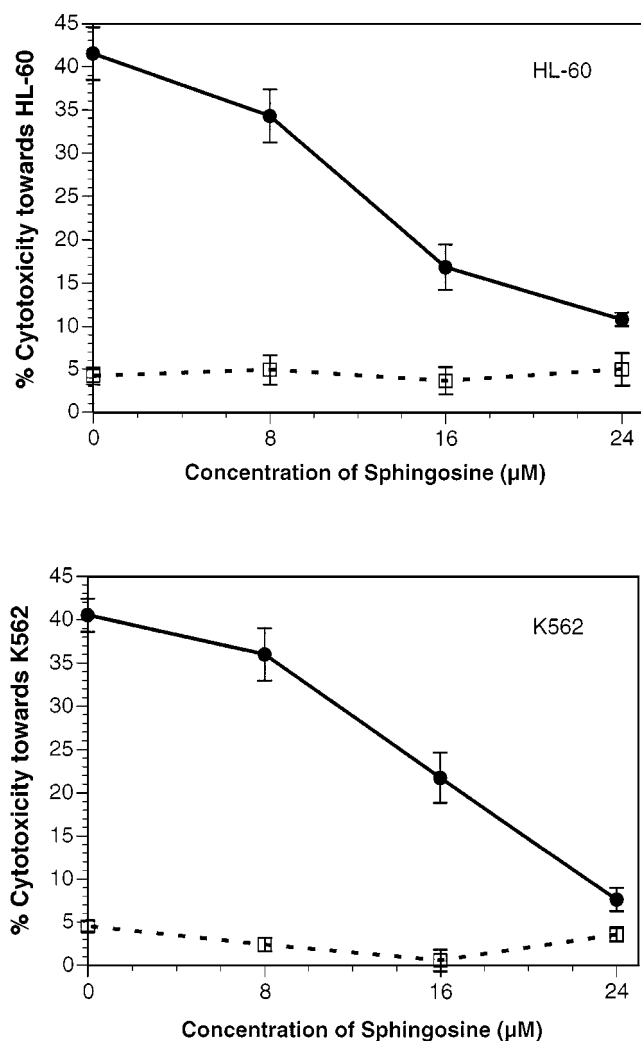

Fig. 2. Inhibition of monocyte activation by increasing concentrations of sphingosine. Monocytes were either unactivated or activated with IL-2 (1000 U/ml) for 18 h. At the beginning of this activation phase, sphingosine was added to the monocytes at the indicated concentrations. Monocyte-mediated cytotoxicity against HL-60 or K562 cells was assessed as in Fig. 1. Results are means±SE from three independent experiments, each with triplicate sets of monocyte cultures. At concentrations of 16 μM and 24 μM, sphingosine significantly blocked activation by IL-2 for leukemic cell lysis ($P<0.01$). □ no inhibitor, ● IL-2-activated.
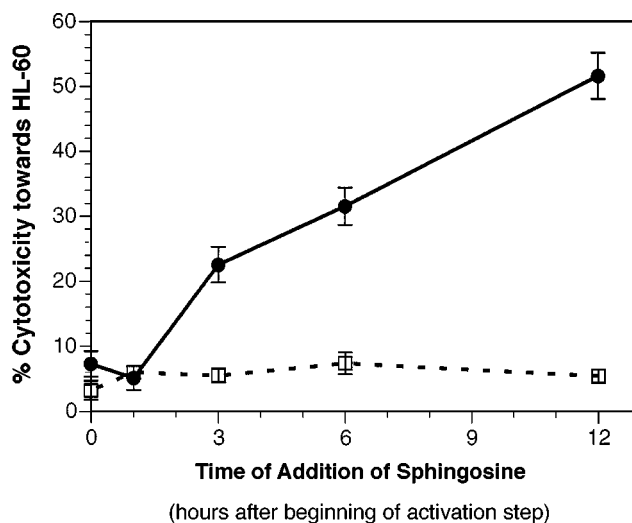

Fig. 3. Time course of the inhibition by sphingosine of monocyte activation for killing HL-60 cells. Sphingosine (25 μM) was added to monocytes at the indicated time after addition of IL-2 (1000 U/ml) to monocytes. After a total incubation time of 18 h, the monocyte medium was replaced with fresh complete medium, and the HL-60 leukemic cells were added. Then the cytolytic activity of the monocytes against HL-60 cells was assessed as in Fig. 1. Results are means±SE from triplicate sets of monocyte cultures. Monocytes activated with IL-2, but never exposed to sphingosine, showed 51±1% killing, whereas monocytes exposed to neither IL-2 nor sphingosine showed 5±1% killing. This experiment is representative of three independent experiments, each with triplicate cultures. A delay of 3 h or longer before adding sphingosine, after the beginning of activation with IL-2, prevented sphingosine from completely blocking activation of monocytes ($P<0.02$). □ no inhibitor, ● IL-2-activated.

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Fig. 5. Sphingosine prevented the enhancement of PKC activity in IL-2-activated monocytes. Monocytes were treated with IFN-γ (100 U/ml) or IL-2 (1000 U/ml), in the presence or absence of an inhibitor, either sphingosine (25 μM) or H7 (25 μM), for 30 min. After the 30 min incubation, monocytes were collected, lysed, and assessed for PKC activity. Results are means±SE from duplicate monocyte cultures. IFN-γ (100 U/ml) or IL-2 significantly increased PKC activity ($P<0.02$). Sphingosine or H7 significantly decreased the PKC activity of activated monocytes ($P<0.01$). ■ no inhibitor, □ H7, ▨ sphingosine.
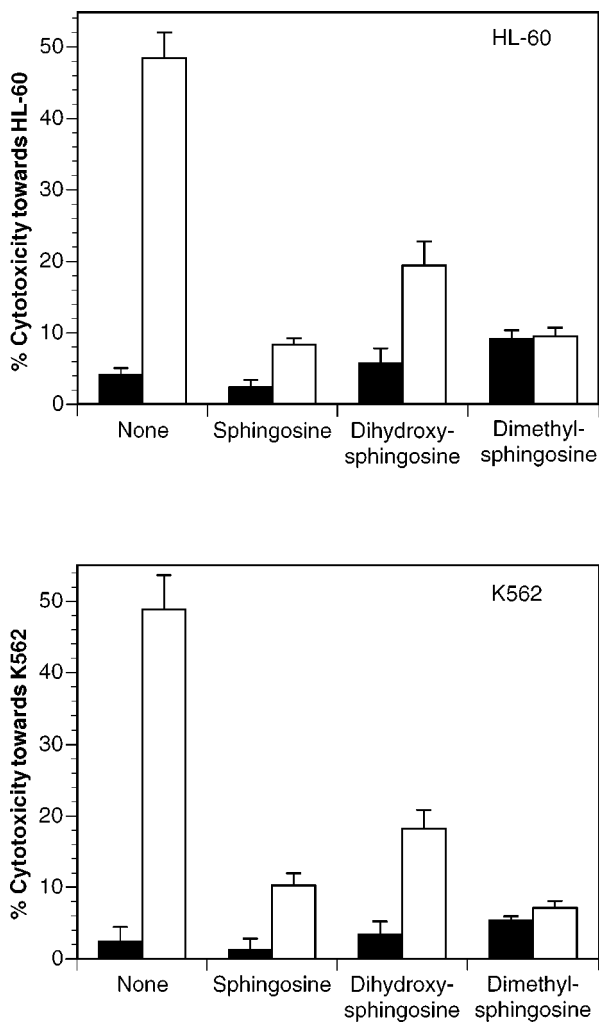
Fig. 4. Effect of analogs of sphingosine on monocyte activation for lysis of leukemic cells. Monocytes were either unactivated or activated with IL-2 (1000 U/ml) for 18 h. At the beginning of the activation phase, either sphingosine (25 μM), dihydrosphingosine (20 μM), or N,N-dimethylsphingosine (15 μM) was added to the monocytes. The cytolytic activity of monocytes towards HL-60 or K562 cells was assessed as in Fig. 1. Results are means±SE from three independent experiments, each with triplicate sets of monocyte cultures. All three inhibitors significantly inhibited the activation of monocytes by IL-2 ($P<0.01$). ■ unactivated, □ IL-2-activated.

(15 μM), H7 (25 μM), or DMSO (0.1%). However, these reagents were toxic to monocytes at concentrations higher than those that we used (data not shown).

We examined the time course of the effect of sphingosine. As shown in Fig. 3, the earlier sphingosine was added to monocytes, after the beginning of the activation phase with IL-2, the greater the inhibition. Almost the
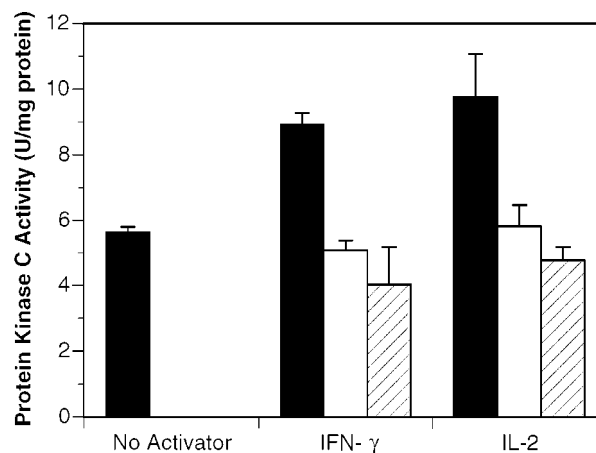
same inhibition was seen when sphingosine was added 1 h after addition of IL-2, as when the sphingosine was added together with the IL-2. In contrast, sphingosine added at 12 h after addition of IL-2 no longer inhibited the activation, or the killing of HL-60 cells.

We also tested some bioactive analogs of sphingosine. Dihydrosphingosine (20 μM) or N,N-dimethylsphingosine (15 μM), like sphingosine (25 μM), inhibited the killing of HL-60 or K562 cells by IL-2-activated monocytes, although the extent of inhibition varied (Fig. 4). These sphingosine analogs, at the concentrations used, were not toxic to monocytes as judged from the MTT assay (data not shown).

Sphingosine is known to inhibit PKC.[28] We examined whether sphingosine inhibited PKC activity in IL-2-activated monocytes under our conditions. Enhanced PKC activity was seen in monocytes after a 30 min treatment of monocytes with IL-2 (1000 U/ml) or IFN-γ (100 U/ml). This enhancement of PKC activity by the activators was completely blocked by sphingosine (25 μM) or H7 (25 μM) (Fig. 5).

The involvement of PKC in the mechanism by which sphingosine inhibited killing of leukemic cells was supported by experiments in which the effect of sphingosine was blocked by adding a known PKC activator, PMA. The results of these experiments are as follows: mono-

cytes activated by IL-2 killed 33±3% of HL-60 cells in the absence of PMA and 30±3% of HL-60 cells in the presence of 1 μM PMA; monocytes activated with IL-2 in the presence of 25 μM sphingosine but with no PMA killed only 9±1% of HL-60 cells, as expected; but monocytes activated with IL-2 in the presence of sphingosine and in the presence of PMA killed 27±3% of HL-60 cells (n=3). This showed that the PKC activator PMA could reverse the effects of sphingosine, suggesting that the inhibitory effects of sphingosine involved interfering with activation of PKC.

DISCUSSION

In this study, we demonstrated that sphingosine and its analogs inhibited activation of monocytes by IL-2 or IFN-γ. As a result, the cytokine-treated monocytes could no longer kill leukemic cells (Figs. 1 and 4). Although sphingosine has been shown to be a potent inducer of apoptosis in leukemic cells,[29] in our experiments the leukemic cells were not exposed to sphingosine, because the sphingosine was washed away from the monocyte cultures before the leukemic cells were added. The monocytes were exposed to sphingosine, but sphingosine did not appear to induce apoptosis in monocytes, because sphingosine and the analogs, at the concentrations used, had no effect on monocytes, as assessed by MTT assay, a sensitive indicator of cell metabolism and viability.

The time course study showed that sphingosine, when added to monocytes early during the activation phase, effectively inhibited the activation by IL-2. In contrast, sphingosine added 12 h later failed to inhibit activation and killing (Fig. 3). These results suggest that sphingosine might inhibit an early event in the activation of monocytes by IL-2, such as activation of PKC.

The increase in PKC activity in monocytes is a relatively early event in their activation; LPS-induced PKC activity in monocytes reaches a maximum around 30 min after addition of LPS.[31] PKC plays an important role in intracellular signaling processes in growth, apoptosis, and activation of a variety of cells.[32] Sphingosine has been shown to inhibit PKC activity in cells.[28] With the PKC assay, we confirmed that sphingosine and H7 did inhibit IFN-γ-enhanced or IL-2-enhanced PKC activity in monocytes (Fig. 5). Other studies showed that LPS-induced TNF-α and IL-1β production by monocytes is inhibited by H7[32] and sphingosine.[33] Thus, PKC might be involved in the mechanism by which sphingosine inhibits killing of leukemic cells. Experiments which showed that the PKC activator PMA blocked the inhibition of killing by sphingosine also suggested that sphingosine interfered with PKC.

In our recent study, we found that N-acetylsphingosine (C₂-ceramide) had no effect on the activation of mono-

cytes by IFN-γ or LPS, as assessed by use of the same cytotoxicity assay system as used here.[34] In contrast, here we found that sphingosine did inhibit monocyte activation by IFN-γ or IL-2. These results are reasonable, because sphingosine inhibits PKC activity,[28] whereas C₂-ceramide does not.[35]

Exogenously added sphingosine can be metabolized to ceramide and sphingosine-1-phosphate in cells.[36] These metabolites, and sphingosine, are considered to be second messengers in signal transduction in cells. We did not assess the metabolism of sphingosine, so there might have been a contribution of sphingosine metabolites to the inhibition of monocyte activation.

Several substances produced by macrophages have been reported to be responsible for the lysis of tumor cells, including oxygen radicals, nitric oxide, neutral proteases, C3a, arginase, TNF-α, and IL-1β.[37] We showed that oxygen radicals were not involved in leukemic cell lysis by activated monocytes.[34] Nitric oxide is a major effector molecule for tumor cell killing by murine macrophages. However, nitric oxide is barely detectable in human macrophages and monocytes,[23, 38] except under special conditions.[39]

TNF-α appears to be involved in lysis of leukemic cells, but other unknown factors are also critical, because the leukemic cells that we used are resistant to being killed by TNF-α alone.[23] However, the ability of sphingosine to inhibit production of TNF-α by monocytes might be an important mechanism by which sphingosine inhibited lysis of leukemic cells. Nevertheless, the effects of sphingosine are far-ranging, involving cytolysis, cytokines, and oxygen radicals, as well as inhibition of both serine/threonine and tyrosine phosphorylation.[40] Therefore, the mechanisms of sphingosine inhibition of leukemic cell lysis are not likely to be restricted to inhibition of cytokine release.

Earlier work, which we confirmed, showed that sphingosine inhibits priming for enhanced release of O_2^-.[41] Thus, the effects of sphingosine were not restricted to interfering with cytolysis, but also included interfering with oxygen radicals, which are not required for cytolysis. This suggests that sphingosine interferes with signal transduction mechanisms that control activation of monocytes for several independent functions, and that the effects of sphingosine are not limited to, and may not even affect, the processes directly involved in cytolysis. In fact, sphingosine was not present during the period when cytolysis was taking place, having been washed away from the monocytes before the leukemic cells were added. However, the question of whether sphingosine directly affects the mechanisms of cytolysis is presently hard to clarify, because these mechanisms remain unknown.

In summary, we showed that sphingosine and its analogs, as well as the PKC inhibitor H7, inhibited the activation of monocytes by IL-2 and IFN-γ, blocking their

ability to lyse leukemic cells. Recently, sphingosine and its analogs have been shown to be potent anti-tumor agents.[30] However, we show here that sphingosine also interfered with the ability of monocytes to kill leukemic cells. Therefore, as potential anti-tumor agents, sphingosine, or drugs that affect sphingosine metabolism, might have undesirable consequences for host defense.

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