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Induction of Tissue Factor Production but not the Upregulation of Adhesion Molecule Expression by Ceramide in Human Vascular Endothelial Cells

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Hirokawa, M., Kitabayashi, A., Kuroki, J. & Miura, A.B. *Induction of Tissue Factor Production but not the Upregulation of Adhesion Molecule Expression by Ceramide in Human Vascular Endothelial Cells.* Tohoku J. Exp. Med., 2000, **191** (3), 167–176 —— Binding of tumor necrosis factor-α (TNF-α) to p60 TNF-α receptor induces the activation of sphingomyelinase to generate ceramide, which in turn activates certain protein kinases and phosphatases, resulting in various TNF-α-mediated biological effects. We have investigated the role for the sphingomyelin/ceramide pathway in the TNF-α-induced upregulation of adhesion molecule expression and tissue factor production of human endothelial cells. TNF-α stimulated human umbilical vascular endothelial cells (HUVECs) to upregulate the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and HLA class I molecules in addition to the induction of procoagulant tissue factor production. C2-ceramide, a highly cell-permeable ceramide analog, was able to stimulate HUVECs to produce tissue factor activity as well as TNF-α. However, C2-ceramide did not stimulate HUVECs to upregulate the expression of VCAM-1, ICAM-1 and HLA class I molecules. These results suggest that there exist both the ceramide-dependent and -independent pathways in TNF-α signal transduction system in human vascular endothelial cells. —— human endothelial cells; TNF-α; ceramide; tissue factor; adhesion molecules

Although tumor necrosis factor-α (TNF-α) has been described to inhibit the growth of certain transformed cells and has been expected to be an active anti-tumor agent, in vivo administration of TNF-α encounters unfavorable side effects including endotoxin shock-like syndrome and coagulopathy, which fact limits its application to clinical uses (Schiller et al. 1991). It has been reported that these adverse effects are partly dependent upon its vascular effects (Nawroth

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and Stern 1986; Nawroth et al. 1986). It has been recently demonstrated that the sphingomyelin/ceramide pathway is involved in TNF-α signaling and cell-permeable ceramides can induce apoptosis in TNF-α-sensitive tumor lines such as HL60 and U937 cells (Kolesnick and Golde 1994; Pushkareva et al. 1995). Moreover, we have observed that a cell-permeable ceramide analog also has a growth-inhibitory effect against tumor cells lacking TNF-R with a distinct mechanism from that seen in HL60 and U937 cells (Kuroki et al. 1996; Kawabata et al. 1999). Therefore, cell-permeable ceramide analogs might be a candidate for an active anti-tumor agent.

In our preliminary experiments, we have made an interesting observation that both TNF-α and a cell-permeable ceramide induce apoptosis in HL60 cells, but only TNF-α can induce differentiation of this cell line (unpublished observation). This result prompted us to test the possibility that there might exist distinct signal transduction pathways for TNF-α in human endothelial cells. If this is the case, one might be able to avoid the serious side effects of TNF-α by using ceramide analogs. In this communication, we present the data suggesting that the sphingomyelin/ceramide pathway may not be involved in the TNF-α-mediated regulation of adhesion molecule expression in human endothelial cells.

Materials and Methods

HUVEC

Human umbilical vascular endothelial cells (HUVECs) were obtained from Kurabo-Clonetics (Osaka). These cells were grown in modified MCDB131 medium supplemented with 2% fetal calf serum (FCS), 10 ng/ml human epidermal growth factor (hEGF), 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, 0.05 μg/ml amphotericin B and 0.4% bovine brain extract according to the manufacturer's instruction.

Cytokines and reagents

Recombinant human TNF-α was obtained from Upstate Biotechnology Inc. (UB1, Lake Placid, NY, USA). C2-ceramide and native ceramide were purchased from Matreya (Pleasant Gap, PA, USA). C2-ceramide is a very cell-permeable sphingolipid, addition of which to HL60 and U937 cells has been reported to result in inhibition of proliferation (Okazaki et al. 1990; Obeid et al. 1993). These compounds were dissolved in ethanol and were added to the cultures so that the final concentration of ethanol became 0.1%. Sphingomyelinase (SMase) derived from *Staphylococcus aureus* was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Assay for tissue factor activity

Total cellular tissue factor activity was determined by a standard one-stage clotting assay as previously described (Bevilacqua et al. 1986). HUVECs were

grown to subconfluency in 24 well culture plates and were stimulated with various reagents. Then, the cells were washed three times with warm phosphate buffered saline (PBS) and harvested by scraping after three times freezing-thawing procedures. The harvested cells were resuspended in PBS, and 100 μl of cell suspension was mixed with 100 μl of $CaCl_2$ (40 mM) and 100 μl of citrated normal human platelet-poor plasma (Baxter, Deerfield, IL, USA). Clotting times were measured at 37°C with a FibroSystem (BBL/Becton-Dickinson, Franklin Lakes, NJ, USA). Tissue factor activity was determined from the standard curves obtained by using standard rabbit brain thromboplastin (Baxter). Thromboplastin activity giving 60 seconds of clotting time was arbitrarily defined as 1000 mU. Each data represents the mean $\pm$ S.E. of triplicate cultures. This experiment was repeated twice more with similar results.

Monoclonal antibodies

Mouse-derived monoclonal antibodies (mAbs) used in this study were as follows: anti-CD54 (ICAM-1; 15.1, IgG1; Ancel, Bayport, MN, USA); anti-CD106 (VCAM-1; IgG1, PharMingen, San Diego, CA, USA); anti-HLA class I (W6/32, IgG2a, ATCC, Manassas, VA, USA); anti-HLA class II (L243, IgG2a, ATCC). Irrelevant mouse IgG (a mixture of IgG1, IgG2a, IgG2b and IgG3) was purchased from Coulter (Hialeah, FL, USA). Monoclonal mouse anti-human TNF-R p60 and rat anti-human TNF-R p80 antibodies were purchased from Endogen (Cambridge, MA, USA) and Genzyme (Cambridge, MA, USA), respectively.

Flow cytometric analysis for adhesion molecules

Cells were harvested by the treatment with trypsin/ethylenediaminetetraacetic acid (EDTA) and then free cells were stained with various mAbs. After staining, the cells were analyzed by using a Cytron (Ortho Diagnostics, Tokyo).

Assay for apoptosis

Apoptosis of the cells was assessed by flow cytometric analysis (Kuroki et al. 1996; Kawabata et al. 1999). Human-derived HL60 (acute myelogenous leukemia) and U-937 (monocytic leukemia) cells were provided by Fujisaki Cell Center (Okayama). After the treatment with various stimuli, the cells were washed twice with PBS and were fixed with 80% ethanol/PBS. After fixation medium was removed by centrifugation, 500 μl of PBS was added to each sample and the cells were treated by DNase-free RNase A (20 μg/ml) for 30 minutes at 37°C. Then, supernatants were removed and the cells were stained with 500 μl of propidium iodide (50 μg/ml; Sigma) for 5 to 10 minutes. Flow cytometric analysis was performed using a Cytron with a cell cycle program (version 1.4). Apoptotic cells were defined as cells containing less than 2N DNA.

Reverse transcriptase-polymerase chain reaction (RT-PCR) for IL-6

Total cellular RNA was prepared according to the method previously described (Chomczynski and Sacchi 1987; Kitabayashi et al. 1995). Oligo-dT was used as primers for cDNA synthesis with the use of a kit according to the manufacturer's instruction (Amersham Pharmacia Biotech., Uppsala, Sweden). PCR primers for the detection of human IL-6 and β-actin were purchased from Clonetics (Palo Alto, CA, USA). Thirty cycles of amplification were performed under the following condition by using a thermal cycler: denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 2 minutes.

SDS-PAGE and immunoblotting for phosphotyrosine

HUVECs were stimulated with either TNF-α or C2-ceramide and cell lysates were prepared to look for the appearance of tyrosine phosphorylated proteins. Sample preparation, sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed according to the methods previously reported (Hirokawa et al. 1995, 1996). Briefly, samples were electrophoresed on 9% SDS-polyacrylamide gels. The resolved proteins were transferred to polvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and probed with anti-phosphotyrosine mAb 4G10 (UBI). The blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel, Durham, NC, USA) followed by incubation with enhanced chemiluminescence solution (Amersham, Arlington Heights, IL, USA). Immunoreactive bands were visualized on X-OMAT AR films (Eastman Kodak, Rochester, NY, USA). Molecular weight determination of immunoblotted proteins was done by using the biotinylated SDS-PAGE standards kit (Bio-Rad, Hercules, CA, USA).

Results

C2-ceramide has been described as being highly cell-permeable and exogenous addition of C2-ceramide to cultures induces apoptotic cell death in HL60 and U-937 cells as well as TNF-α (Okazaki et al. 1990; Obeid et al. 1993). We have confirmed that C2-ceramide (20 μM) induces apoptosis in HL60 and U937 cells in our hands (Fig. 1). Moreover, exogenous SMase, which has been reported to induce an increase in ceramide levels in HL60 cells (Yang et al. 1993), also induced apoptotic cell death. We have firstly examined the capability of C2-ceramide and SMase to stimulate HUVECs to upregulate the expression of adhesion receptors. TNF-α stimulated HUVECs to upregulate the expression of VCAM-1, ICAM-1 and HLA class I molecules as previously reported by others (Fig. 2) (Pohlman et al. 1986). However, exogenous addition of C2-ceramide did not provide this effect (Fig. 2). We also looked for the effect of SMase on the expression of adhesion receptors on HUVECs and found that exogenous addition of SMase did not upregulate adhesion molecules on HUVECs (Fig. 3).

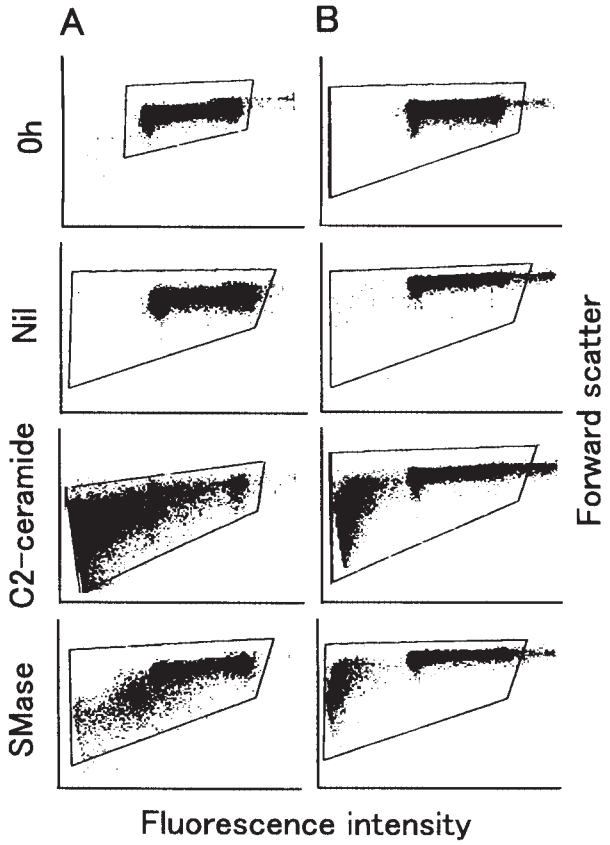

Fig. 1. Induction of apoptosis in HL60 (A) and U937 (B) cells by C2-ceramide and sphingomyelinase (SMase.) C2-ceramide and SMase were added to cultures at the concentration of 20 μM and 0.01 U/ml, respectively. The cells were incubated for 18 hours and then were analyzed. "Nil" means absence of any additives.

To exclude the possibility that the failure of C2-ceramide to induce the upregulation of adhesion receptors on endothelial cells might be due to the poor incorporation of C2-ceramide into the cells, we examined the effect of C2-ceramide on the procoagulant tissue factor production by HUVECs. It has been well described that TNF-α stimulates the production of tissue factor activity from human endothelial cells through their p60 and p80 TNF-α receptors (TNF-R) (Nawroth and Stern 1986; Martin et al. 1993; Kirchhofer et al. 1994; Schmid et al. 1995). Tissue factor activity production induced by TNF-α was evident after 3 hours of incubation, reached the maximal level after 6 hours and declined thereafter (Fig. 4). C2-ceramide induced the production of tissue factor activity with the similar kinetics to TNF-α (Fig. 4). We also examined the cytokine gene expression in HUVECs following the stimulation with TNF-α and C2-ceramide. RT-PCR analysis demonstrated that IL-6 gene expression was induced by TNF-α but not C2-ceramide (data not shown), which data appeared to be consistent with the results of adhesion receptor experiments.

Above data suggest the presence of alternative pathways for TNF-α signaling in addition to the sphingomyelin/ceramide pathway in human endothelial cells. We have tested this hypothesis by examing the protein phosphorylation patterns

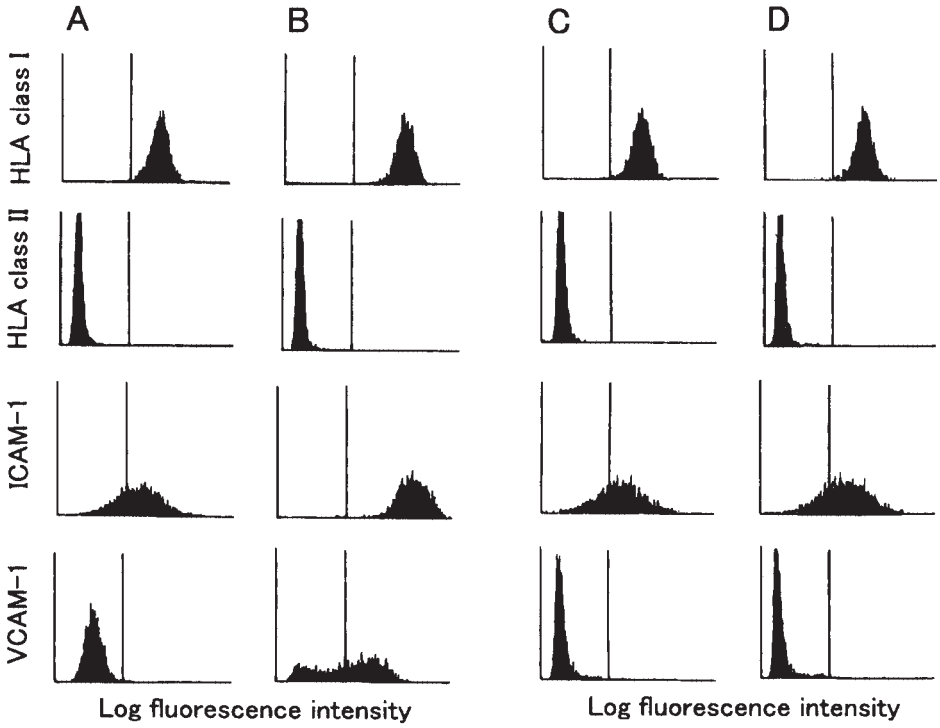

Fig. 2. Upregulation of adhesion molecules on human endothelial cells induced by TNF-α but not C2-ceramide. A, no additive; B, TNF-α; C, Ceramide; D, C2-ceramide. TNF-α and ceramides were added to cultures at the concentration of 20 ng/ml and 20 μM, respectively. The cells were harvested after 18 hours of incubation. Although the data were not shown, the expression pattern of adhesion molecules on HUVECs after 18 hours of culture in the absence of any additives was essentially the same as that before starting cultures.

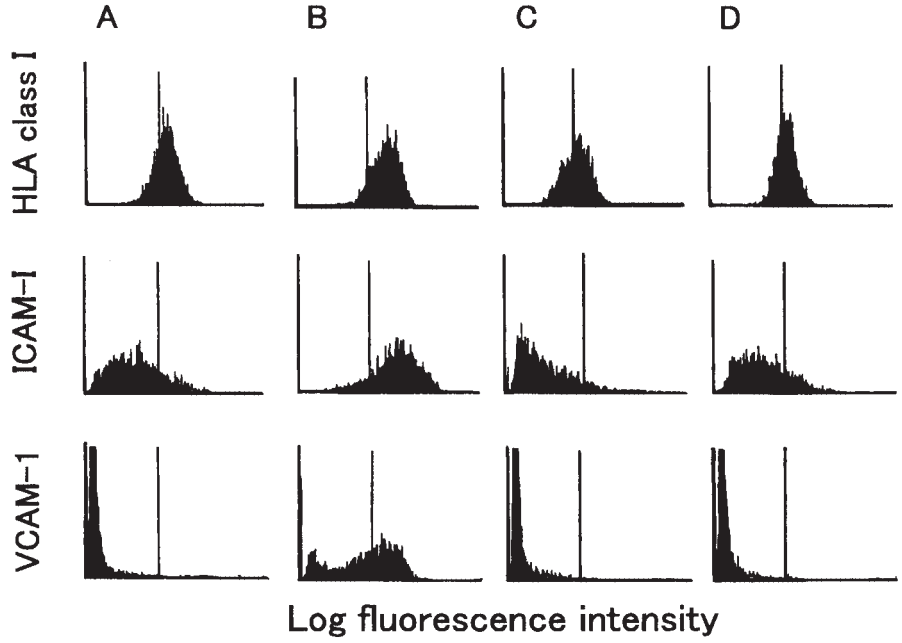

Fig. 3. Failure of exogenous SMase to induce the upregulation of adhesion molecules on HUVECs. A, no additive; B, TNF-α; C, C2-ceramide; D, SMase. Ceramides and SMase were added to cultures at the final concentration of 20 μM and 0.01 U/ml. The cells were incubated for 18 hours. This experiment was repeated once with the similar results.

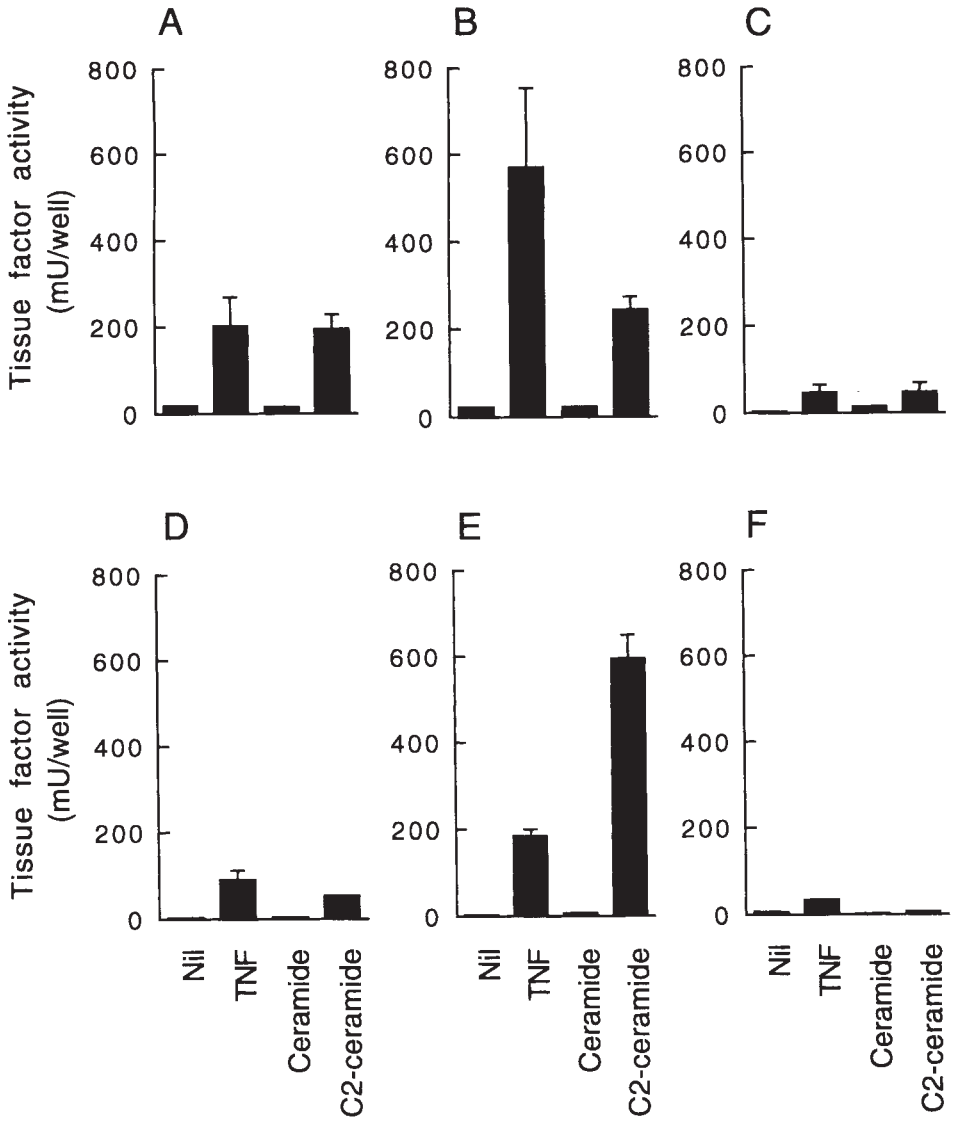

Fig. 4. Induction of tissue factor production from HUVECs by TNF-α and C2-ceramide. TNF-α was added to the cultures at the concentration of 20 ng/ml. Ceramides were dissolved in ethanol and were added at the final concentration of 20 μM. The final concentration of ethanol in cultures was 0.1% (vol/vol). Results of two representative experiments are shown.
Exp. 1: A (3 hours), B (6 hours) and C (18 hours).
Exp. 2: D (3 hours), E (6 hours) and F (18 hours).

in HUVECs following the stimulation with TNF-α and C2-ceramide, since TNF-α-induced activation of the sphingomyelin/ceramide pathway leads to increased tyrosine phosphorylation of p42 mitogen-activated protein kinase in certain cell lines (Raines et al. 1993). However, neither TNF-α nor C2-ceramide induced the significant changes in the protein phosphorylation pattern of tyrosine residues in HUVECs (Fig. 5).

Discussion

To our knowledge, this is the first report describing the functional differences between TNF-α and its cell-permeable second messenger, C2-ceramide. There are

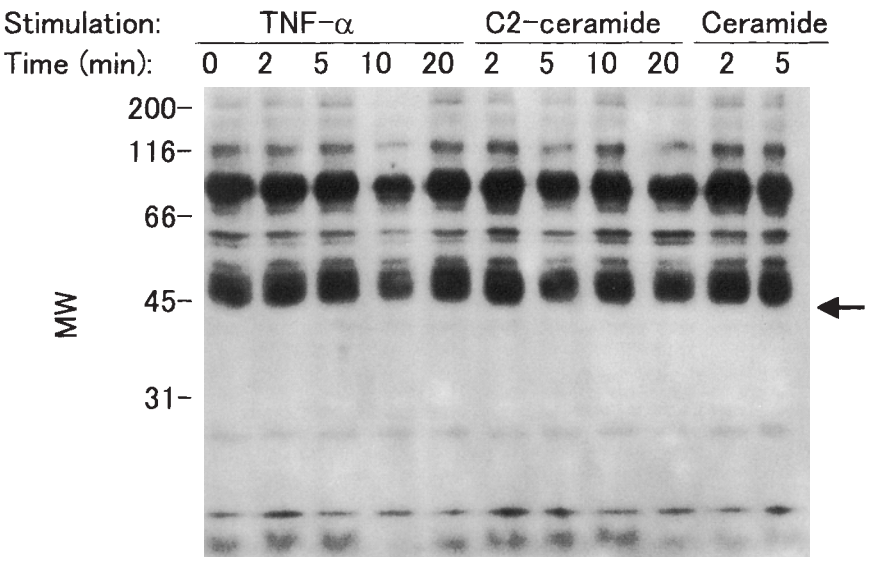

Fig. 5. Comparison of the protein tyrosine phosphorylation patterns following the stimulation with TNF-α and C2-ceramide. No significant increase in protein phosphorylation of tyrosine residues was seen in either stimulation.

at least two possible explanations for our observations. Firstly, subunits of TNF-R may be responsible for distinct functions. Since the sphingomyelin/ceramide pathway is activated following the ligation of TNF-α to p60 TNF-R (Kolesnick and Golde 1994; Pushkareva et al. 1995), the induction of adhesion receptor expression might be mediated via p80 TNF-R. Although we have tested this hypothesis by using mAbs specific for each subunit of TNF-R, we do not yet have conclusive data. Both anti-TNF-R antibodies (2 μg/ml), which were available in our laboratory, did not induce the upregulation of adhesion receptors, which might be due to the lack of agonistic effects of antibodies used in this study. Secondly, there might exist more than one post-receptor signaling pathways following the binding of TNF-α to p60 TNF-R. This is supported by the recently reported observation that the sphingosine kinase pathway through the generation of sphingosine 1-phosphate is involved in mediating TNF-α-induced expression of adhesion molecules on human endothelial cells (Xia et al. 1998).

There is a report, which appears to be against our findings, that cell-permeable ceramide induces the upregulation of ICAM-1 on keratinocytes (Wakita et al. 1996). The distinct biological effects of ceramide on adhesion receptor expression may be explained by the different cell-types for the experiments.

In the clinical aspect, the results obtained in the present study suggest that the cell-permeable ceramide is the alternative to TNF-α for the treatment of cancer patients. One might be able to avoid certain adverse effects resulting from TNF-α/endothelial cell interactions.

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